

# Mitochondrial Thymidine Kinase and the Enzymatic Network Regulating Thymidine Triphosphate Pools in Cultured Human Cells\*

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In non-proliferating cells mitochondrial (mt) thymidine kinase (TK2) salvages thymidine derived from the extracellular milieu for the synthesis of mt dTTP. TK2 is a synthetic enzyme in a network of cytosolic and mt proteins with either synthetic or catabolic functions regulating the dTTP pool. In proliferating cultured cells the canonical cytosolic ribonucleotide reductase (R1–R2) is the prominent synthetic enzyme that by *de novo* synthesis provides most of dTTP for mt DNA replication. In non-proliferating cells p53R2 substitutes for R2. Catabolic enzymes safeguard the size of the dTTP pool: thymidine phosphorylase by degradation of thymidine and deoxyribonucleotidases by degradation of dTMP. Genetic deficiencies in three of the participants in the network, TK2, p53R2, or thymidine phosphorylase, result in severe mt DNA pathologies. Here we demonstrate the interdependence of the different enzymes of the network. We quantify changes in the size and turnover of the dTTP pool after inhibition of TK2 by RNA interference, of p53R2 with hydroxyurea, and of thymidine phosphorylase with 5-bromouracil. In proliferating cells the *de novo* pathway dominates, supporting large cytosolic and mt dTTP pools, whereas TK2 is dispensable, even in cells lacking the cytosolic thymidine kinase. In non-proliferating cells the small dTTP pools depend on the activities of both R1-p53R2 and TK2. The activity of TK2 is curbed by thymidine phosphorylase, which degrades thymidine in the cytoplasm, thus limiting the availability of thymidine for phosphorylation by TK2 in mitochondria. The dTTP pool shows an exquisite sensitivity to variations of thymidine concentrations at the nanomolar level.

Mammalian cells contain two separate pools of deoxyribonucleotides: a cytosolic-nuclear pool used for the synthesis of nuclear DNA and a mitochondrial pool for mitochondrial (mt)<sup>3</sup>

DNA synthesis. In the former pool dNTPs are produced from ribonucleotides by the *de novo* pathway, where ribonucleotide reductase and thymidylate synthase are key enzymes, and by phosphorylation of deoxyribonucleosides via the salvage pathway. mt dNTPs derive from the salvage of deoxyribonucleosides catalyzed by mt kinases and from import of deoxyribonucleotides preformed in the cytosol. Although the mt inner membrane is impermeable to nucleotides, cytosolic and mt dNTP pools communicate via specific transporters. mt carriers for deoxyribonucleotides have been cloned in yeasts (1, 2) and biochemically characterized in mammalian mitochondria (3, 4). Furthermore, early observations on the incorporation of precursors into mtDNA (5) and our own studies on the dynamics of the mt dTTP pool in cultured human cells (6, 7) provide functional evidence for mt import of thymidine nucleotides from the cytoplasm and demonstrate that in cycling cells the main source of mt dTTP is cytoplasmic *de novo* synthesis.

Nuclear DNA synthesis is restricted to the S phase of the cell cycle. Ribonucleotide reductase is induced at the transition between G<sub>1</sub> and S so that *de novo* synthesis of cytosolic dNTPs starts concurrently with nuclear DNA replication when the request for precursors is high. Also cytosolic thymidine kinase (TK1) is induced in S phase and contributes to the production of dTTP. Thus cytosolic dNTP pools expand in S phase supporting the needs of nuclear DNA polymerases and then strongly decrease at the exit of mitosis as anaphase-specific proteolysis removes R2, the small subunit of ribonucleotide reductase (8), and TK1 (9).

A second stable R2 subunit of ribonucleotide reductase has been recently discovered and named p53R2, because its expression is regulated by the tumor suppressor p53, a transcription factor involved in the DNA damage response (10, 11). However, p53R2 is present at a low level also in undamaged cycling cells and is the only small subunit of RNR present in quiescent fibroblasts in culture together with R1 (12); the large subunit is needed to make a functional ribonucleotide reductase. Thus it has now emerged that cells outside S phase have the potential to carry out some *de novo* synthesis of dNTPs. The level of such synthesis is very low compared with that in cycling cells (13), in agreement with the small size of cytosolic dNTP pools in resting cells. However, the R1-p53R2 variant of ribonucleotide reductase fulfills an important function *in vivo*, as demonstrated by the mtDNA depletion arising in the skeletal muscle of patients with inactivating mutations in *RRM2B*, the gene coding for p53R2, and in several organs of knockout mice (14).

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<sup>3</sup> The abbreviations used are: mt, mitochondrial; 5BU, 5-bromouracil; cdN, cytosolic 5'-deoxynucleotidase; FCS, fetal calf serum; HU, hydroxyurea; mdN, mitochondrial 5'-deoxynucleotidase; shRNA, short hairpin RNA; siRNA, short interfering RNA; TdR, thymidine; TK2, mt thymidine kinase; TP, thymidine phosphorylase; RNAi, RNA interference; BVDU, bromovinyldeoxyuridine.

mtDNA synthesis is not limited to the S phase of the cell cycle but takes place also in differentiated cells (15) where nuclear DNA replication has stopped. The intramitochondrial salvage pathway for dNTP synthesis starts with the phosphorylation of imported deoxyribonucleosides by the two mt deoxyribonucleoside kinases, thymidine kinase 2 (TK2) and deoxyguanosine kinase, two constitutively expressed enzymes encoded by two nuclear genes (16, 17). The two enzymes have substrate specificities that allow for the phosphorylation of all four deoxyribonucleosides required for DNA synthesis. Isotope-flow experiments with radioactive deoxyribonucleosides indicate that the intramitochondrial salvage pathway is constitutively active (7, 18). Indeed, until the discovery of the pathology linked to p53R2 deficiency, it was considered the main provider of dNTPs for mtDNA replication in quiescent and differentiated cells where the cytosolic pool is very low. We have recently shown that, in cultured human fibroblasts, the size of mt dNTP pools also changes with the proliferation state of the cells, remaining within a few percentage points of the cytosolic pools (7). The quantitative changes of mt dNTP pools from cycling to quiescent cells are accompanied by a stronger relative decrease of mt dTTP that alters the proportions of the four dNTPs in the mt pool. The specific behavior of thymidine triphosphate underscores the existence of a separate regulation of this precursor and confirms the importance of dTTP for the maintenance of genomic stability. Two mt diseases in humans depend on mutations of genes involved in thymidine metabolism that by altering the mt dTTP pool destabilize the mt genome. Mutations of thymidine phosphorylase (TP), a catabolic enzyme that degrades thymidine and deoxyuridine in the cytosol, cause mt neurogastroencephalomyopathy, a multisystemic syndrome characterized by multiple mutations, deletions, and depletion of mtDNA (19). The enzyme loss of function leads to an increased mt dTTP pool that reduces the fidelity of mtDNA synthesis. The acute myopathic form of mtDNA depletion syndrome is instead associated with mutations in TK2 and is attributed to muscle-specific shortage of mt dTTP (20). A similar tissue-specific mtDNA depletion is caused by deoxyguanosine kinase deficiency, where the target tissues are instead liver and brain (21). Thus, although in both cases the genetic defect is present in all somatic cells, the mt phenotype becomes manifest only in selected tissues, suggesting that the unaffected cells can compensate the mt enzyme deficiency by extramitochondrial pathways.

TK2 shares with TK1 the preferred substrates, *i.e.* thymidine and deoxyuridine, and in addition phosphorylates deoxycytidine, which is however a much better substrate for the cytosolic deoxycytidine kinase (22). Although the two thymidine kinases have the same affinity for thymidine at substrate concentrations below 10  $\mu\text{M}$  ( $K_m$  values of  $\sim 0.5 \mu\text{M}$ ), the  $V_{\text{max}}$  is 20-fold higher for TK1 (23). In cycling cells where TK1 is expressed, TK2 accounts only for a small percentage of total TK activity (5, 24). In non-cycling cells instead, TK2 is the only thymidine kinase and in cultured quiescent fibroblasts its activity is up-regulated (24), suggesting that its role becomes more important when dTTP synthesis in the cytosol is turned down. We previously showed that both TK1 and TK2 participate with two related deoxyribonucleotidases, cdN and mdN, in two futile or

“substrate” cycles that in the cytosol and mitochondria, respectively, modulate the relative concentrations of thymidine and its monophosphate, dTMP, keeping under control the dTTP pools in the two compartments (25, 26). Both TK2 and mdN are constitutively expressed (7, 22); therefore, the activity of TK2 is curbed by that of the deoxynucleotidase that catalyzes the opposite reaction. With the experiments presented here we explored the role of TK2 in the maintenance of the mt dTTP pool in relation to that of other two players in the metabolic network: ribonucleotide reductase and TP. We wished to find out under which conditions TK2 function is limiting for the synthesis of mt dTTP and outline a possible basis for the tissue-specific phenotype of TK2 deficiency. Our approach was to silence the TK2 expression by RNA interference, in cycling cells expressing TK1 or devoid of TK1 and in quiescent cells where both TK1 and the S-phase-specific ribonucleotide reductase are inactive, and to measure mt dTTP pool size and the incorporation of exogenous thymidine into this pool. In quiescent fibroblasts we dissected the functional interactions of R1-p53R2 ribonucleotide reductase and TP with TK2 by chemically inhibiting the enzymes in TK2-silenced and non-silenced cells. The availability of thymidine in the extracellular compartment was an important factor in the regulation of the small dTTP pool of quiescent cells.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Cell Growth**—The established human tumor cell lines HEK 293, Ost TK1<sup>-</sup>, and HOS TK1<sup>+</sup> and the human skin fibroblast lines C63 and C72 were grown in Dulbecco's modified Eagle's medium with 7–10% heat-inactivated fetal calf serum (FCS) and antibiotics. To maintain quiescent cultures of human fibroblasts we transferred contact inhibited cells to medium with 0.1% dialyzed FCS. All cell lines were periodically checked for mycoplasma contamination by the Venor GEM PCR-based method (Minerva Biolabs). We determined cell numbers with a Coulter counter and cell-cycle distribution by flow cytometry.

**Preparation of Plasmids for Expression of shRNAs and Transfection Protocols**—For the down-regulation of TK2 by RNAi we chose seven oligonucleotide sequences present in TK2 cDNA (Imgenex). We produced *in vitro* the corresponding siRNAs with the Silencer siRNA Construction Kit from Ambion and tested their ability to silence TK2 expression in transiently transfected HEK 293 cells. After 48 h of transfection we extracted total cellular RNA from siRNA-transfected and mock transfected cultures to measure the level of TK2 mRNA by real-time PCR (see below). We chose the two most effective sequences, KF (AAGGTCTATGGCTCATGTCTG) and KG (AAATGCCTGCTGCTGCCAAGT), and designed two 64-mer oligonucleotides each containing inverted repeats of the target sequence separated by a 9-bp linker. We cloned the two oligonucleotides in the tetracycline-inducible plasmid pTER (27) and controlled each construct by sequencing. To obtain inducible expression of the shRNAs we prepared a stable clone of Ost TK1<sup>-</sup> cells transfected with plasmid pcDNA6/TR (Invitrogen), coding for a tetracycline repressor that is inactivated by tetracycline. We selected stable clones of pcDNA6/TR Ost TK1<sup>-</sup> cells in the presence of 6  $\mu\text{g}/\text{ml}$  blasticidin (Invitro-

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gen). We tested the ability of the clones to produce the repressor by transiently transfecting them with reporter plasmid pcDNA4TO/LacZ (Invitrogen) in the presence or absence of 2  $\mu\text{g}/\text{ml}$  doxycycline (Sigma). One positive clone named Ost 1.25 was chosen for further work. To down-regulate TK2 in TK1<sup>-</sup> cells we prepared inducible clones from cultures of clone Ost 1.25 transfected with the pTER-KF and pTER-KG constructs, whereas in the case of HOS TK1<sup>+</sup> cells we prepared constitutive clones without repressor. The protocols for plasmid transfections and isolation of clones were those described previously (26).

**Transfections with siRNAs**—We employed two pools of four different siRNAs obtained from Dharmacon, the siGENOME SMART pool reagent to target the mRNA of human TK2 (NM\_004614) and the siCONTROL Non-targeting siRNA pool as a negative control.

Skin fibroblast were seeded at  $0.75 \times 10^6$  cells per 10-cm dish in Dulbecco's modified Eagle's medium plus 10% FCS and cultured for 4 days until  $\sim 90\%$  confluent. Transfection was done by adding siRNAs (final concentration, 50 nM) mixed with 0.036 ml of DharmaFECT 1 in 1.8 ml of Opti-MEM to 7.2 ml of Dulbecco's modified Eagle's medium with 10% dialyzed FCS without antibiotics. Three days later the medium was diluted 1:1 with fresh medium supplemented with 10% dialyzed FCS, and the cells were kept for an additional 4 days in the presence of 25 nM siRNAs. The same cultures underwent a second transfection with 25 nM siRNAs in Dulbecco's modified Eagle's medium with 0.1% dialyzed FCS, and the isotope experiments were performed 3 days later. By this protocol the cells remained in the presence of the siRNAs for a total of 10 days without replating.

**mRNA Extraction, Reverse Transcription, PCR, and Real-time PCR**—We extracted total RNA from  $0.5 \times 10^6$  cells with TRIzol reagent (Invitrogen) and Phase Lock gel Heavy (Eppendorf, Italy). We checked the quality of the RNA samples by capillary electrophoresis (RNA 6000 Nano LabChip, Agilent Bioanalyzer 2100, Agilent Technologies). We prepared cDNAs by reverse transcription as described (26). Real-time PCR assays were performed in 96-well optical plates with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using the following parameters: 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C and 60 s at 60 °C. The amplification reactions were in 0.025 ml containing 0.2  $\mu\text{M}$  primers in PCR buffer (Power Sybr Green PCR master mix, Applied Biosystems) and the cDNA sample. To standardize the expression of individual target genes in HOS TK1<sup>+</sup> and Ost TK1<sup>-</sup> cells we chose as reference hydroxymethylbilane synthase that was expressed at a level similar to TK2. In the case of skin fibroblasts we used as reference succinate dehydrogenase complex subunit A that maintained the same expression level during cell proliferation and quiescence. Primers for the three subunits of human ribonucleotide reductase, p53, succinate dehydrogenase complex subunit A, hydroxymethylbilane synthase were from previous publications (28–31). TK2 primers were 5'-GCCCGGTGTTCAGTGGTTT-3' and 5'-GGCAGCAGTTCAAGCAGGA-3'. We performed each real-time PCR assay with three different amounts of cDNA in triplicate to control the amplification efficiency of each gene by standard curves. Each cDNA preparation

was analyzed at least three times. We employed the comparative Ct method (32) for data processing.

**Immunoblotting**—We prepared and quantified cell protein lysates as described by Pontarin *et al.* (13). Gel electrophoresis was run with a 12% polyacrylamide gel for protein R2 and p53R2, 9% for p53, and 6% for R1. We loaded onto the gels two or three different aliquots of each protein to establish a range of proportionality for the signal of the antibodies. We used 10–20  $\mu\text{g}$  for protein R1 and R2, 8–16  $\mu\text{g}$  for p53, 2–4  $\mu\text{g}$  for p53R2, and 0.5–1  $\mu\text{g}$  for  $\beta$ -actin. We detected the subunits of human ribonucleotide reductase and  $\beta$ -actin (used as internal calibrator) with the antibodies used previously (13). The antibody against human p53 was a mouse monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA) used at 1/1200 dilution. All secondary antibodies were horseradish-peroxidase conjugated and were developed by using an ECL advanced kit (Amersham Biosciences). We quantitated the signals with Kodak 1D image analysis software.

**Isotope Experiments**—The procedures for the isotope experiments, separation of cytosolic and mt dTTP pools, and determination of the specific radioactivity of dTTP were detailed earlier (6). Before addition of [<sup>3</sup>H]TdR we substituted the medium with fresh medium with 10% (cycling cells) or 0.1% dialyzed FCS (quiescent cells) and left the cultures to equilibrate for 1–2 h. All manipulations took place in a 37 °C room to avoid thermal shocks. We incubated HOS TK1<sup>+</sup> cells with [<sup>3</sup>H]TdR (20,000 cpm/pmol, PerkinElmer Life Sciences) 48 h from seeding ( $0.8 \times 10^6$  cells per 10-cm plate) and inducible Ost TK1<sup>-</sup> cells after incubation with 2  $\mu\text{g}/\text{ml}$  doxycycline for 4 days, during which the cells were trypsinized and replated once. At the end of [<sup>3</sup>H]TdR incubations we moved the cultures to a cold room and separated cytosolic and mt dNTPs by differential centrifugation of cell homogenates as described before (6). We extracted the dNTP pools with 60% methanol and dissolved the pellet of the combined nuclear and mt fraction in 0.3 M NaOH to measure the incorporation of radioactivity into macromolecular DNA. Total cellular dNTP pools were extracted with ice-cold 60% methanol for 1 h from the cells still attached to the plates (7). We determined the size of dNTP pools and the specific radioactivity of the dTTP pool in isotope experiments by a DNA-polymerase assay (6, 33).

**Enzymatic Assays**—We assayed TK2 activity in whole cell and mt extracts with 0.2  $\mu\text{M}$  [<sup>3</sup>H]BVDU as the substrate and the total thymidine kinase activity in extracts of cycling TK1<sup>+</sup> cells with 1  $\mu\text{M}$  [<sup>3</sup>H]TdR (24). In extracts of quiescent fibroblasts we used [<sup>3</sup>H]TdR and a specific inhibitor of TK2 (24) to ascertain the presence of TK1 activity. We determined TP activity as described by Marti *et al.* (34), with modifications. For each sample we prepared 4 tubes: 2 aliquots (6–12  $\mu\text{g}$ ) of protein extract in 0.02 ml of 0.1 M Tris-arsenate buffer, pH 6.5, containing 10 mM TdR and 2 parallel blank reactions in buffer without TdR. After 1 h at 37 °C we stopped the reactions with 0.1 ml of 0.3 M NaOH. In the blanks 10 mM TdR was added only after the addition of NaOH. The  $A_{300\text{ nm}}$  of all samples was measured to calculate the specific activity of TP as described previously (34). We express TK2 activities as picomoles of product/min/mg of protein and TP activities as nanomoles/h/mg.



We prepared protein extracts as described previously (24) by adding protease inhibitor mixture (Roche Applied Science) to the lysis buffer. We measured protein concentration by the colorimetric procedure of Bradford (35) with bovine serum albumin as standard.

For the preparation of mitochondrial extracts we resuspended  $1 \times 10^8$  cells in 12 ml of extraction buffer containing 0.21 M mannitol, 0.07 M sucrose, 10 mM Tris HCl, pH 7.5, 0.2 mM EGTA, 2 mg/ml digitonin (Sigma), and 1 tablet of protease inhibitor mixture from Roche Applied Science. We homogenized the cells by passing them four times through a syringe needle (22 gauge  $\times$  30 mm). We sedimented first the nuclei by centrifuging the homogenate at  $1,000 \times g$  for 10 min at 4 °C, and then the mitochondria by centrifuging the supernatant at  $15,000 \times g$  for 20 min. After washing twice with 4 ml of the same buffer, we resuspended the mitochondrial pellet in 0.25 ml of lysis buffer containing 0.2 M NaCl. We centrifuged the lysate at  $19,000 \times g$  for 20 min at 4 °C to precipitate membrane debris and used the supernatant for the enzymatic assays.

## RESULTS

### Contribution of TK2 to the dTTP Pool in Cycling Cells

**Preparation of Plasmids to Silence TK2 by RNA Interference—**To study the role of TK2 in the maintenance of mt dTTP we developed an siRNA reagent to knock down enzyme expression in cultured human cells. We tested in transient transfections with HEK 293 cells the ability of several *in vitro* synthesized siRNAs to down-regulate TK2 mRNA. Two siRNAs targeting the 3'-untranslated region, named KF and KG, reduced the level of TK2 mRNA to 35 and 50% relative to mock transfected controls. We made plasmid constructs to express the two sequences as shRNAs in stable clones and compared their silencing effects in cells devoid of TK1 to simplify the detection of TK2 activity. We transfected cultures of clone Ost TK1<sup>-</sup> 1.25, which contains the tetracycline repressor, with plasmids pTER-KF, pTER-KG, or the empty vector used as control, isolated stable clones of each kind, and measured the level of TK2 mRNA by real-time PCR. Positive pTER-KF clones had 35% of the control mRNA level, and pTER-KG clones had 38%. In all RNAi clones TK2 activity in the presence of doxycycline was ~10% of the control (whose specific enzyme activity was 0.7 pmol/min/mg of protein). The KF and KG sequences were equally effective in reducing TK2 expression both at the mRNA and protein level; therefore, in some of the further experiments we employed only the KF construct.

**Silencing of TK2 in TK1-proficient Cells—**From the TK1-proficient HOS cell line we isolated four stable pTER-KF clones in which TK2 mRNA was 30–40% of the control. We prepared whole cell and mitochondrial extracts from cultures of these four RNAi clones and four control clones transfected with the empty vector and measured the total thymidine kinase and TK2 activity in both fractions (Table 1). In the control clones, the specific enzyme activity of TK2 was 3.5-fold higher in mitochondria than in whole cell extracts. In the RNAi clones both values were close to the background determined previously in the presence of a specific TK2 inhibitor (24). Thus TK2 activity was virtually abolished by the shRNA produced in these cells.

**TABLE 1**

**Total thymidine kinase activity and TK2 activity in clones of HOS TK1<sup>+</sup> cells with TK2 silencing and in control clones**

We measured total thymidine kinase activity (corresponding to TK1 plus TK2 activity) by the phosphorylation of  $1 \mu\text{M}$  [<sup>3</sup>H]TdR and TK2 activity by the phosphorylation of  $0.2 \mu\text{M}$  [<sup>3</sup>H]BVDU in extracts from four control and four RNAi clones. We used two different aliquots of each extract to check for proportionality in the assays. Values are means  $\pm$  S.D.

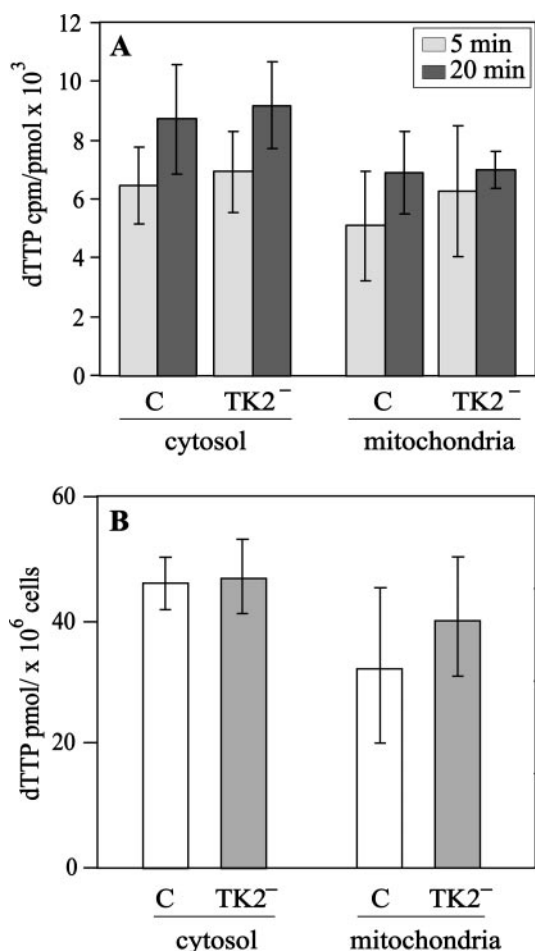
Cells	Kinase activity			
	Whole cell extracts		mt extracts	
	[ <sup>3</sup> H]TdR	[ <sup>3</sup> H]BVDU	[ <sup>3</sup> H]TdR	[ <sup>3</sup> H]BVDU
	<i>pmol/min/mg protein</i>			
Control clones	304 $\pm$ 37	0.38 $\pm$ 0.04	8.4 $\pm$ 2.2	1.4 $\pm$ 0.3
RNAi clones	244 $\pm$ 26	0.03 $\pm$ 0.01	5.2 $\pm$ 1.5	0.1 $\pm$ 0.07

However, the total phosphorylation of [<sup>3</sup>H]TdR was identical in the two sets of clones both in cytosolic extracts where it reflected the high TK1 activity, and in mitochondrial extracts where a minor ( $\leq 2\%$ ) contamination with cytosol masked the decrease of TK2 activity in the RNAi clones. Clearly in cycling cells that express TK1 the contribution of mitochondrial TK2 to the overall cellular thymidine kinase activity is negligible.

We next examined in the pTER-KF clones whether the loss of TK2 influenced the size of the mt dTTP pool and the incorporation of [<sup>3</sup>H]TdR into mt dTTP. Because the activity of TK1 and the size of dNTP pool are strongly dependent on the cell cycle, we took great care to perform all isotope experiments under similar growth conditions. Cytofluorometric analyses indicated that the mean frequency of S-phase cells was  $46 \pm 6\%$  in the RNAi clones and  $44 \pm 6\%$  in the controls. We incubated parallel cultures of four RNAi and four control clones with  $0.3 \mu\text{M}$  [<sup>3</sup>H]TdR for 5 or 20 min and measured the incorporation of isotope into cytosolic and mt dTTP (Fig. 1). The specific radioactivity of dTTP increased between 5 and 20 min and was at both time points ~20% lower in mitochondria than in the cytosol, with no difference between control and silenced clones (Fig. 1A). In the cytosol the specific radioactivity of dTTP was after 20 min about half that of the precursor thymidine, reflecting the very efficient salvage of the deoxynucleoside by TK1. Also the size of the dTTP pools was not affected by TK2 silencing with the mt pools containing ~1% of the dTTP present in the cytosol (Fig. 1B). We showed earlier (6) that dTTP represents only one-third of the total thymidine phosphates in mitochondria but close to 90% in the cytosol. The incorporation of dTMP into DNA was identical in the two series of clones ( $5.0 \pm 0.3$  pmol/min in the control clones and  $5.2 \pm 1.2$  pmol/min in the RNAi clones), as calculated from the incorporation of radioactivity into DNA and the specific radioactivity of dTTP. In conclusion, the absence of TK2 did not affect any aspect of the metabolism of thymidine in proliferating cells expressing TK1 in the cytoplasm.

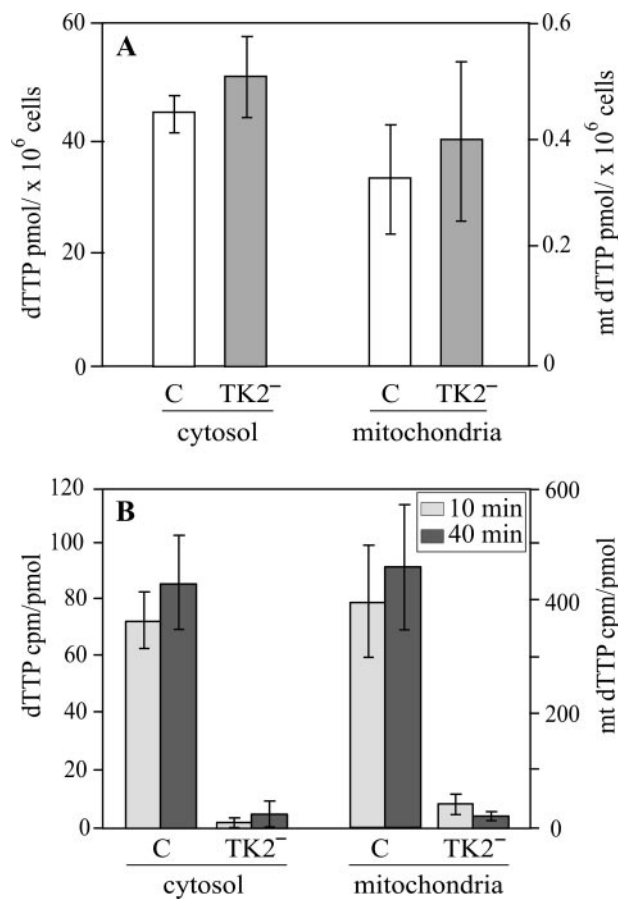
**Silencing of TK2 in TK1-deficient Cells—**The experiments with HOS TK1<sup>+</sup> cells had shown that TK2 is dispensable in cycling cells that contain an active cytosolic thymidine kinase. We asked the question if the mitochondrial thymidine kinase becomes relevant for the maintenance of mt dTTP in cells lacking the cytosolic enzyme and performed a similar thymidine incorporation experiment with clones of TK1-deficient Ost cells where TK2 was down-regulated. We incubated two RNAi

## TK2 and dTTP Pool Regulation



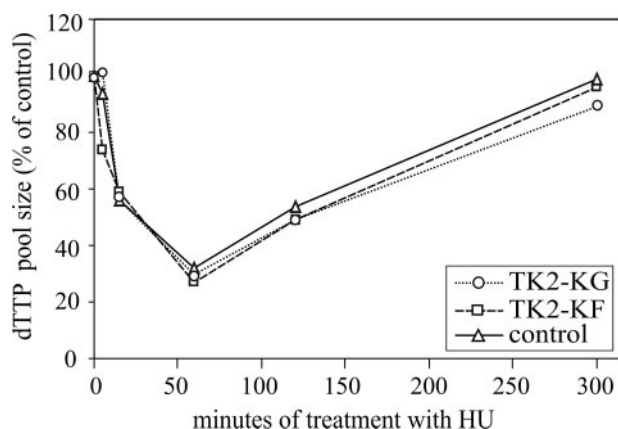
**FIGURE 1. TK2 silencing does not affect the synthesis of dTTP from [<sup>3</sup>H]TdR in cycling TK1-proficient cells.** We incubated proliferating cultures of four stable clones of HOS TK1<sup>+</sup> cells transfected with plasmid pTER-KF (TK2<sup>-</sup>) and four clones transfected with the empty vector (C) with 0.3 μM [<sup>3</sup>H]TdR (20,000 cpm/pmol). After 5 and 20 min we separated mt and cytosolic dTTP pools and measured their specific radioactivity (A) and size (B) by the DNA polymerase assay. Bars indicate ±S.D.

clones and two controls for 4 days with 2 μg/ml doxycycline to induce the expression of the shRNAs. We then measured cytosolic and mt dTTP pool sizes and the incorporation of 1 μM [<sup>3</sup>H]TdR into dTTP. All clones grew similarly and at the time of the experiment had the same frequency of S-phase cells: 46% the controls and 44% the RNAi clones. In the RNAi clones TK2 mRNA was decreased by 60%, and TK2 enzyme activity amounted to only 8% of the control value. Nevertheless, the size of the dTTP pool of the RNAi clones was unchanged (Fig. 2A). As in the case of HOS TK1<sup>+</sup> cells the mt pool was ~1% of the cytosolic pool, and their absolute values were similar to those of the TK1-proficient cells. The lack of effect of TK2 silencing on the mt dTTP pool size contrasted with a very strong effect on the incorporation of radioactive TdR into dTTP (Fig. 2B). In the control clones the specific radioactivity of mt dTTP was ~5-fold higher than in cytosolic dTTP (notice the difference in scale of the *ordinates* in Fig. 2B). In the RNAi clones dTTP-specific radioactivity was only ~5% of the controls in both mitochondria and cytosol. The higher specific radioactivity of dTTP in the mitochondria than in the cytosol of control cells reflects the site of TdR phosphorylation. In the TK1-defi-



**FIGURE 2. TK2 silencing in cycling TK1-deficient cells blocks the phosphorylation of [<sup>3</sup>H]TdR but leaves the sizes of cytosolic and mt dTTP pools unchanged.** We incubated proliferating cultures of two doxycycline-inducible clones (TK2<sup>-</sup>) of Ost TK1<sup>-</sup> cells transfected with either plasmid pTER-KF or plasmid pTER-KG and 2 clones transfected with the empty vector (C) with 1 μM [<sup>3</sup>H]TdR (20,000 cpm/pmol) for 10 and 40 min. After each time we separated mt and cytosolic dTTP pools and measured their size (A) and specific radioactivity (B) by the DNA polymerase assay. Bars indicate ±S.D.

cient cells the silencing of TK2 almost eliminated the salvage of thymidine but left the size of mt dTTP pool unchanged, providing further evidence for the view that in cycling cells the major supplier of mt dTTP is cytosolic *de novo* synthesis (6). We thus asked how inhibition of ribonucleotide reductase affects the dTTP pool of TK1-deficient cells with normal and down-regulated TK2 activity. To this purpose we treated two TK2-silenced clones and one control clone with the specific inhibitor of ribonucleotide reductase hydroxyurea (HU) for between 5 min and 5 h and compared the size of their dNTP pools. In contrast to what occurs in TK1-proficient cells, where the dTTP pool is scarcely changed by treatment with HU (13, 36), in Ost TK1-deficient cells 3 mM HU caused a fast drop of the cellular dTTP pool. However, TK2 down-regulation did not affect the kinetics of the decrease (Fig. 3). The dTTP pool size reached a minimum of ~30% of the initial value after 60 min of treatment and then progressively recovered returning almost to the pre-treatment level after 5 h of continuous exposure to HU. As the dATP pool remained strongly inhibited also after 5 h (not shown), inactivation of HU or induction of new ribonucleotide reductase do not appear to be involved in the dTTP recovery that remains unexplained. However, these results confirm



**FIGURE 3. Silencing of TK2 in cycling TK1-deficient cells does not enhance the depletion of the dTTP pool induced by hydroxyurea.** We treated proliferating cultures of two clones of Ost TK1<sup>-</sup> cells expressing different anti-TK2 shRNAs and a control clone with 3 mM hydroxyurea for 5–300 min. We measured the size of the dTTP pools at various time points. Data are shown as percentages of dTTP pool sizes in control cultures of each clone (47 pmol/10<sup>6</sup> non-silenced cells, 31 pmol/10<sup>6</sup> TK2-KG cells, and 41 pmol/10<sup>6</sup> TK2-KF cells).

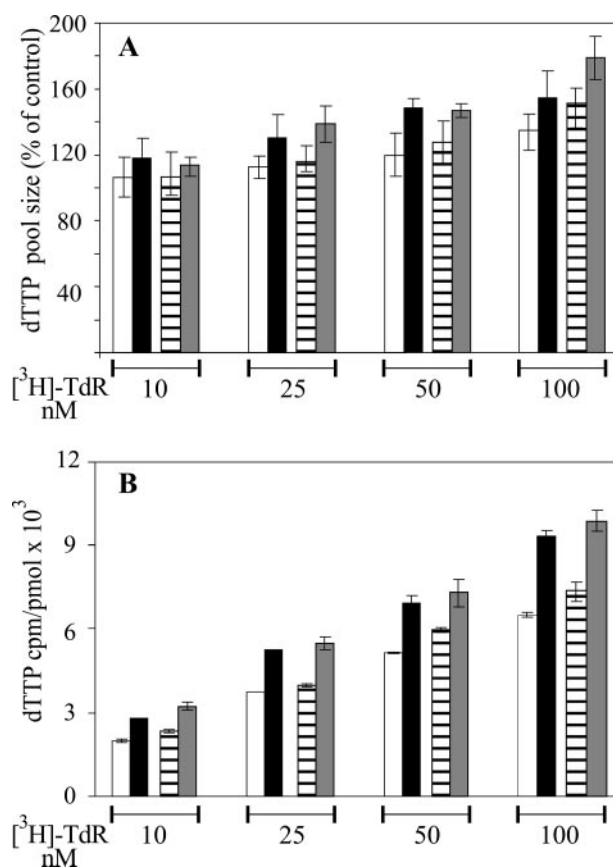
the different behavior of the two pools in cells treated with HU reported earlier (13, 36). In separate analyses of the mt dTTP pools we found that after 2 h of HU treatment mt dTTP had decreased to 43% in the RNAi clones and to 52% in the control clone (not shown) suggesting that the residual cytosolic dTTP was sufficient to sustain the mt pool in the presence of HU, independently of the level of TK2 activity.

From these experiments with actively growing cells we conclude that, although expressed both in cycling and quiescent cells, TK2 is not required to maintain the mt dTTP pool in the former when dNTP synthesis in the cytosol is up-regulated. To examine the effects of TK2 silencing in a non-proliferating cell system we moved from the two tumor cell lines used so far that lack normal contact inhibition of growth to normal human fibroblasts, which can be brought to a quiescent state in culture.

#### Contribution of TK2 to the dTTP Pool in Quiescent Cells

**Thymidine Salvage by TK2 and the Influence of Thymidine Phosphorylase**—To analyze the effects of TK2 silencing during quiescence we chose a line of non-transformed skin fibroblasts that in the presence of low serum can be kept quiescent for extended periods of time in contrast to the cell lines used in the previous experiments.

In quiescent cells both TK1 and the cell-cycle regulated small subunit of ribonucleotide reductase R2 are down-regulated (8, 9), thus TK2 becomes the only kinase responsible for salvage of thymidine and the R1-p53R2 complex the only form of ribonucleotide reductase producing dNTPs *de novo*. The thymidine that feeds the salvage pathway is derived from dephosphorylation of intracellular dTMP catalyzed by deoxynucleotidases or from the extracellular milieu. Both sources of thymidine are likely to be sparse in postmitotic cells and tissues. In humans the concentration of thymidine circulating in the blood is below the detection limit of the common analytical methods (50 nM). We investigated if low concentrations of extracellular thymidine are effectively phosphorylated by quiescent cells and if they affect the size of the dTTP pool.

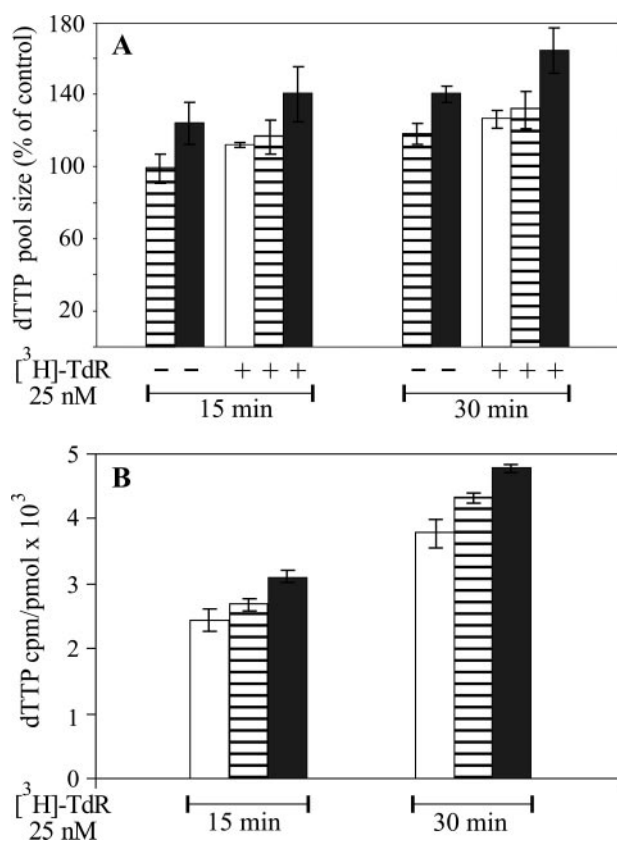


**FIGURE 4. TK2-mediated salvage of extracellular thymidine at nanomolar concentrations expands the dTTP pool of quiescent human fibroblasts.** We incubated quiescent cultures of C63 (black and white bars) and C72 (striped and shaded bars) fibroblasts with 10–100 nM [<sup>3</sup>H]TdR (20,000 cpm/pmol) for 15 (white and striped bars) or 30 (black and shaded bars) min. We measured the size (A) and specific radioactivity (B) of the dTTP pool by the DNA polymerase assay. In A dTTP pool sizes are expressed as percentages of the original sizes before addition of TdR to the medium (1.3 pmol/million C63 cells and 2.3 pmol/million C72 cells). Bars show the range of determinations.

In quiescent cultures of two skin fibroblast lines (C63 and C72) with undetectable TK1 activity we tested the incorporation of 10–100 nM [<sup>3</sup>H]TdR into the dTTP pool. We incubated the cells for 15 and 30 min in fresh medium with 0.1% dialyzed serum and increasing concentrations of labeled thymidine and measured the total cellular dTTP pool size, expressed in Fig. 4A as percent variation relative to the controls incubated in the absence of thymidine. In both cell lines the dTTP pool increased slightly but progressively with the concentration of thymidine and the length of incubation. The specific radioactivity of dTTP (Fig. 4B) was identical in the two cell lines at each TdR concentration and passed from 1/7 to ~1/2 of the specific radioactivity of the [<sup>3</sup>H]TdR added to the medium, indicating that salvage of extracellular thymidine was competing with endogenous dTTP synthesis with increasing efficiency at higher concentrations of thymidine. In fact the specific radioactivity of dTTP increased more than pool size, revealing that normal quiescent fibroblasts strictly regulate the size of their dTTP pool. These results suggest not only that TK2 in quiescent cells contributes to the maintenance of the dTTP pool and at very low concentrations of thymidine, but also that the extent of this effect highly depends on the concentration of the nucleoside.

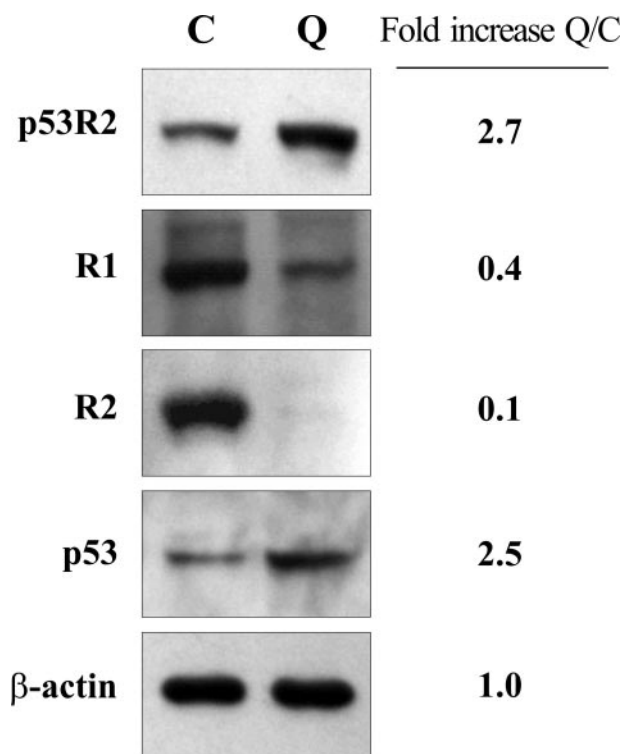


## TK2 and dTTP Pool Regulation



**FIGURE 5. Inhibition of TP promotes thymidine salvage by TK2.** We incubated quiescent cultures of C63 skin fibroblasts with 10 (striped bars) or 100 (black bars)  $\mu\text{M}$  5BU or no inhibitor (control, white bars) for 15 min. Then we added to the medium of the controls and part of the 5BU-treated cultures 25 nM [<sup>3</sup>H]TdR (20,000 cpm/pmol) for 15 or 30 min and incubated two sets of 5BU-treated cultures for the same times in the absence of TdR. In A, dTTP pool sizes are expressed as percentages of the original dTTP pool in untreated cells (1.9 pmol/million cells). B, specific radioactivity of dTTP in the cultures incubated with [<sup>3</sup>H]TdR. Bars show the range of determinations.

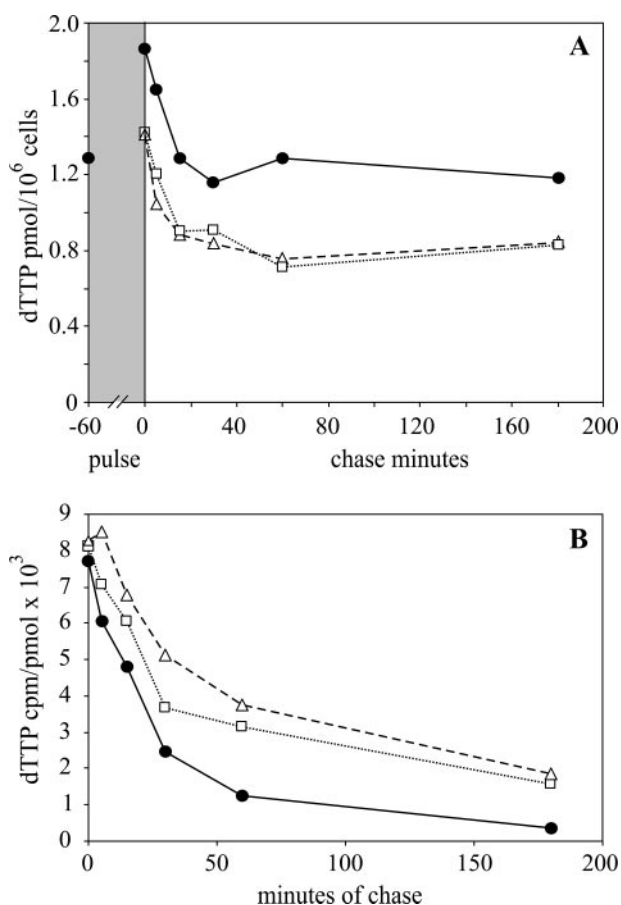
The actual amounts of [<sup>3</sup>H]TdR phosphorylated were different in the two lines. The original dTTP pool size was 1.3 pmol/million cells in C63 fibroblasts and 2.3 in C72 cells and after 30-min incubation with 100 nM thymidine the pools reached 1.9 and 4.2 pmol/million cells, respectively. The two fibroblast lines were derived from different donors and several factors may influence their nucleotide content, but we noticed that they differed in their level of TP activity. In whole cell extracts we found a specific enzyme activity of 820 nmol/h/mg of protein in C63 fibroblasts and 260 nmol/h/mg in C72 fibroblasts. The influence of TP on the dTTP pool of cultured cells is not easily predicted, because the presence of equilibrative nucleoside transporters in the plasma membrane and the large volume of the extracellular compartment remove the thymidine substrate from the enzyme when the concentration gradient is directed outward from the cell. We tested the effects of 5-bromouracil (5BU), a cell-permeable inhibitor of TP, on the size of the dTTP pool in quiescent C63 fibroblasts incubated in fresh medium with 0.1% dialyzed serum in the absence or presence of 25 nM [<sup>3</sup>H]TdR (Fig. 5). The cultures received either 10 or 100  $\mu\text{M}$  5BU 15 min before addition of [<sup>3</sup>H]TdR for 15 or 30 min. The variations of dTTP pool size relative to the untreated control (Fig. 5A) show that 100  $\mu\text{M}$  5BU already in the absence of



**FIGURE 6. The p53R2 small subunit of ribonucleotide reductase is up-regulated in quiescent skin fibroblasts.** Immunoblots of protein extracts from cycling (C) and quiescent (Q) cultures of C63 fibroblasts. We loaded the same amounts of C and Q protein extracts to detect each individual protein, but due to different sensitivities of the primary antibodies we used different aliquots for the different proteins (see "Experimental Procedures").  $\beta$ -Actin was the loading control. Comparisons were done within the proportionality range of the signals.

added TdR after 15 min caused a small increase of the dTTP pool that became more marked after 30 min. This increase indicates that quiescent fibroblasts contain a small pool of internal thymidine derived from catabolism of endogenous thymidine nucleotides even when the extracellular medium is devoid of nucleosides and that TP competes with TK2 for their shared substrate. As in the previous experiment (Fig. 4A), the addition of 25 nM TdR caused a small, time-dependent expansion of the cellular dTTP pool that was enhanced by the inhibition of TP with 100  $\mu\text{M}$  5BU. The specific radioactivity of the dTTP pool (Fig. 5B) increased with time (as in Fig. 4B) and with the dose of the TP inhibitor proving that also the lower concentration of 5BU exerted an effect on the dynamics of the pool. These experiments indicate that TP in normal quiescent cells contributes to a control of the dTTP pool size by affecting the intracellular concentration of thymidine, the substrate of TK2.

**Ribonucleotide Reduction in Quiescent Human Fibroblasts—**The discovery of p53R2 led to the realization that also quiescent cells in the absence of R2 have a limited capacity for the *de novo* synthesis of dTTP (12, 13). We compared by Western blotting the levels of the three subunits of ribonucleotide reductase in protein extracts from cycling cultures of C63 fibroblasts and cultures that had been maintained in the quiescent state for 10 days with 0.1% serum after reaching confluence (Fig. 6). We also measured the level of p53, the transcription factor that induces p53R2 expression. In comparison to the cycling fibroblasts, the quiescent cells had lost the R2 small subunit, p53R2



**FIGURE 7. HU-sensitive *de novo* synthesis of dTTP occurs in quiescent skin fibroblasts.** Pulse-chase experiment with 100 nM [ $^3\text{H}$ ]TdR in cultures of C63 fibroblasts maintained for 10 days in 0.1% FCS after reaching confluence. All cultures received a 60-min pulse with [ $^3\text{H}$ ]TdR, followed by a 180-min chase without TdR in the medium. At 50 min of the pulse we added 3 (squares) or 15 (triangles) mM HU to two sets of cultures. The treatment continued during whole chase. A third set of cultures without HU was the control (circles). A, dTTP pool sizes. The point on the ordinate indicates the size of dTTP pool in control cells before addition of TdR. At time 0 the radioactive medium was replaced with TdR-free medium plus or minus HU. B, decline of dTTP specific radioactivity during the chase.

was increased almost 3-fold and the large subunit R1 was decreased 2-fold. The induction of p53 protein, a signal of the quiescent state (37, 38), was not due to the incubation in low serum, because we found a similar induction in quiescent cultures of C63 fibroblasts maintained in 10% serum for 10 days after reaching confluence. The immunoblots suggest that the R1-p53R2 variant of ribonucleotide reductase was responsible for the *de novo* synthesis of dNTPs in quiescent C63 fibroblasts. To test this assumption we examined the effects of HU in such cells by a pulse-chase experiment with 100 nM [ $^3\text{H}$ ]TdR. Three parallel sets of cultures received a 60-min pulse of labeled thymidine and were then shifted to fresh medium without thymidine for a 180-min chase. After 50 min of the pulse two sets of cultures received 3 or 15 mM HU, and the treatment continued throughout the chase. The third set without HU served as a control. The size and specific radioactivity of the dTTP pool were measured in all sets at different times during the chase (Fig. 7, A and B). In the controls the dTTP pool increased  $\sim 30\%$  from 1.3 to 1.9 pmol/million cells during the 60-min pulse and returned to the pre-pulse value during the first 15 min of the

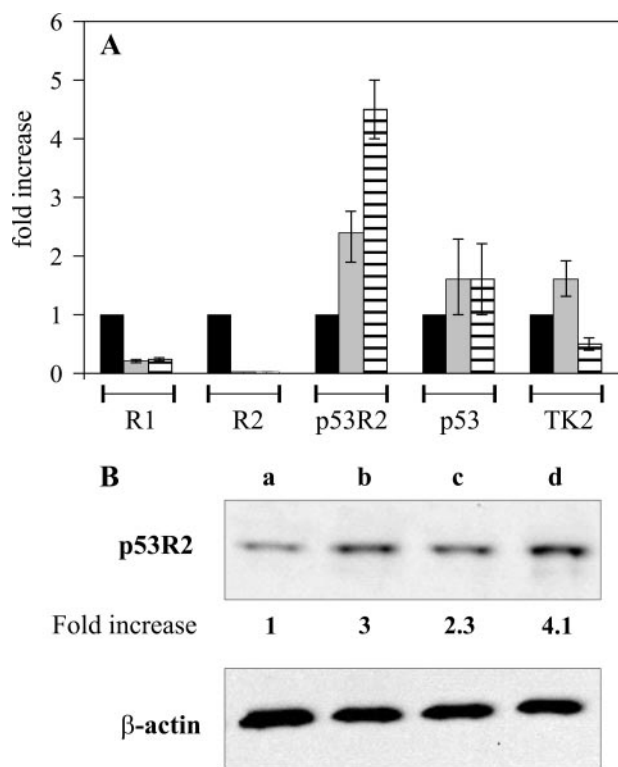
chase (Fig. 7A), decreasing at a rate of 0.04 pmol/min/million cells. The HU-treated cells started the chase with a content of dTTP lower than the controls, because HU was present during the final 10 min of the pulse and had set off a decrease of the dTTP pool that continued during the first 40 min of the chase until the pool stabilized at  $\sim 60\%$  of the control size with either dose of HU (Fig. 7A).

The specific radioactivity of dTTP in the controls decreased rapidly during the first 30 min of the chase, followed by a slower continuous decline (Fig. 7B). The continued loss of radioactivity from the dTTP pool after stabilization of its size indicates that the pool turned over and labeled dTTP was replaced by new non-radioactive dTTP produced *de novo*. From the pool size and specific radioactivity we calculate that the turnover rate in the controls was 0.09 pmol/min/million cells between 15 and 30 min and 0.03 pmol/min/million cells during the rest of the chase. In the HU-treated cells the turnover was slower, especially at the higher concentration of inhibitor. However, after 30 min of chase the specific radioactivity was almost halved and continued to decrease until 180 min, which means that *de novo* synthesis of dTTP was not completely blocked by HU. In conclusion the data in Figs. 6 and 7 demonstrate that quiescent skin fibroblasts contain an active ribonucleotide reductase consisting of the R1-p53R2 complex, produce dTTP *de novo* and maintain a small dTTP pool that is slowly turning over in the absence of extracellular deoxynucleosides.

**Silencing of TK2 in Quiescent Human Fibroblasts**—The above experiments with C63 fibroblasts showed that during quiescence these cells contain interlocking enzyme activities that together influence the pool size of dTTP. Our interest was to investigate the role of TK2 in this network unraveling the contributions of the three enzymes, TK2, ribonucleotide reductase, and TP. We used RNA interference to silence TK2 and the specific inhibitors HU and 5BU to turn off ribonucleotide reductase and TP.

To silence TK2 we transfected cultures of C63 fibroblasts close to confluence with a pool of siRNAs targeted against TK2 mRNA or a pool of non-targeting siRNAs (negative control). We exposed the cells to the siRNAs for a total of 10 days. In the three experiments performed according to this protocol the quiescent state of the transfected fibroblasts was demonstrated by the lack of measurable TK1 activity in cell extracts (not shown) and by a low percentage of S phase cells, 1% in the controls and 3.5% in the TK2-silenced cells. In the cells transfected with the negative control siRNAs real-time PCR analysis detected an increased expression of TK2 mRNA (Fig. 8A) in agreement with our previous observation that TK2 activity increases when fibroblasts pass from the growing to the quiescent state (24). In the cultures transfected with the anti-TK2 siRNA the residual level of TK2 mRNA was even lower (50%) than in cycling fibroblasts (Fig. 8A). In the silenced cells the mean specific enzyme activity was 8% of the control value. In the transfected cultures we measured also the mRNAs of the three subunits of ribonucleotide reductase and p53 mRNA and compared their levels with those of corresponding mRNAs derived from cycling cultures. The changes from cycling to quiescent cells for the mRNAs of these proteins were similar to those found by immunoblotting (Fig. 6), with R2 disappearing,

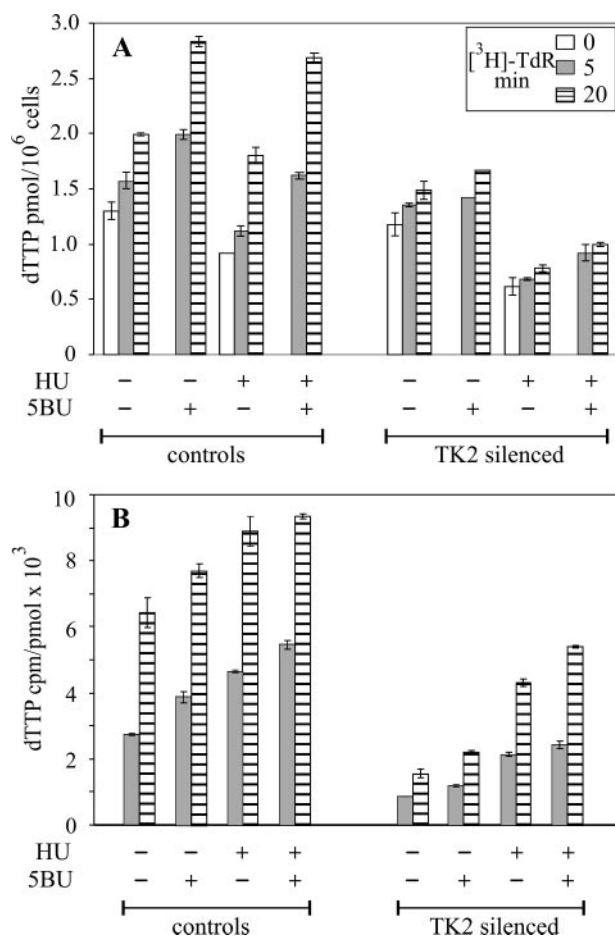




**FIGURE 8. Expression of the ribonucleotide reductase subunits, p53 and TK2, in control and TK2-silenced quiescent human fibroblasts.** *A*, we measured by real-time PCR the levels of the mRNAs for ribonucleotide reductase subunits R1, R2, and p53R2, for p53 and TK2 in cycling and quiescent skin fibroblasts. We extracted total RNA from proliferating control fibroblasts (black bars) and from quiescent fibroblasts transfected with negative control (shaded bars) or anti-TK2 (striped bars) siRNAs. We retrotranscribed the RNAs and analyzed aliquots of the cDNAs as indicated under "Experimental Procedures." The level of each mRNA is expressed as -fold increase relative to that measured in cycling cells. *B*, we examined by immunoblotting the abundance of p53R2 in extracts of control cycling (*a*) and quiescent (*b*) fibroblasts and in extracts of quiescent fibroblasts transfected with negative control (*c*) and anti-TK2 (*d*) siRNAs.  $\beta$ -Actin was the loading control. Bars show  $\pm$  S.D.

R1 decreasing, and p53 and p53R2 increasing. The quiescent TK2-silenced and non-silenced cells behaved similarly except for p53R2 mRNA that was 2-fold higher in the TK2-silenced cells. Also the immunoblots showed a small increase of p53R2 in the TK2-silenced cells (Fig. 8*B*). However, more experiments are needed to clarify the question whether p53R2 is indeed up-regulated in cells lacking a normal level of TK2.

To examine the relative roles of TK2 and ribonucleotide reductase in the synthesis of dTTP in quiescent fibroblasts and the influence of TP on TK2 activity, we used the following protocol. Cultures of skin fibroblasts were exposed for 10 days to either anti-TK2 siRNAs or negative control siRNAs. On the day of the experiment both groups of cultures received fresh medium with 0.1% dialyzed serum for 30 min. Then sets of each group received either no inhibitor (control) or 3 mM HU or 100  $\mu$ M 5BU or both inhibitors. After 30 or 45 min of treatment 25 nM [ $^3$ H]TdR was added to control and inhibitor-treated cultures that were incubated further for 20 or 5 min. By this procedure all the inhibitor-treated plates were exposed to the drugs for the same length of time, *i.e.* 50 min altogether. One set of HU-only and one of no-inhibitor plates did not receive [ $^3$ H]TdR to determine their dTTP pools in the absence of extracellular nucleoside. The results are summarized in Fig. 9 that com-



**FIGURE 9. Influence of extracellular thymidine on the decrease of the dTTP pool caused by TK2 silencing and inhibition of ribonucleotide reductase and TP in quiescent skin fibroblasts.** We transfected for 10 days quiescent cultures of C63 skin fibroblasts with anti-TK2 or negative control siRNAs. We then replaced the cultures with fresh medium with 0.1% dialyzed serum and added to different sets of cultures 3 mM HU, 100  $\mu$ M 5BU, both inhibitors, or no inhibitor. During the last 20 (striped bars) or 5 (shaded bars) min of treatment we incubated the cultures with 25 nM [ $^3$ H]TdR. Empty bars represent incubations without TdR. *A*, dTTP pool sizes; *B*, specific radioactivity of the dTTP pools. controls, cells transfected with negative control siRNAs; TK2 silenced, cells transfected with anti-TK2 siRNAs. Bars show the range of determinations.

pare dTTP pool sizes (Fig. 9*A*) and specific radioactivities (Fig. 9*B*) under the different conditions.

A clear difference was evident between TK2-silenced cells and non-silenced fibroblasts. Each pairwise comparison reveals that in the TK2-silenced cells dTTP pool size was lower than in the non-silenced controls (Fig. 9*A*) and that the incorporation of exogenous [ $^3$ H]TdR into dTTP was reduced (Fig. 9*B*). In the absence of added TdR or inhibitors the dTTP pool of TK2-silenced cells was only 10% reduced relative to the control (Fig. 9*A*). However, from the increase in pool size caused by 20-min incubation with 25 nM [ $^3$ H]TdR we calculate that the dTTP pool had increased at a rate of 0.03 pmol/min in non-silenced cells and of 0.016 in the TK2-silenced fibroblasts. When TP was inhibited by 5BU the rate increased to 0.08 pmol/min in the non-silenced cells and to 0.025 pmol/min in the silenced cells. These results indicate that, although strongly reduced, TK2 still retained some activity in the silenced fibroblasts. Inhibition of ribonucleotide reduction by HU caused a larger decrease of the



## TK2 and dTTP Pool Regulation

paring cell lines from patients and healthy individuals. Only in non-dividing cells was TK2 activity limiting for the maintenance of mt dTTP. In cycling cells the activities of the R1-R2 ribonucleotide reductase together with TK1 prevailed so that TK2 down-regulation did not affect the size of mt dTTP pool or the incorporation of exogenous thymidine into the pool (Fig. 1). Surprisingly, even when TK2 was the only thymidine kinase, was its contribution to mt dTTP dispensable in cycling cells. In Ost TK1<sup>-</sup> cells [<sup>3</sup>H]TdR phosphorylation was almost abolished by TK2 down-regulation, yet the size of the mt dTTP pool was unaffected (Fig. 2). These results agree with previous observations that mt dTTP in proliferating cells is provided by cytosolic *de novo* synthesis (5, 6).

The situation changes during quiescence (Fig. 10). Very little dTTP is required for DNA synthesis, and the size of the dTTP pool accordingly shrinks to a few percent of the size in S-phase cells (7). Now TK1 is absent but the activity of TK2 increases relative to cycling fibroblasts (24) (Fig. 8B). Ribonucleotide reduction continues at a greatly reduced rate (13), catalyzed by R1-p53R2 in place of R1-R2 (Figs. 6 and 7). The catabolic 5'-deoxynucleotidases are not cell cycle-regulated, and their activities remain unchanged (7). The availability of extracellular thymidine for salvage by TK2 is unknown. The concentration of TdR in the blood is <50 nM, but the presence in the genome of two separate genes for thymidine kinase suggests that their substrate is available and is used. Considering the low *in situ* activity of TK2 compared with that of TK1 in cycling cells (5, 24), it was questionable if low thymidine concentrations would be sufficient to detect phosphorylation by TK2 alone.

We now show that in quiescent fibroblasts even 10 nM thymidine sufficed to expand the dTTP pool slightly by TK2-mediated salvage (Fig. 4). The degree of phosphorylation depended on the concentration of TdR (Fig. 4) and was more evident from the progressive increase of dTTP specific radioactivity than from the changes in pool size. One factor that contributed to keep dTTP pool size in check was the catabolic activity of TP (Fig. 5). Thus TK2 action is balanced in the cytosol by TP that degrades the substrate preventing its intramitochondrial phosphorylation (see Fig. 10). In earlier work we showed that mdN, the mt deoxynucleotidase (26), affects the phosphorylation of thymidine by degrading the product of the TK2 reaction. In the experiments reported here the activity of deoxynucleotidases in quiescent fibroblasts appears from the turnover of the dTTP pool observed during the chase after a pulse with radioactive TdR (Fig. 7B). The continuous decline of dTTP-specific radioactivity without a decrease in pool size in the absence of TdR in the medium was due to dephosphorylation of dTMP by cdN and mdN coupled with *de novo* synthesis of new non-labeled dTMP (see Fig. 10). Thus a series of different factors influences the formation of dTMP by TK2 in addition to the actual activity of the enzyme: the influx of thymidine from the extracellular environment, the competing activity of TP, and the degradation of dTMP by the 5'-deoxynucleotidases.

Which of the two pathways, *de novo* synthesis or salvage, influences the size of the dTTP pool most in quiescent fibroblasts? To address this question we inhibited, separately or in combination, TK2 by siRNA silencing and ribonucleotide

reductase and TP by chemical inhibitors (Fig. 9). None of the methods used was 100% effective, but the observed changes in the size of the dTTP pool may model situations occurring *in vivo* in cell types where gene expression of one or other relevant enzyme is modified. The pool size of dTTP depended on the availability of extracellular thymidine. When TdR was not added to the medium, silencing of TK2 caused a smaller decrease of the dTTP pool than inhibition of ribonucleotide reductase by HU. Interestingly, the combined inhibition of both enzymes had a more than additive effect (Fig. 9A), suggesting a functional interaction with TK2 counteracting the activity of deoxynucleotidases. When TdR was added to the medium, TK2-mediated salvage compensated for the inhibition of ribonucleotide reductase, suggesting that, when TdR is available, TK2 may substitute for impaired *de novo* synthesis of dTTP. In fact, the role of TK2 became more and more influential as the concentration of extracellular TdR increased, as shown by the progressively higher specific radioactivity of dTTP in cells incubated with increasing concentrations of radioactive TdR (Fig. 4B). These results concern total cellular dTTP in quiescent fibroblasts, but we obtained similar results when we separated the mt dTTP pool, substantiating previous data (7, 18).

The present experiments demonstrate how the dTTP pool of quiescent fibroblasts is modulated by the interconnected activities of the five enzymes indicated in the scheme in Fig. 10 and highlight the important role played by the availability of thymidine. At physiological low concentrations, thymidine contributes to the maintenance of the dTTP pool through salvage by TK2. On the other hand, salvage of excess circulating thymidine caused by TP deficiency disrupts the regulation of the dTTP pool and causes mtDNA abnormalities (18, 19).

In the skin fibroblasts used here both TK2 and TP were relatively highly expressed. The balance between the various components of the network in all probability shows considerable variations between different tissues. Thus skeletal muscle contains much less TK2 than fibroblasts (39, 40) and no detectable TP (41). To understand the metabolic mechanism underlying the tissue-specific phenotypes caused by genetic loss of enzymes in the network it is necessary to consider and investigate the whole network. Given the non-strict correlation between enzyme activities and mRNA levels, expression profiling in different organs may give only approximate information that needs to be corroborated by measurements of enzyme activities (40). The development of specific enzymatic assays that permit the discrimination between enzymes with overlapping substrate specificities makes this approach amenable (24, 42). A more challenging task is the evaluation of TdR in the target tissues.

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