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Mitochondrial Deoxyribonucleotides, Pool Sizes, Synthesis, and Regulation*

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We quantify cytosolic and mitochondrial deoxyribonucleoside triphosphates (dNTPs) from four established cell lines using a recently described method for the separation of cytosolic and mitochondrial (mt) dNTPs from as little as 10 million cells in culture (Pontarin, G., Gallinaro, L., Ferraro, P., Reichard, P., and Bianchi, V. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 12159–12164). In cycling cells the concentrations of the phosphates of thymidine, deoxycytidine, and deoxyadenosine (combining mono-, di-, and triphosphates in each case) did not differ significantly between mitochondria and cytosol, whereas deoxyguanosine phosphates were concentrated to mitochondria. We study the source and regulation of the mt dTTP pool as an example of mt dNTPs. We suggest two pathways as sources for mt dTTP: (i) import from the cytosol of thymidine diphosphate by a deoxynucleotide transporter, predominantly in cells involved in DNA replication with an active synthesis of deoxynucleotides and (ii) import of thymidine followed by phosphorylation by the mt thymidine kinase, predominantly in resting cells. Here we demonstrate that the second pathway is regulated by a mt 5′-deoxyribonucleotidase (mdN). We modify the *in situ* activity of mdN and measure the transfer of radioactivity from [^{3}H]thymidine to mt thymidine phosphates. In cycling cells lacking the cytosolic thymidine kinase, a 30-fold overproduction of mdN decreases the specific radioactivity of mt dTTP to 25%, and an 80% decrease of mdN by RNA interference increases the specific radioactivity 2-fold. These results suggest that mdN modulates the synthesis of mt dTTP by counteracting in a substrate cycle the phosphorylation of thymidine by the mt thymidine kinase.

The synthesis of DNA requires a supply of deoxyribonucleotides. In eukaryotic cells the bulk of DNA is located in the nucleus and replicated once during the S phase, which in cycling cultured cells occupies approximately one-third of the cell cycle. Terminally differentiated cells or resting cells in culture only rarely or never replicate their DNA. In cycling cells the synthesis of deoxyribonucleotides from ribonucleotides occurs mostly during S phase, simultaneously with nuclear DNA replication (1). In resting cells the *de novo* synthesis of deoxyribonucleotides is shut down. In addition to nuclear DNA, eukaryotic cells also contain mitochondrial (mt)[1] DNA that represents ~5% of the total cellular DNA (2). For its replication mt DNA requires only a correspondingly small fraction of the total deoxyribonucleoside triphosphates (dNTPs) of the cell. Replication is, however, not coordinated with that of nuclear DNA and also requires dNTPs outside the S phase (3). This requirement may become particularly critical in resting cells.

It is generally believed that special dNTP pools in mitochondria not in equilibrium with cytosolic dNTP pools serve the synthesis of mt DNA. Seminal reports from Clayton's laboratory (4, 5) demonstrated early that radioactive thymidine was preferentially incorporated into mt DNA in cultured cells that could phosphorylate the nucleoside only inside mitochondria because they lacked a cytosolic thymidine kinase (TK1$^-$). This result suggests strongly that thymidine phosphates formed by the mt thymidine kinase (TK2) (6, 7) are at least partially sequestered in mitochondria to serve preferentially for mt DNA synthesis. Subsequent work by others argued for a strict separation of mt and cytosolic pools. In HeLa cells whose *de novo* synthesis of dTMP was blocked by amethopterin, only the size of the cytosolic dTTP pool decreased, whereas mt dTTP actually increased, suggesting a complete separation of the metabolism of the two pools (8). We could, however, not confirm this result and found instead that amethopterin decreases the size of both pools, suggesting a communication between them (9).

Such a communication was further emphasized by our kinetic isotope chase experiments with [^{3}H]thymidine in TK1$^+$ and TK1$^-$ cultured cells (9) that demonstrated a rapid influx of *de novo* synthesized thymidine phosphates into mitochondria from the cytosol and an equally rapid movement in the opposite direction of nucleotides synthesized inside mitochondria by phosphorylation of thymidine. Our results suggested that although mitochondrial and cytosolic dNTPs do form separate kinetic pools that can be synthesized independently, they are rapidly interconverted. To be able to do these experiments, we devised a novel method for the quantitative separation of mt and cytosolic dNTP pools that made possible separate analyses of the two pools (9). With this method that was validated in several independent ways, we can measure the size of mt dNTP pools from as little as 10^7 cultured cells.

mt dNTP pools are fed by two separate potential pathways that transport deoxynucleosides (or deoxynucleotides) from the cytosol into mitochondria, as exemplified for dTTP in Fig. 1: (i) a deoxynucleotide (dTDP) is introduced by a deoxynucleotide transporter (10, 11) or (ii) a deoxynucleoside (thymidine) is introduced, probably by equilibrative nucleoside transporter 1

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[1] The abbreviations used are: mt, mitochondrial; dNTP, deoxyribonucleoside triphosphate; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase; cdN, cytosolic 5′-deoxyribonucleotidase (formerly dNT-1); mdN, mitochondrial 5′-deoxyribonucleotidase (formerly dNT-2); dTDP, thymidine diphosphate; RNAi, RNA interference.

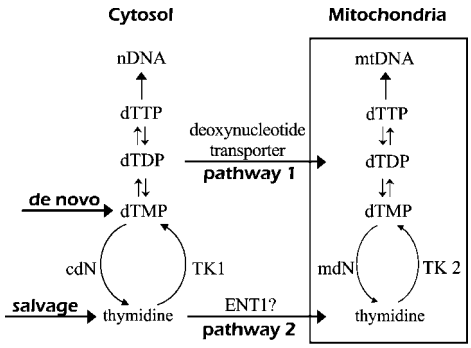


FIG. 1. **Hypothetical scheme for the interrelation of cytosolic and mitochondrial dTTP pools.** The cell requires dNTPs for the synthesis of both nuclear and mitochondrial DNA. The two processes occur independently of each other and use separate pools localized to separate compartments in the cell. Cytosolic dNTPs, exemplified in the figure by dTTP, arise *de novo* by reduction of ribonucleotides or by salvage of deoxyribonucleosides. Mitochondrial dTTP arises by direct import from the cytosol of dTDP by a deoxynucleotide carrier or by import of thymidine, possibly via the nucleoside carrier equilibrative nucleoside transporter 1 (*ENT1*), followed by intramitochondrial phosphorylation by TK2. Two separate substrate cycles, one in the cytosol and the other in mitochondria, regulate the level of dTMP. In each case a 5′-deoxyribonucleotidase counteracts the activity of a kinase. In the cytosol, the two enzymes are TK1 and cdN; in mitochondria the two enzymes are TK2 and mdN.

(12), and phosphorylated by a mt kinase (TK2). The first pathway relies mainly on the *de novo* synthesis of deoxyribonucleotides in cells replicating their nuclear DNA. The second pathway is not related to nuclear DNA replication but relies on the availability of thymidine that can be salvaged from the extracellular milieu. We hypothesized recently from experiments with cycling cells in culture that in dividing cells the vast majority of mt dNTPs enters mitochondria via the first pathway but that in nondividing cells with no *de novo* synthesis of deoxynucleotides the second pathway becomes important (9). Genetic mitochondrial diseases affecting enzymes of either pathway attest to the importance of both and support our hypothesis: (i) the deoxynucleotide carrier is involved in Amish microcephaly (13), demonstrating its importance for prenatal brain growth, and (ii) TK2 is involved in mitochondrial DNA depletion myopathy, a disease that appears in early childhood (14).

How are the two pathways regulated? In both instances the concentration of the cytosolic metabolites required for transport into mitochondria must be an important factor. For the first pathway the concentration of dTDP is tightly linked to the cell cycle, depending largely on the activity and allosteric regulation of the enzyme ribonucleotide reductase (15). In a secondary manner the cytosolic substrate cycle (Fig. 1) between TK1 and a cytosolic 5′-deoxyribonucleotidase (cdN (16)) is also a contributing factor (17). The availability of thymidine for the second pathway is an unknown factor. It is, however, not linked to the cell cycle but to thymidine concentration in the bloodstream. Interestingly, a large increase in thymidine concentration in blood because of the genetic loss of the enzyme thymidine phosphorylase is connected with increased mutations in mt DNA leading to mitochondrial neurogastrointestinal encephalomyopathy (18, 19). It is a fair assumption that the mt disease depends on a high concentration of intra-mt thymidine, indicating the importance of the second pathway for mt dNTP pools.

The present work concerns the regulation of the second pathway by mdN (formerly dNT2 (16)), an intra-mt 5′-deoxyribonucleotidase with a pronounced specificity for the dephosphorylation of dTMP and dUMP (20). We have suggested (Fig. 1) that mdN together with TK2 forms a substrate cycle that regulates the concentration of dTMP (and dUMP) inside mitochondria. We proposed that mdN counteracts the activity of TK2 by dephosphorylating dTMP and thus inside the mitochondria is a guardian against the overproduction of thymidine phosphates. Here we test this hypothesis in cycling cells in culture that either overproduce mdN or have lost a large part of the activity of the enzyme by RNA interference. We analyze the effects of changed mdN activity on cell growth, dNTP pool size, and DNA synthesis and compare in isotope experiments with labeled thymidine the ability of genetically manipulated and control cells to transform the nucleoside to thymidine phosphates. Our results support a role for mdN in the regulation of the mt dTTP pool.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Growth—The established human tumor lines OSTTK1$^-$ and HOSTK1$^+$ and the mouse fibroblast lines 3T3TK1$^-$ and 3T3TK1$^+$ were as in earlier experiments (9). From OSTTK1$^-$ we prepared a cell line that could be induced by ponasterone to overproduce mdN by procedures identical to those described for a similar inducible line of 293 cells (21). After recloning we obtained clone OSTTK1$^-$ 20.7, which gave highly reproducible overproduction of mdN after induction with graded amounts of ponasterone. This clone was the one used here for all experiments with overproducing OSTTK1$^-$ cells.

The original OSTTK1$^-$ line was used to prepare a stable cell line with a specific mdN gene silencing response. We chose two separate 19-mers present in the coding sequence of human mdN to design two 64-mer synthetic oligonucleotides (Sigma) as described by Brummelkamp *et al.* (22) and cloned them into the pSuper plasmid (22) for the construction of two targeting vectors. We first tested both plasmids by transient transfection in 293 cells for their ability to interfere with mdN mRNA as determined by real time PCR analysis. The small interfering RNA sequence (R6, GATGTTCAAGTACTGTCCC) gave the best interference and was chosen for construction of a stably transfected cell line. We cloned a zeocin cassette (pSV40/Zeo; Invitrogen) into pSuper and pSuperR6 to give plasmids pSuperZeo and pSuperZeoR6, respectively. We used the two plasmids to prepare stable clones by transfection of OSTTK1$^-$ cells with Metafectene (Biontex, Munich, Germany). After a 48-h recovery period, we started selection with 75 μg/ml zeocin during a 2–3-week period replacing the media every 3 days. We picked 30 individual clones in each group and tested them by real time PCR. In 40% of the clones mdN mRNA was in repeated analyses decreased to between 15 and 30% of the controls. We used four clones from each group for our experiments.

All of the cells were grown and manipulated as described earlier (9, 17, 21). They were periodically checked for mycoplasma contamination by a PCR-based procedure (Minerva Biolabs, Berlin, Germany). The amount of cells was determined with a Coulter counter. The distribution of cells in the cell cycle was analyzed by a computerized fluorescence-activated cell sorter from Becton Dickinson (Milano, Italy). All of the quantitative values for pool sizes, protein, radioactive incorporation, and enzyme activities are given per million cells to make possible comparisons between experiments.

RNA Extraction, Reverse Transcription PCR, and Real Time PCR—We extracted total RNA from 1.5×10^7 cells with TRIzol reagent (Invitrogen) and tested its integrity by electrophoresis on 1% formaldehyde-agarose gels. We treated the RNA for 30 min with DNase I (Dna-free™; Ambion). After inactivation of DNase I, we transcribed the RNA (2.5 μg in 20 μl) with Super-Script II reverse transcriptase (Invitrogen) and oligo(dT)$_{15}$V primer (V = G + A + C). We diluted the resulting cDNA with water to 0.25 ml and used it as a template for the real time PCR analysis (qPCR Core Kit for SYBR Green; Eurogentec, Belgium) to determine the expression of mdN mRNA in each original clone. We amplified mdN cDNA with primers d2RT5 (GAGGAGTTGAGGCCACTGTTCA) and d2RT6 (ATAAGGGGTGCTGTGGCTGAG). We excluded contamination of RNA by genomic DNA by PCR analysis before reverse transcription. As a standard housekeeping gene with an expression level similar to mdN, we chose hydroxymethyl-bilane synthase, amplified with the primers given in Ref. 23.

Real time PCR assays were performed on cDNA samples in 96-well optical plates with a GeneAMP 5700 sequence detection system (Applied Biosystem) using the following parameters: 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 60 s. The amplification reactions were performed in 25 μl containing 0.2 μM primers and 0.2 mM dNTPs in PCR buffer with 2 mM $MgCl_2$ and 0.6 units of Hot Gold Star enzyme (Eurogentec), SYBR Green at 1:66000 dilution, and 1–8 μl cDNA. The PCR data were captured using Sequence Detector Software. Each assay was done with three different amounts of cDNA in triplicate

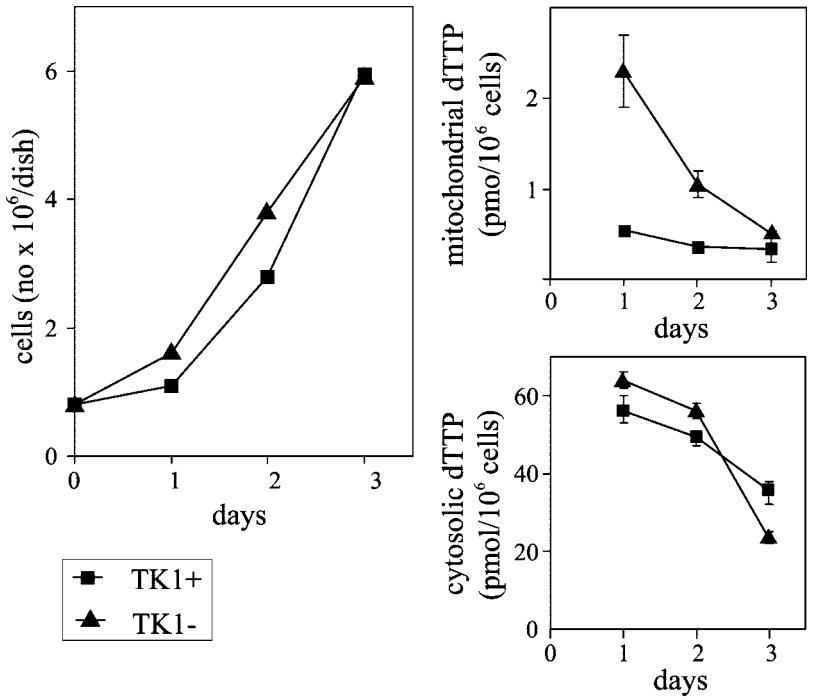


FIG. 2. **Mitochondrial and cytosolic dNTP pools during the growth of OSTTK1$^-$and HOSTK1$^+$cells.** The cells were seeded on 10-cm dishes. Between 1 and 3 days after seeding, we determined the number of cells with a Coulter counter, separated mt and cytosolic nucleotide pools, and determined on duplicate samples the size of the dTTP pools. The *bars* indicate the range of the determinations.

to obtain standard curves to ensure a similar efficiency of amplification. Hydroxymethyl-bilane synthase and mdN gave equal PCR efficiency. The data were analyzed with the comparative C_t method where the amount of target is normalized to the endogenous reference (24).

Isotope Experiments with [^{3}H]Thymidine, Separation, and Analyses of dNTP Pools—The procedure for the isotope experiments, separation of cytosolic and mt dTTP pools, and determination of the radioactivity in thymidine phosphates and specific radioactivity of dTTP were detailed earlier (9, 21). Briefly, we incubated cells for the indicated time periods with labeled thymidine and, after washing with ice-cold phosphate-buffered saline, separated cytosolic and mt dTTP by differential centrifugation, combining nuclear and mt fractions for mt dTTP. We extracted thymidine phosphates with 60% methanol and analyzed them by ion exchange chromatography to separate thymidine from thymidine phosphates (dTTP + dTDP + dTMP). The total radioactivity in each compartment was determined.

Analytical Methods—We determined the activities of mdN and TK2 by earlier described assays (6, 7, 20, 21). We used the specific inhibitors DBP-T (25) and bromovinyldeoxyuridine (26), respectively, to distinguish the activities of the mt enzymes from similar cytosolic enzymes (cdN and TK1). Specific enzyme activity is defined as units/mg of protein. One unit is 1 μmol of product formed during 1 min.

We determined the size of dNTP pools and the specific radioactivity of the dTTP pool in isotope experiments by a DNA-polymerase assay described in detail earlier (17, 27). Briefly, we calculated the unknown amount of the dNTP from the incorporation of an excess of [α-^{32}P]dATP into a synthetic polymer by DNA polymerase using an appropriate standard curve with the dNTP. We calculated the specific radioactivity of dTTP from the incorporation of ^{3}H and ^{32}P into DNA using a standard curve with [^{3}H]dTTP and [α-^{32}P]dATP.

We calculated the rate of DNA synthesis, expressed as pmol dTMP/min, from the cpm incorporated from labeled thymidine into DNA during a given time period, divided by the specific radioactivity of the cytosolic dTTP during that period. The statistical significance of differences between controls and RNAi-inhibited cells was assessed by Mann-Whitney *U* nonparametric tests because of the low number of samples.

RESULTS

Size of mt dNTP Pools—Whereas the size of cytosolic dNTP pools of cultured cells has been the subject of many investigations, reliable systematic studies of mt dNTP pools are not available. During previous isotope flow experiments (9), we measured the sizes of mt dTTP pools in different cell lines employing a new method for their separation from cytosolic dTTP but at that time did not report the values. We now applied this methodology also to the other dNTP pools and here report analyses of all four mt pools from four earlier described cell lines (HOSTK1$^+$, OSTK1$^-$, 3T3TK1$^+$, and 3T3TK1$^-$).

The size of cytosolic dNTP pools of cultured cells changes with the growth phase (28–30). We now show that also the size of the mt dTTP pool varies with the growth phase (Fig. 2). Both the cytosolic and the mt dTTP pools of OSTTK1$^-$ and HOSTK1$^+$ cells were largest during early growth, when more cells are in S phase, than at the end of the growth curve. For mitochondria, the difference was 2-fold in TK1$^+$ cells and 5-fold in TK1$^-$ cells, in the cytosol the differences were smaller. During early growth 3.5% of the total dTTP was in mitochondria in TK1$^-$ cells, the corresponding value for TK1$^+$ cells was 1%.

Table I reports the pool sizes of all four mt and cytosolic dNTPs from four different cell lines determined in exponentially growing cultures with ~35% of the cells in S phase. For OSTTK1$^-$ and HOSTK1$^+$ cells, the size of the dTTP pool was determined on four separately grown cultures. Table I gives the means of the four measurements with standard deviations to demonstrate the reproducibility of the results. The dTTP pools of the 3T3 cells are the means from two individual cell cultures; all other values are from duplicate analyses of single cultures. In the last column of Table I we give the calculated averages for each dNTP with standard deviations.

Among cytosolic dNTPs dGTP was by far the smallest pool, and also the dATP pool was smaller than the two pyrimidine pools (1, 31). This is in accordance with earlier reports. Also among mt dNTPs the dGTP pool was smallest, but the asymmetry between the four pools was not pronounced, with dTTP, dATP, and dCTP having similar pool sizes. Compared with the large variations of the dTTP pool during cell growth (Fig. 2) variations for each mt dNTP were small between cell lines. Table II shows the distribution between mitochondria and cytosol for each dNTP. The average values from the four cell lines (last column in Table II) show that the mt pools amount to between 3 and 7% of the corresponding cytosolic pools. In this comparison purines, and in particular dGTP, are more concentrated in mitochondria than pyrimidines. Nevertheless also in mitochondria dGTP is the smallest pool in absolute terms (Table I). We compare here only the size of the triphosphate

TABLE I
Size of mt and cytosolic dNTP pools of four cell lines

We separated mt and cytosolic dNTP pools from individual cell lines and analyzed their content of the four dNTPs. The last column gives the average values for the four lines with their standard deviations. The values for the dTTP pools of HOSTK1$^+$ and OSTTK1$^-$ cells are the average values from determinations on four separately grown cultures with their standard deviations.

	HOS TK1$^+$	OST TK1$^-$	3T3TK1$^+$	3T3TK1$^-$	Averages
	pmol/10^6 cells	*pmol/10^6 cells*	*pmol/10^6 cells*	*pmol/10^6 cells*	*pmol/10^6 cells*
Mitochondria					
dTTP	0.68 ± 0.26	1.6 ± 0.22	0.52	0.70	0.88 ± 0.49
dATP	0.45	1.2	0.45	0.88	0.75 ± 0.43
dCTP	0.74	0.89	0.56	0.81	0.75 ± 0.14
dGTP	0.27	0.56	0.39	0.37	0.40 ± 0.12
Cytosol					
dTTP	43 ± 9	37 ± 7	19	29	32 ± 10
dATP	11	30	11	18	17.5 ± 9
dCTP	19	29	20	42	27.5 ± 11
dGTP	5.8	6.2	5.5	6.2	5.9 ± 0.3

TABLE II
Ratios between mt and cytosolic dNTPs of the four cell lines

The ratios were calculated from the values for each dNTP in Table I. The last column shows the average values for individual dNTPs with standard deviations. The values are given as *mitochondrial dNTP/cytosolic dNTP* × 10^2.

	HOS TK1$^+$	OST TK1$^-$	3T3 TK1$^+$	3T3 TK1$^-$	Averages
dTTP	1.6	4.3	2.7	2.4	2.8 ± 1.1
dATP	4.1	4.0	4.1	4.9	4.3 ± 0.4
dCTP	3.9	3.1	2.8	1.9	2.9 ± 0.8
dGTP	4.8	9.0	7.1	6.0	6.7 ± 1.8

pools, which is somewhat misleading because the level of nucleoside phosphorylation differs between the cytosol and mitochondria (see "Discussion").

Effects of Overproduction of mdN—We had found earlier (21) that overproduction of mdN in cells containing an active cytosolic TK1 did not measurably affect cytosolic dTTP pools. Here we focus our experiments on mt dTTP by changing the experimental protocol in two ways: (i) We used cultured tumor cells devoid of the cytosolic TK1 (OSTTK1$^-$ cells) that only phosphorylated [^{3}H]thymidine inside mitochondria by TK2; any formed [^{3}H]dTMP was therefore immediately available for dephosphorylation by mdN in the postulated intra-mt substrate cycle between TK2 and mdN (Fig. 1). (ii) We studied the effects of mdN overproduction on the separated mt and cytosolic dTTP pools.

In preliminary experiments we treated inducible OSTTK1$^-$ cells with 1–4 μM ponasterone for up to 72 h and determined the effects on cell growth, mdN activity, dNTP pool sizes, and incorporation of radioactive thymidine (data not shown). At high concentrations the inducer had a profound effect on all parameters giving after 4 days a 100-fold increase in mdN activity and a varying decrease in the size of all four dNTP pools. The most sensitive parameter appeared to be the incorporation of [^{3}H]thymidine that decreased already at the earliest time point in cells treated with as little as 1 μM ponasterone. We therefore conducted three experiments with lower concentrations of ponasterone and short induction time. First we show the data from the most detailed experiment. We treated cells for 24 h with 0.8, 1.5, or 2.5 μM ponasterone for 24 h before pulsing the cultures with [^{3}H]thymidine and investigated the effects of mdN overproduction on cell growth, the size of dNTP pools, and the utilization of the radioactive thymidine for dTTP and DNA synthesis.

Induction with increasing concentrations of ponasterone gave a linear increase in the specific enzyme activity (units/mg of protein) of mdN, starting from a value of ~4 × 10^{-3} in the control to 0.12 at the highest concentration of the inducer (Fig. 3). Cell growth was not inhibited, and after 24 h of induction ~35% of the cells were in the S phase at all inducer concentrations (Fig. 3). Also the sizes of the four dNTP pools were

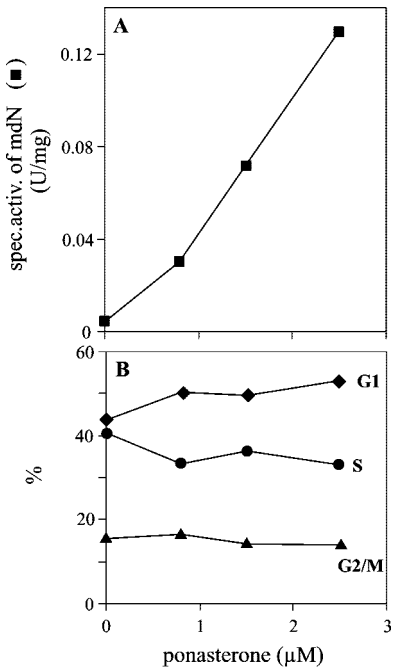


FIG. 3. **Specific activity of mdN after induction of OSTTK1$^-$ cells with different concentrations of ponasterone.** The cells were treated with the indicated concentration of the inducer for 24 h. *A*, specific activity (*spec.activ.*) of mdN (units·mg protein^{-1}) in cellular extracts. *B*, distribution of cells in the three phases of the cell cycle determined by flow cytometry.

unaffected (data not shown). We found, however, a clear inhibition of the phosphorylation of [^{3}H]thymidine. The total radioactivity present in thymidine phosphates was decreased in a dose-dependent manner (Fig. 4), both in mitochondria and in the cytosol. At the highest concentration of ponasterone the radioactivity had decreased to ~50% in mitochondria and to 40% in the cytosol. The radioactivity was present in the sum of dTMP + dTDP + dTTP and thus measured the total phosphorylation of thymidine. We found earlier that dTTP represents ~80% of the radioactivity in the cytosol, whereas in mitochon-

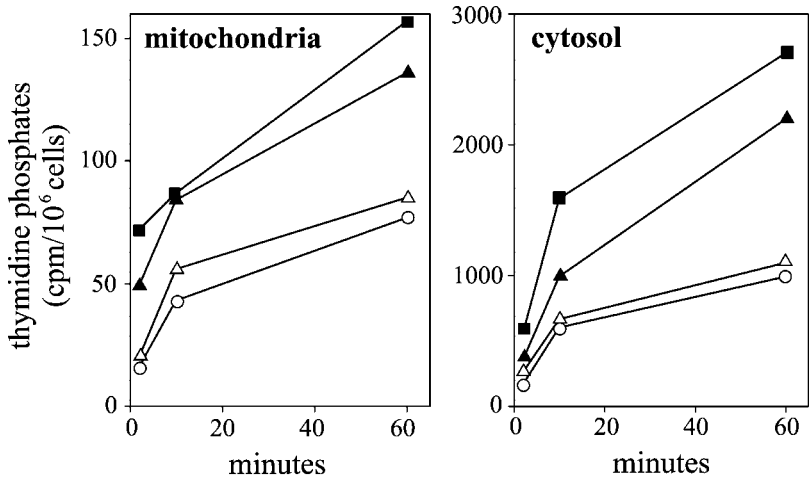


FIG. 4. **Total radioactivity in thymidine phosphates after induction of OSTTK1⁻cells with different concentrations of ponasterone.** After 24 h of induction with ponasterone (■, control; ▲, 0.8 μM; △ 1.5 μM; ○, 2.5 μM), we incubated cells with 1 μM [^{3}H]thymidine (20,000 cpm/pmol) for the indicated times, separated mitochondrial from cytosolic thymidine phosphates (dTMP + dTDP + dTTP), and determined in each case the total incorporated radioactivity.

dria ~50% is in dTDP, and dTTP accounts for only ~25% (9). As earlier observed in TK1⁻ cells (9), the total radioactivity of thymidine phosphates in the cytosol far exceeded the radioactivity in the mt pools at all time points.

We found no significant change in the total radioactivity of the cellular thymidine pool (data not shown), indicating that ponasterone treatment did not decrease the availability of thymidine but affected a step between thymidine and thymidine phosphates. Cell extracts from ponasterone-treated and control cells had the same TK2 activity (data not shown), suggesting that the lower incorporation of radioactivity in the thymidine phosphate pool was not caused by a change in the rate of thymidine phosphorylation but by a subsequent loss of isotope from dTMP because of higher mdN activity in the induced cells.

We arrive at the same conclusion from measurements of the specific radioactivity of dTTP (Fig. 5). This parameter is a function of both the synthesis of radioactive dTTP (and therefore of dTMP) from [^{3}H] thymidine and the dilution of radioactivity by nonlabeled dTTP synthesized *de novo*. After 60 min of incubation of the cells treated with the highest inducer concentration, the specific radioactivity of both the mt and cytosolic dTTP pools was only 20–25% of that in the control, with cells treated with lower concentrations of inducer occupying intermediate positions (Fig. 5). These results suggest that ponasterone treatment had decreased the synthesis of dTTP from labeled thymidine, resulting in a larger dilution of the specific activity of dTTP by *de novo* synthesized nonlabeled dTTP.

Also labeling of DNA from [^{3}H]thymidine was decreased in a dose-dependent manner in ponasterone-treated cells (Fig. 6). However, this does not mean that the rate of DNA synthesis was decreased because the radioactivity was incorporated from dTTP and not from thymidine. In calculating the rate of DNA synthesis, we must therefore correct for the decreased specific activity of dTTP in the induced cells. When we divide the linear incorporation of [^{3}H]thymidine (between 10 and 60 min) with the average specific radioactivity of the cytosolic dTTP pool during the same period, we obtain the following rates (pmol dTMP·min^{-1}·10^6 cells^{-1}) at increasing ponasterone concentrations: 2.4 (control), 2.1, 2.1, and 2.5. Thus, ponasterone treatment did not significantly decrease the rate of DNA synthesis.

In two additional less extensive experiments, we further determined the effect of mdN overproduction on the phosphorylation of labeled thymidine in mitochondria. In one case, at lower ponasterone concentrations, mdN overproduction was smaller than in the above described experiment; in the other case, at higher concentrations, it was greater. In both cases the results fit well into those from the first experiment. Fig. 7 brings together data from the three experiments concerning

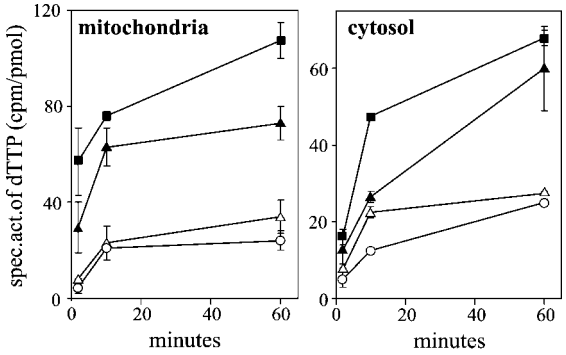


FIG. 5. **Specific radioactivity of the dTTP pool of OSTTK1⁻cells after induction with different concentrations of ponasterone.** The results are from the same experiment as in Fig. 4 but now show the effects of ponasterone (■, control; ▲, 0.8 μM; △ 1.5 μM; ○, 2.5 μM) on the specific radioactivities (duplicate values) of mt and cytosolic dTTP from each time point.

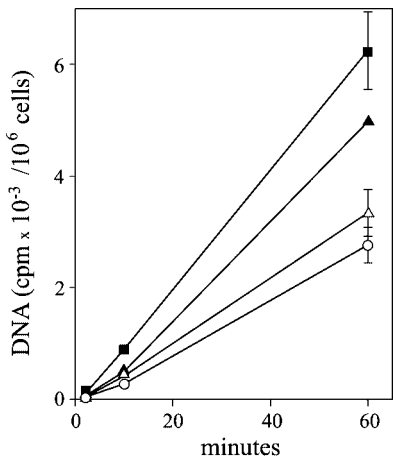


FIG. 6. **Incorporation of [^{3}H]thymidine into DNA after induction of OSTTK1⁻cells with different concentrations of ponasterone.** The results are from the same experiment as in Fig. 4 but now show the effects of ponasterone (■, control; ▲, 0.8 μM; △ 1.5 μM; ○, 2.5 μM) on the radioactivity incorporated from thymidine into DNA.

the specific radioactivity of dTTP in mitochondria and the cytosol after 60 min of incubation. In both cases there is an asymptotic decrease in the specific activity at increasing overproduction of mdN that levels off at ~20% of the control value.

Effects of Decreased mdN Activity on the Incorporation of [^{3}H]Thymidine into Thymidine Phosphates—We prepared stable lines of OSTTK1⁻ cells with decreased mdN activity by

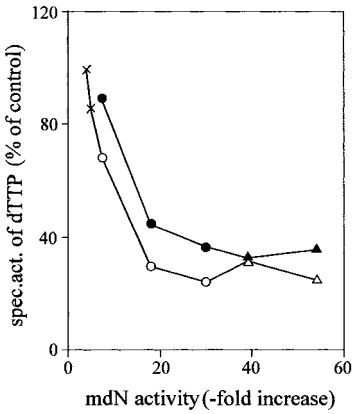


FIG. 7. **Decrease of the specific activity of dTTP in mitochondria and cytosol at increasing overproduction of mdN.** The figure combines the data from three independent experiments with OSTTK1$^-$ cultures after 24 h of induction with various concentrations of ponasterone. We report the specific activity of mt and cytosolic dTTP after 60 min of incubation with [^{3}H]thymidine at different degrees of mdN overproduction. Experiment 1 (×, mt dTTP) used 0.3 and 0.6 μM ponasterone; experiment 2 (○, mt dTTP; ●, cytosolic dTTP) used 0.8, 1.5, and 2.5 μM and is identical to the experiment in Fig. 5; experiment 3 (△, mt dTTP; ▲, cytosolic dTTP) used 1.0 and 2.0 μM ponasterone. The *abscissa* gives the increase in mdN activity over the noninduced controls. The *ordinate* shows the percentage of specific activity of dTTP relative to the noninduced control. In experiment 1, 100% corresponds to 130 for mt dTTP; in experiment 2, 100% is 105 for mt dTTP and 65 for cytosolic dTTP; and in experiment 3, 100% is 85 for mt dTTP and 57 for cytosolic dTTP.

transfection with a plasmid vector expressing a small interfering RNA targeted to mdN-mRNA as described under "Experimental Procedures." For the experiments described below, we used four independent clones containing between 15 and 28% of the mdN-mRNA of control cells as measured repeatedly by real time reverse transcription PCR. Clones with the empty vector served as controls. We found no significant effect of RNAi inhibition on cell growth or the size of dTTP pools (not shown). On the average 34% of the controls and 39% of the RNAi-inhibited cells were in the S phase at the time of the isotope incorporation experiments.

The incorporation into mt thymidine phosphates increased in the RNAi-inhibited cells, after both 10 and 60 min of incubation as shown in Fig. 8. The average values for mt pools, expressed as cpm·10^6 cells^{-1}, were at 10 min 299 ± 78 for the controls and 567 ± 141 after RNAi inhibition and at 60 min 371 ± 70 for controls and 707 ± 96 after RNAi inhibition. The statistical significance of the increase was demonstrated by Mann-Whitney *U* nonparametric tests ($p = 0.021$ at both time points). The corresponding cytosolic values were not significantly different.

In Fig. 9 we show the effect of RNAi treatment on the specific activity of the dTTP pools of mitochondria and the cytosol. In both instances RNAi treatment increased the values. For the mitochondrial pools the average increase was from 113 ± 27 to 282 ± 75 at 10 min and from 135 ± 36 to 238 ± 34 at 60 min. For the cytosolic pools the corresponding increases were from 33 ± 7 to 57 ± 12 and from 53 ± 17 to 85 ± 21. In all cases the increases were statistically highly significant ($p = 0.001$ for mitochondrial pools; $p = 0.003$ and 0.007 for cytosolic pools).

We also determined the effect of RNAi inhibition on the rate of DNA synthesis. Fig 10 gives for each clone the rate of dTTP incorporation into DNA calculated as described above by dividing the incorporation of [^{3}H]thymidine by the specific activity of the cytosolic dTTP pool. There was no significant difference between the average values for the two groups of clones (3.5 in controls *versus* 3.9 in RNAi-inhibited cells).

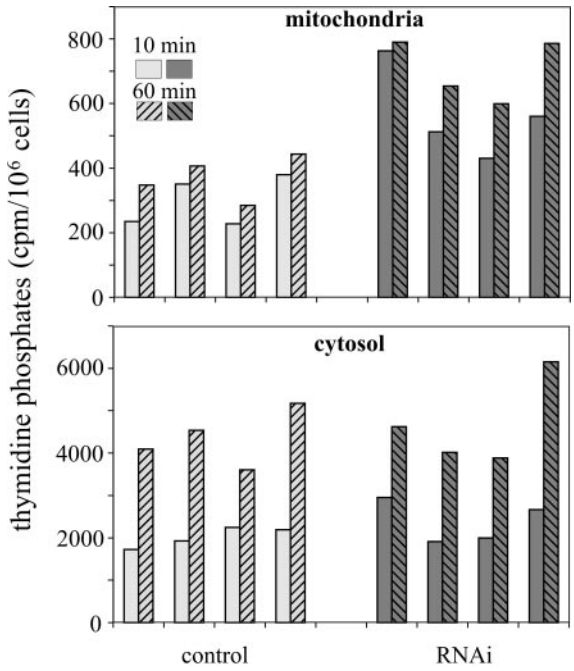


FIG. 8. **Effect of mdN silencing by RNAi on the incorporation of [^{3}H]thymidine into mitochondrial and cytosolic thymidine phosphates of OSTTK1$^-$cells.** Four individual clones, stably transfected with a vector expressing small interfering RNA directed against mdN, were incubated with [^{3}H]thymidine for 10 or 60 min, after which time we determined the total radioactivity of the thymidine phosphate pools of mitochondria and cytosol. As controls we used four individual clones transfected with the empty vector. The increase in radioactivity in the mitochondrial pools of RNAi clones is statistically significant at both times. There was no significant difference between the cytosolic pools.

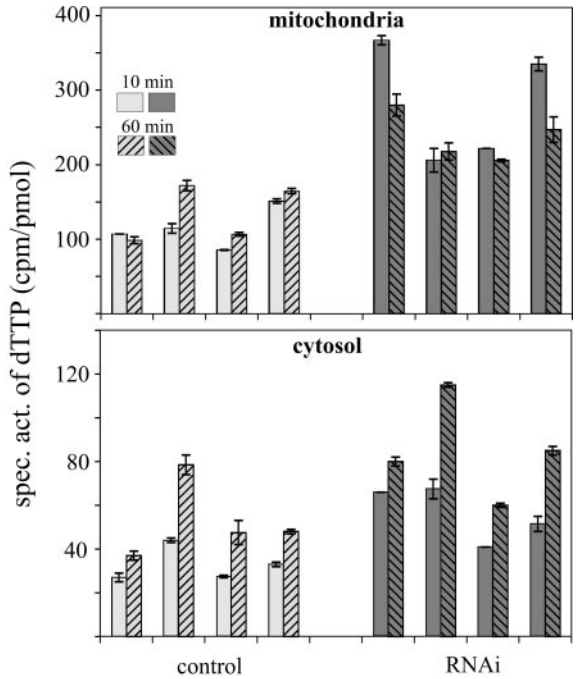
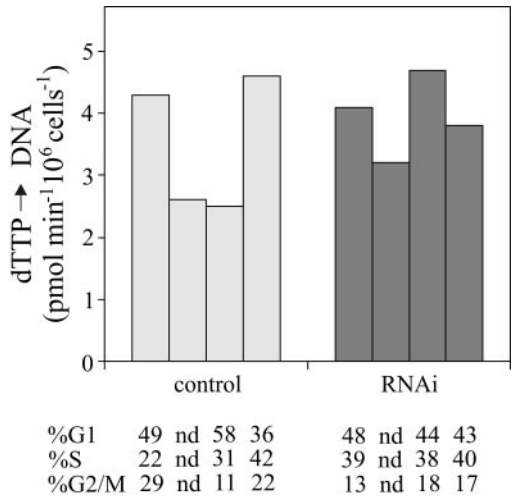


FIG. 9. **Effect of RNAi-silencing of mdN on the specific radioactivity of dTTP in OSTTK1$^-$cells.** The data are from the same experiment as Fig. 8 and show the values for the specific radioactivity of dTTP determined at 10 or 60 min. The difference between the RNAi and control groups is statistically highly significant both in mitochondria and the cytosol.

DISCUSSION

Different laboratories report divergent values for the sizes of cellular (*i.e.* cytosolic) dNTP pools in cultured cells. One reason



%G1	49	nd	58	36	48	nd	44	43
%S	22	nd	31	42	39	nd	38	40
%G2/M	29	nd	11	22	13	nd	18	17

FIG. 10. **Effect of RNAi-silencing of mdN on the rate of DNA synthesis.** In the experiment of Fig. 8 we measured the total incorporation of [^{3}H]thymidine into cellular DNA between 10 and 60 min. From this value we calculated the rate of DNA synthesis (pmol dTTP incorporated into DNA during 1 min by 10^6 cells). We found no difference in the rate of DNA synthesis between RNAi-silenced cells and controls. The small *table* below the graph shows the distribution of cells between the three phases of the cell cycle determined by flow cytometry. *nd*, not done.

for the diversity is that pool sizes change during the cell cycle (1, 28–30), and this effect was not always taken into account. As an example, synchronized 3T3 cells contained 100 pmol of dTTP/10^6 cells during the S phase and 5 pmol/10^6 cells during $G_{o,}$ with the S phase pool in addition turning over twice as fast as the G_o pool (29). As demonstrated here (Fig. 2) a similar, albeit much smaller, effect is seen during the growth of human tumor cell cultures and affects both cytosolic and mt dTTP. Also the other three mt dNTPs behave similarly to dTTP (data not shown). The pools were largest during early growth when the cultures contained a larger proportion of S phase cells. Interestingly, cytosolic and mt dTTP pools behaved similarly, even though mt DNA replication is independent of nuclear DNA replication and not coordinated with the cell cycle (3).

We made all further pool analyses under defined growth conditions, with ~35% of the cells in S phase. Repeated analyses of both mt and cytosolic dTTP pools of separately grown cultures then showed only small variations for each tumor cell line (Table I), demonstrating the reproducibility of our measurements. For mt dNTPs variations between different cell lines were moderate, with the exception of human OSTTK1$^-$ cells, whose pools were approximately double the size of the other three cell lines (Table I). Within each line, mt pool sizes of dATP, dCTP, and dTTP were roughly the same, whereas the dGTP pool was smaller, approximately half the size of the others. These data agree reasonably well with results from HeLa cells published originally (8) but not with more recent data published by the same laboratory (32).

In the cytosol the size of each dNTP varied more from one cell line to the other (Table I). The two pyrimidine pools were always larger than the two purine pools. The last column of Table I gives the average pool sizes for all four dNTPs, showing that dTTP + dCTP constituted 70% of all dNTPs and dGTP only 7%. These results, and in particular the small dGTP pool, agree with many earlier analyses (1, 31).

How large a fraction of an individual dNTP is located inside mitochondria? The data vary considerably between the different cell lines (Table II), mostly depending on variations between the cytosolic pools. The average values for each dNTP calculated in the last column of Table II suggest that mitochondria contain 3% of dTTP and dCTP, 4% of dATP, and 7% of dGTP of each respective cytosolic pool. The difference between dGTP and the other dNTPs is statistically significant ($p < 0.04$). A similar asymmetry for dGTP was reported for HeLa cells (8).

Estimates of the relative volume of mitochondria in cultured cells vary, but assuming a value of 10% of the cytosolic volume (33), our above calculations would suggest that dNTPs are excluded from mitochondria. However, the percentage values in Table II are for triphosphates; monophosphates and diphosphates were not determined. The levels of phosphorylation of nucleosides differ widely between mitochondria and the cytosol. In isotope experiments with ^{3}[H]thymidine (9), we found in the cytosol consistently 80% of the radioactivity in dTTP and 20% in dTDP, whereas mitochondria contained ~25% dTTP and dTMP each and ~50% dTDP. Thus, measurements of dTTP account for 80% of the total thymidine phosphate pool in the cytosol but only for 25% in mitochondria. Because ATP, ADP, and AMP were similarly distributed, such a distribution is probably also valid for the other dNTPs. When we on this assumption include monophosphates and diphosphates in our calculations, mitochondria contain 10% of the total thymidine and deoxycytidine phosphates, 13% of deoxyadenosine phosphates, and 23% of deoxyguanosine phosphates. This changes the picture and suggests that the first three deoxynucleotides are evenly distributed between cytosol and mitochondria, whereas deoxyguanosine phosphates are concentrated to mitochondria.

As a general conclusion it therefore appears from data in Tables I and II that mt dNTP pools reflect the size of cytosolic pools and that in cycling cells most deoxynucleotide pools are quite evenly distributed between mitochondria and the cytosol. dGTP apparently forms an exception and accumulates in mitochondria. This asymmetry depends on a small cytosolic dGTP pool and not on a large dGTP pool in mitochondria.

What is the cellular machinery responsible for the distribution? As discussed in the introduction and shown in Fig. 1, we envisage two separate pathways as sources for the mt dTTP pool, one dependent on the deoxynucleotide transporter and the other on equilibrative nucleoside transporter 1. The size of the mt dTTP pool depends on the regulation of both pathways. The present work concerns the regulation of the second pathway, specifically the question to what extent the mt 5′-deoxyribonucleotidase mdN is involved in its regulation. To this purpose we investigated how the *in situ* activity of mdN affects the utilization of [^{3}H]thymidine for the synthesis of thymidine phosphates. The human tumor cell line OSTTK1$^-$ phosphorylates thymidine exclusively by the intra-mt TK2, and therefore all radioactivity found in thymidine phosphates and DNA originates in mitochondria. After transfection with an appropriate plasmid we increased the activity of mdN by induction of the cells with ponasterone up to 30-fold. In the other direction, we decreased mdN activity by RNA interference to 20% of the controls. In both instances we found in isotope experiments that the incorporation of thymidine into thymidine phosphates and DNA as well as the specific radioactivity of the mt and cytosolic dTTP pools depended on the activity of mdN; increasing mdN activity decreased the phosphorylation of thymidine (Figs. 4–7), and decreasing mdN activity gave an increase (Figs. 8 and 9). In all experiments the activity of TK2 was not affected. Our results strongly suggest a participation of mdN in an intra-mt substrate cycle where the enzyme counteracts the activity of TK2 (Fig. 1). The effects on the cytosolic pools and on incorporation into DNA are secondary and were caused by a decreased or increased export of radioactive nucleotides from mitochondria to the cytosol. In accordance with this interpre-

tation, changes in mdN activity gave more clear-cut effects in mitochondria than in the cytosol.

In an earlier experiment (21) overproduction of mdN by $TK1^+$ 293 cells did apparently not affect the phosphorylation of thymidine. In these cells the phosphorylation of thymidine was, however, almost exclusively catalyzed in the cytosol by TK1, whereas in the present experiments with $TK1^-$ cells, thymidine was phosphorylated in mitochondria, and the resulting dTMP was directly available for dephosphorylation by intra-mt mdN. This difference in all probability is the reason for the apparently contradictory results and supports the existence of an intra-mt substrate cycle between TK2 and mdN.

Overproduction of mdN up to a certain level gave a graded decrease of thymidine incorporation. Within this range we found, however, no discernible effect on cell growth, DNA synthesis, or the size of the mt and cytosolic dTTP pools, not even when the specific radioactivity of dTTP was decreased to 25%. Also when we lowered mdN activity by RNA interference, resulting in an increased dTTP specific radioactivity, the size of the dTTP pool did not change. Thus, the level of mdN activity does not affect the *concentration* of dTTP in mitochondria or in the cytosol. For mitochondria this means that any decrease in the phosphorylation of thymidine was compensated for by an increased import of dTDP from the cytosol. Note that the changed mdN activity was not limited to the 60 min of the isotope experiment. It lasted for a considerable time, in the RNA interference experiments for many cell generations. Clearly in cycling cells a decrease or increase in pathway 2 is buffered by pathway 1; the deoxynucleotide transporter compensates for changes in the substrate cycle. mdN does regulate the intra-mt phosphorylation of thymidine, but in cycling cells the pathway in which mdN participates is of minor importance compared with the import of dTDP. The answer to the question of whether in terminally differentiated cells the intra-mt substrate cycle assumes a greater importance, as we have suggested, requires further work.

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