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Human Mitochondrial 5′-Deoxyribonucleotidase

OVERPRODUCTION IN CULTURED CELLS AND FUNCTIONAL ASPECTS*

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Deoxynucleoside triphosphates (dNTPs) used for mitochondrial DNA replication are mainly formed by phosphorylation of deoxynucleosides imported into mitochondria from the cytosol. We earlier obtained evidence for a mitochondrial 5′-nucleotidase (dNT2) with a pronounced specificity for dUMP and dTMP and suggested that the enzyme protects mitochondrial DNA replication from excess dTTP. In humans, accumulation of dTTP causes a mitochondrial genetic disease. We now establish that dNT2 *in vivo* indeed is located in mitochondria. The native enzyme shows the same substrate specificity and affinity for inhibitors as the recombinant dNT2. We constructed ponasterone-inducible cell lines overproducing dNT2 with and without the green fluorescent protein (GFP) linked to its C terminus. The fusion protein occurred in mitochondria mostly in an inactive truncated form, with only a short C-terminal fragment of dNT2 linked to GFP. No truncation occurred when dNT2 and GFP were not linked. The cell mitochondria then contained a large excess of active dNT2 with or without the mitochondrial presequence. After removal of ponasterone overproduced dNT2 disappeared only slowly from the cells, whereas dNT2-mRNA was lost rapidly. Overproduction of dNT2 did not lead to an increased excretion of pyrimidine deoxyribonucleosides, in contrast to overproduction of the corresponding cytosolic deoxynucleotidase, suggesting that the mitochondrial enzyme does not affect overall cellular deoxynucleotide turnover.

Mammalian 5′-nucleotidases form a heterogeneous group of enzymes differing in primary structure, cell localization, and tissue and substrate specificity. They dephosphorylate various nucleotides to nucleosides and inorganic phosphate. Originally they were distinguished from their enzyme activity, more recently via cloning of their cDNAs resulting in the clear definition of 7 different species (1–7). One enzyme is a ubiquitous ectonucleotidase anchored to the surface of the plasma membrane (1). Five enzymes occur in the cytosol (2–6), and one in mitochondria (7). The mitochondrial nucleotidase and one of the cytosolic enzymes are structurally related and show a preference for the hydrolysis of deoxyribonucleotides and were for this reason named deoxyribonucleotidases (dNTs),[1] with dNT1 in the cytosol (6) and dNT2 in mitochondria (7).

We are investigating the regulation of deoxyribonucleoside triphosphate (dNTP) pools in relation to DNA synthesis and became first interested in the physiological function of dNT1 and cloned its cDNA (6). We obtained evidence that dNT1 participates in the regulation of pyrimidine but not purine dNTP pools via cytosolic substrate cycles (8). Subsequently we discovered the related dNT2, which in its cDNA contains a presumptive mitochondrial presequence, and obtained strong evidence that the enzyme was localized in mitochondria (7). We overproduced dNT2 in *Escherichia coli*, purified the recombinant protein close to homogeneity, and found that its substrate specificity was narrower than that of dNT1 and essentially limited to dephosphorylation of dTMP and dUMP. This specificity suggested that dNT2 participates in the regulation of thymidine phosphate metabolism in mitochondria. The existence of a mitochondrial thymidine kinase (TK2) (9, 10), separate from the cytosolic thymidine kinase (TK1) (11) is well established. The enzyme functions in the normal synthesis of mitochondrial phosphates via import of thymidine from the cytoplasm. The importance of this pathway was recently underscored by the finding that malfunctions of TK2 cause a genetic disease characterized by depletion of mitochondrial DNA (12). In addition, one or several transport systems for deoxynucleoside di- and/or triphosphates may contribute to import of thymidine phosphates from the cytosol. We hypothesized dNT2 to counteract accumulation of dTTP. The requirement for a protection from an excess of dTTP is illustrated by a mitochondrial genetic disease (MNGIE) caused by the loss of the cytosolic thymidine phosphorylase (13). A defect in this catabolic enzyme leads to accumulation of thymidine and presumably dTTP.

Thus human mutants show that both lack and overproduction of thymidine phosphates lead to malfunction of mitochondrial DNA synthesis suggesting that a stringent control of the mitochondrial thymidine phosphate pool is of critical importance.

The mitochondrial localization and the substrate specificity of dNT2 suggest that the enzyme plays a role in this context. We suggest that dNT2 is the catabolic arm of an intramitochondrial substrate cycle with TK2 representing the anabolic arm.

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[1] The abbreviations used are: dNTs, deoxyribonucleotidases; dNTPs, deoxyribonucleoside triphosphates; dNT2, mitochondrial 5′(3′) deoxyribonucleotidase; GFP, green fluorescent protein; dNT1, cytosolic 5′(3′) deoxyribonucleotidase; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase; BPE-T, (1-[2-deoxy-3,5,-*O*-(2-bromo-1-phosphono)ethylidene-β-D-*threo*-pentofuranosyl]thymine); PMcH-U, ((+/-)-1-*trans*-(2-phosphonomethoxycyclohexyl)uracil); TBS, Tris-buffered saline; DLU, digital light units; AP, alkaline phosphatase.

This puts dNT2 in a position to brake the accumulation of intramitochondrial dTTP.

In our earlier experiments the mitochondrial localization and biochemical behavior of dNT2 was deduced from the properties of the cloned cDNA and the recombinant protein (7). We now establish the occurrence of dNT2 in mitochondria and the identity of some properties of the native enzyme with those of the recombinant protein. We also describe the construction of various cell lines stably transfected with vectors containing cDNA constructs of dNT2 and characterize the overproduced protein products. Finally, we study the effects of overproduction of dNT2 on the metabolism of pyrimidine deoxyribonucleotides.

EXPERIMENTAL PROCEDURES

Materials—The inhibitors BPE-T (14) and PMcH-U (15) were generous gifts from Dr Jan Balzarini, Rega Institute, Katholieke Universiteit, Leuven, Belgium. ^{3}H-labeled dUMP and uridine were obtained from Amersham Biosciences. Ponasterone A, zeocin, and G418 were from Invitrogen. Soluble digitonin from Sigma was not completely soluble. Before use, we suspended the compound at the indicated concentration, "dissolved" it for 2 h at 37 °C with occasional vortexing, and clarified the suspension by centrifugation. The expression vectors for mammalian cells were from Invitrogen (pIND and pcDNA3) and CLONTECH (pIRES2-EGFP). Antibodies were from Santa Cruz Biotechnology (anti-adenine nucleotide transporter), from CLONTECH (anti-GFP), from Sigma (AP-conjugated anti-rabbit and anti-chicken secondary antibodies) and from Chemicon (AP-conjugated anti-guinea pig Ig). Pure recombinant dNT1 and dNT2 were prepared as described earlier.

Bacterial Strains and Mammalian Cell Lines—E. coli DH5α was used for routine transformations and plasmid preparation according to standard procedures (16). *E. coli* BL21 (DE3) p*lysS* was the host strain for the expression of recombinant proteins from sequences subcloned in pET20b (Novagen).

Most of the experiments were done with human HeLa or 293 cells. Clone 293-2-100 was previously isolated in our laboratory (17). The cytosolic thymidine kinase (TK1)-deficient mouse 3T3 TK$^-$ cell line was from Dr. Edgard Wawra (University of Vienna, Vienna Biocenter, Wien, Austria). Cells were grown in Dulbecco's modified Eagle's medium with 7.5% heat-inactivated fetal calf serum and antibiotics in a humified incubator with 5% CO_2 at 37 °C. Absence of Mycoplasma contamination was periodically checked by an immunoassay (Mycoplasma Detection Kit, Roche Molecular Biochemicals).

Vector Construction and Transfection of Cells—We prepared four new constructs for expression of dNT2 in mammalian cells. One (pdNT2) derived from the ecdyson-inducible pIND vector (Invitrogen) and contained the dNT2 coding sequence by itself, three were constitutive vectors. Two of these derived from pcDNA3 (Invitrogen) and contained the dNT2 sequence either alone (pc3dNT2) or fused to the GFP sequence (pc3dNT2-GFP). The third constitutive construct (pIRES-dNT2) produced dNT2 and GFP as separate proteins from the same mRNA. The inserts for the new constructs were obtained from the complete dNT-2 cDNA (7) by PCR reactions run with reverse primer NT2.10 5′-CTGAGGATCCAGCCCCACAGAGGA-3′ and forward primer NT2.16 5′-CATCAAGCTTCTGGGCCATGATCCG-3′ (pdNT2, pc3dNT2) or forward primer NT2.33 5′-CATCCTCGAGCTGGGCCATGATCCG-3′ (pIRES-dNT2). The sequence coding for the dNT2-GFP fusion protein obtained from p1dNT2-GFP (7) was subcloned as a *Hin*dIII/*Bam*H1 fragment into vector pcDNA3 to give pc3dNT2-GFP.

Semiconfluent mammalian cell cultures were transfected by calcium phosphate precipitation and either analyzed after 48–72 h (transient transfections) or selected with G418 600–800 μg/ml starting 24 h from transfection. After about 2 weeks clones were picked, expanded, and analyzed by enzymatic assays of crude extracts and by fluorescent microscopy in the case of vectors carrying the GFP-coding sequence.

Induction of Overproduction—Bacteria overproducing dNT2 containing the leader sequence were obtained by transformation with p1M-dNT2 (7) and induction with IPTG. After induction extracts had a dNT2 specific activity of 1.5 and on Western blots reacted with the peptide antibody p77 directed against the leader sequence (see Fig. 3 below).

For inducible overproduction of dNT2 in mammalian cells we used an ecdyson-inducible expression system in 293 cells as previously described (17). Cultures of clone 293-2-100 that constitutively express the intracellular ecdyson receptor (17) were the recipient of the inducible vectors described above. Two stable derivatives of this clone were used for the experiments, *i.e.* clone 293-R transfected with plasmid p1dNT2-GFP and clone 293–4A transfected with plasmid pdNT2. For induction experiments cells were plated at 2–4 $\times$ 10^5 cells per 5-cm Petri dish, and induction with ponasterone A was started after 6–12 h. At different times of induction replicate plates were used for measuring cell growth and preparation of cell extracts for enzyme assays. In chase experiments the inducer was removed after 72 h when fresh medium was added, and the cultures were incubated for up to 48 h to measure the decay of dNT2 expression.

Preparation of Antibodies—We prepared antibodies against mouse dNT1 and human dNT2 from chicken eggs by repeated injections of 0.1 mg of protein into hens, using two animals for each enzyme. Eggs were collected from each animal and antibodies were purified with polyethylene glycol (18) and analyzed by Western blotting. Six weeks after the first immunization the intensity of the bands on Western blots no longer increased. The antibodies were further purified by affinity chromatography. dNT1 or dNT2 (0.4 mg) was electrophoresed on a preparative 9% SDS-PAGE gel, the main protein band was electroblotted to a polyvinylidene difluoride membrane, washed first with Tris-buffered saline (TBS) followed by 0.1 M glycine buffer, pH 2.7, and finally equilibrated with TBS. The membranes were cut into small pieces and incubated overnight with 1.5 ml of antibody (3 mg/ml in TBS). The pieces were first washed with TBS, and the bound antibody was then eluted with 3 $\times$ 0.75 ml of 0.1 M glycine, pH 2.7. Each eluate was immediately neutralized with 0.075 ml of 1 M Tris-HCl, pH 9.0. The antibodies could detect 0.5–1 ng of the cognate protein on Western blots (data not shown). They cross-reacted with the related nucleotidase with a 3–10-fold lower sensitivity.

We also used antibodies prepared by immunization of guinea pigs with 3 peptides that form part of dNT2. The following peptides were used: MIRLGGWCARRLCSAAVPAGC (amino acid residues 1 to 20 + C = p77); GRLRPGLSEKAISIWESKNC (residues 82 to 100 + C = p78); and HSWADDWKAILDSKRPC (residues 212 to 228 = p79). On Western blots these antibodies had a lower sensitivity for dNT2 than the chicken antibody but did not react with dNT1. Compared with the chicken antibody, p77 was 10–20 times less sensitive and p78 and p79 were 3–5 fold less sensitive. We thank Dr. Ewa Björling, Karolinska Institute, MTC, Stockholm, Sweden for their preparation.

Preparation of Cell Fractions—We used both HeLa and 293 cells for this procedure and made all manipulations at close to 4 °C. Our procedure builds with minor exceptions on earlier work for purification of mitochondria from cultured cells (19). We washed the cells growing on 10-cm dishes three times with phosphate-buffered saline (PBS) and then scraped them off the dish using 3 ml of extraction buffer (0.21 M mannitol/0.07 M sucrose/10 mM Tris-HCl, pH 7.5/0.2 mM EGTA) per 3 dishes and sedimented the cell suspension at 800 $\times$ *g* for 5 min. We homogenized the sediment with 0.8 ml of extraction buffer containing 2 mg/ml of digitonin by pushing the suspension vigorously 10 times through a 22G $\times$ 1.5 inch needle. A nuclear fraction was sedimented by centrifugation at 1300 $\times$ *g* for 5 min and washed with 1 ml of extraction buffer. The supernatant from the first centrifugation was transferred to an Eppendorf tube, and a mitochondrial fraction was sedimented for 20 min at 14,000 $\times$ *g*. The supernatant from the second centrifugation is referred to as cytosol and was used directly for enzyme assays and Western blots. For the same purpose, the mitochondrial and the nuclear fractions were extracted as described below for whole cells. To determine the degree of cross-contamination, the three fractions from induced 293–4A cells overproducing dNT2 were analyzed by Western blotting and enzyme analyses for the presence of mitochondria-specific enzymes (Fig. 1). On Western blots the adenine-nucleotide transporter and porin, two mitochondrial membrane-specific enzymes, were found only in the mitochondrial and nuclear fractions. These two fractions also contained other mitochondrial marker enzymes (the matrix citrate synthase and HSP60, and the intermembrane cytochrome *c*). These markers were actually more abundant in the nuclear fraction demonstrating a heavy contamination with mitochondria. The cytosol contained also some HSP60, cytochrome *c,* and citrate synthase demonstrating a minor contamination with mitochondrial non-membrane material. A small amount of lactate dehydrogenase activity in mitochondria and nuclei suggests the presence of some cytosol in these fractions.

Before including digitonin during cell fractionation we investigated its effect on the distribution of marker enzymes and dNT2. At concentrations between 0 and 3 mg/ml we found no effects on the distribution between mitochondria and cytosol. However inclusion of digitonin increased the yield of the mitochondrial fraction and decreased the contamination of the nuclear fraction by mitochondria. Washing the nuclei with extraction buffer or centrifuging them through a sucrose cushion

decreased the amount of mitochondrial marker enzymes and dNT2 in this fraction, but did not eliminate it. We therefore did not use these extra steps but included 2 mg/ml digitonin in our procedure.

Extraction of Cells—Cells growing in monolayer were washed three times with cold PBS and drained carefully. They were scraped off the dish in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100 (0.2 ml per 10-cm dish) and vortexed briefly. After addition of NaCl to a final concentration of 0.2 M and additional vortexing the extract was centrifuged at 14,000 × *g* for 20 min. All operations were at close to +4 °C. Extracts could be stored frozen at −80 °C and reassayed several times without apparent loss of activity.

Enzyme Assays—For routine assays of dNT1 and dNT2 activities, portions of the extracts were incubated with 5 mM [^{3}H]dUMP as described earlier (6, 7). The two inhibitors PMcH-U and BPE-T were used to distinguish between dNT1 and dNT2 (20). In these experiments substrate concentration was 0.2 mM, inhibitor concentration, 1 mM. For the determination of substrate specificity all nucleotides were used at 2 mM concentration, and enzyme activity was calculated from the amount of P_i formed during 30 min of incubation (21). Standard spectrophotometric assays were used for citrate synthase (22) and lactate dehydrogenase (23). For all enzymes, one unit of activity is defined as the formation of 1 μmol of product/min. Specific activity is units/mg of protein. Protein was determined (24) with bovine serum albumin as standard.

Northern Blots—Total RNA was extracted with the TRIZOL reagent (Invitrogen) as described by the manufacturer and quantified by spectrophotometry. 8 μg of the RNA were analyzed by Northern blotting (16). A 737-nt fragment of human dNT2 cDNA was generated from p1M-dNT2 by PCR with forward primer NT2.9 (5′-CATACATATGATCCGGCTGGGCGG-3′) and reverse primer NT2.10 (5′-CTGAGGATCCAGCCCCACAGAGGA-3′) and used as a probe. The dNT2 probe was labeled by random priming (Amersham Biosciences) with [α-^{32}P]dCTP, and hybridization was performed in ULTRAhyb buffer (Ambion). Washed filters were autoradiographed and analyzed by densitometry with the Packard OptiQuant software. A β-actin probe (Ambion) was used to calibrate the amounts of RNA loaded in the different lanes. The Digital Light Units (DLU) of each dNT2 hybridization signal were normalized by dividing the DLU of the dNT2 signal with that of the corresponding β-actin signal.

Isotope Chase Experiments—The procedures for these experiments were described in detail earlier (8) and will be outlined only briefly here. We labeled duplicate sets of ponasterone A-induced and non-induced 293-R cells for 2 h with 1 μM [5,6-^{3}H]uridine (40 Ci/mmole). We continued cell growth for an additional 2 h in non-labeled medium and analyzed samples 0, 1, and 2 h after change of medium. In the cells, we analyzed total isotope incorporation into DNA and the specific activities of the dCTP and dTTP pools, in the medium total isotope incorporation into deoxycytidine, thymidine, and deoxyuridine. From the specific activities of dTTP and dCTP we could then calculate the absolute amount (pmol/min) of cytosine and thymine incorporated into DNA as well as the absolute amount of each pyrimidine deoxyribonucleoside excreted by the cells.

Partial Purification of dNT2 from Rat Brain—Mitochondria from rat brain were prepared by differential centrifugation (25), pooled, and kept frozen at −80 °C until a total of 39 brains had been collected. The mitochondria were then extracted by homogenization in 50 ml of 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2 M NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100 containing 1.25 ml of a solution of mammalian protease inhibitors (Sigma). The supernatant solution after centrifugation at 100,000 × *g* for 40 min (35 ml) contained 110 mg of protein and 0.77 units of 5′-nucleotidase activity determined with dUMP as substrate. Protein was precipitated between 40 and 60% saturation of ammonium sulfate, the precipitate was dissolved in a small volume of buffer A (20 mM Tris-HCl, pH 7.5, 20% glycerol, 2 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) dialyzed extensively against buffer A, with recovery of 20 mg of protein and 0.30 units of 5′-nucleotidase activity. This solution was adsorbed to a 6-ml column of DE52, proteins were eluted with a linear gradient of 0–0.15 M NaCl in buffer A (15 + 15 ml). Nucleotidase activity was eluted in two peaks, one appearing at 40 mM, the second at 70 mM NaCl. The dNT2-specific inhibitor BPE-T (20) inhibited the activity in the first but not the second peak. The material in the first peak (1.1 mg of protein, 0.12 units of nucleotidase activity) was used for the experiments described under "Results."

TABLE I

Substrate specificity of recombinant and native deoxyribonucleatidases

The specific activities (units/mg protein) of the different enzyme preparations were as follows; recombinant human dNT2, 340; native mouse brain dNT2, 0.10; recombinant mouse dNT1, 370; native human placents dNT1, 89. The values for recombinant dNT2 and the two preparations of dNT1 are from earlier publications (6, 7, 26).

Substrate	dNT2		dNT1	
	Native	Recombinant (7)	Native (26)	Recombinant (6)
	Relative activity (%)			
dUMP-5′	100	100	100	100
dTMP-5′	88	50	55	65
dCMP-5′	3	0	1	16
dGMP-5′	10	6	81	45
dAMP-5′	4	2	13	10
dIMP-5′	15	8	240	96
UMP-5′	51	30	9	16
CMP-5′	1	0	0	0
GMP-5′	5	2	5	4
AMP-5′	1	0	0	1
IMP-5′	4	2	22	11
UMP-3′	47	47		35
CMP-3′	0	0		0
UMP-2′	27	18		11
GMP-2′	4	2		1

RESULTS

Substrate Specificity and Inhibition of Recombinant dNT2 and dNT2 from Brain Mitochondria—Our earlier work demonstrated that homogeneous human recombinant dNT2 obtained after overproduction in *E. coli* preferentially dephosphorylates dUMP and dTMP with some activity toward the 5′-, 3′-, and 2′-phosphates of uridine. It was important to ascertain that a native mammalian dNT2 showed a similar substrate specificity. For practical reasons we used a partially purified preparation of dNT2 from rat brain mitochondria for the comparison. The results in Table I show that the substrate specificity of the two enzymes indeed was the same. Both showed the highest activity with nucleotides containing uracil or thymine as the base and preferred deoxyribose to ribose as the sugar moiety. We also found a small dephosphorylation of guanine and hypoxanthine deoxyribonucleotides. For comparison we also show the earlier determined substrate specificities of recombinant and native dNT1. These enzymes also preferentially hydrolyze deoxyribonucleotides but are not specific for pyrimidines.

We also compared the effects of the two inhibitors PMcH-U and BPE-T on the two preparations of dNT2 and on recombinant dNT1. As described earlier (20), PMcH-U strongly inhibits recombinant dNT1 and dNT2 whereas BPE-T inhibits mainly dNT2 with a very weak activity toward dNT1. Both preparations of dNT2 retained only 10% activity in the presence of the two inhibitors (Table II). Thus both recombinant and native dNT2 were similarly affected by the inhibitors. With dNT1, PMcH-U almost wiped out all activity, whereas 90% of enzyme activity remained with BPE-T.

dNT2 Is Located in the Mitochondria of HeLa Cells—After transfection of cultured cells with the cDNA of dNT2 linked by its 3′-terminus to the cDNA of GFP the cell mitochondria showed a strong green fluorescence suggesting a localization of the dNT2-GFP fusion protein in mitochondria. The mitochondrial localization of the fluorescence depended on the mitochondrial presequence of dNT2 (7). However, as described below, the mitochondria contained mostly a truncated form of dNT2 and not the complete enzyme linked to GFP. It became therefore important to demonstrate directly the mitochondrial localization of dNT2.

We determined the dephosphorylation of dUMP in extracts from subcellular fractions prepared as described in "Experi-

TABLE II
Effect of inhibitors on recombinant and native dNT2 and on recombinant dNT1

Two different amounts of the three enzyme preparations were assayed with 0.2 mM [^{3}H]dUMP in the presence and absence of 1 mM PMcH-U or BPE-T.

Source of enzyme	Protein in assay	Enzyme activity				
		No inhibitor	+PMcH-U		+BPE-T	
	mg × 10^6	*units × 10^6*	*units × 10^6*	*remaining activity %*	*units × 10^6*	*remaining activity %*
Recombinant dNT2	0.079	5.9	0.50	8	0.61	10
brain dNT2	0.20	16.3	1.3	8	1.34	8
Recombinant dNT1	300	10.8	1.0	10	1.0	10
	740	30.8	3.0	10	3.9	13
	0.47	6.8	0.00	0	6.1	90
	0.93	16.9	0.50	3	14.5	86

TABLE III
Distribution of dNT1 and dNT2 in cell fractions from HeLa cell

The inhibitory effects of PMcH-U and BPE-T on the dephosphorylation of dUMP by extracts from cell fractions were used to differentiate between the presence of dNT1 and dNT2 PMcH-U inhibits both dNT1 and dNT-2. The sum of dNT1 and dNT2 activities is calculated from the difference in dephosphorylation of dUMP in the absence of this inhibitor and in its presence. BPE-T inhibits only dNT-2, dNT2 activity is calculated from the dephosphorylation of dUMP in absence and in the presence of BPE-T, dNT1 activity is obtained from the difference of the activities in the presence of BPE-T and PMcH-U.

Cell fraction	No inhibitor	+PMcH-U	+BPE-T	dNT-1 + dNT-2	dNT-2	dNT-1
			units × 10^3			
Intact cells	3.00	1.17	2.65	1.83	0.35	1.48
Cytosol	2.82	0.93	2.62	1.89	0.19	1.70
Nuclei	0.12	0.028	0.043	0.092	0.067	0.025
Mitochondria	0.60	0.16	0.20	0.44	0.40	0.04

mental Procedures," either directly or in the presence of one of the two inhibitors PMcH-U or BPE-T. dUMP is a substrate for both dNT1 and dNT2. From the results shown in Table III it appears that the difference in enzyme activity in the absence and presence of PMcH-U gives the sum of dNT1 and dNT2; the difference between the values without and with BPE-T gives the activity of dNT2 alone.

Calculated in this way, 90% of the observed total activity in the mitochondrial fraction was dNT2 whereas the cytosol contained 90% dNT1 (Table III). The nuclear fraction contained mostly dNT2, reflecting the contamination by mitochondria shown earlier in Fig. 1.

In these calculations we assumed that each inhibitor was 100% effective. The inhibitors remove, however, only ~90% of the activity. Additionally, BPE-T is not completely specific for dNT2 but also inhibits dNT1 weakly. These effects do not materially influence the general conclusion that can be drawn from our experiment: dNT1 is present in the cytosol, dNT2 occurs in mitochondria. From the data we can also calculate that ~80% of the total dUMP-dephosphorylating activity of HeLa cells comes from dNT1.

Overproduction of dNT2-GFP Leads to Truncated dNT2—The inducible vector p1dNT2-GFP codes for the dNT2-GFP fusion protein and was used earlier with 293-2-100 cells to demonstrate the mitochondrial localization of dNT2 (7). However, in our hands the ecdyson-inducible system worked unsatisfactorily in HeLa and 3T3 cells. In order to assess the mitochondrial import of dNT2 also in these cell lines, we subcloned the dNT2-GFP coding sequence from p1dNT2-GFP into the constitutive vector pcDNA3. Western blots with extracts from clones of HeLa and 3T3 TK$^-$ cells transfected with the new construct (pc3dNT2-GFP) gave now an unexpected result. In all cases the cells overproduced dNT2 activity ~10-fold and showed a strong fluorescence of mitochondria. However, some of our antibodies gave the strongest signal in a position of 33 kDa in addition to a weaker signal at the 54 kDa position expected for a fusion protein between the complete dNT2 and GFP (Fig. 2). The 33-kDa band was found with an antibody against GFP and the peptide antibody p79, but not with peptide antibodies p77 and p78. p79 is directed against the C terminus, p78 against the central part, and p77 against the leader sequence of dNT2. This immunological specificity and the 33-kDa size of the band suggest that the band arose from a severely truncated form of dNT2, linked by its C terminus to GFP. GFP gives a signal at ~29 kDa. The same results were obtained with inducible 293 cells transfected with p1dNT2-GFP (see below).

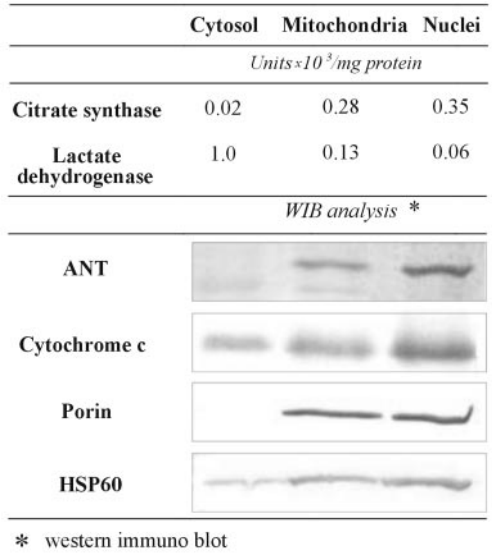

FIG. 1. **Enzyme analyses of cell fractions prepared by differential centrifugation from 293-4A cells overproducing dNT2.** Each cell fraction was analyzed by Western blotting with specific antibodies for the presence of the adenine nucleotide transporter, cytochrome *c*, porin, and HSP60 and by enzyme analyses for citrate synthase (22) and lactate dehydrogenase (23). One unit of enzyme activity is the formation of 1 μmol of product per min.

Overproduction of dNT2 As Such Does Not Result in Truncation—To determine if truncation was caused by the overproduction of dNT2 or by the linkage of dNT2 to GFP we transfected 293 cells with a different vector (pIRES-dNT2) in which

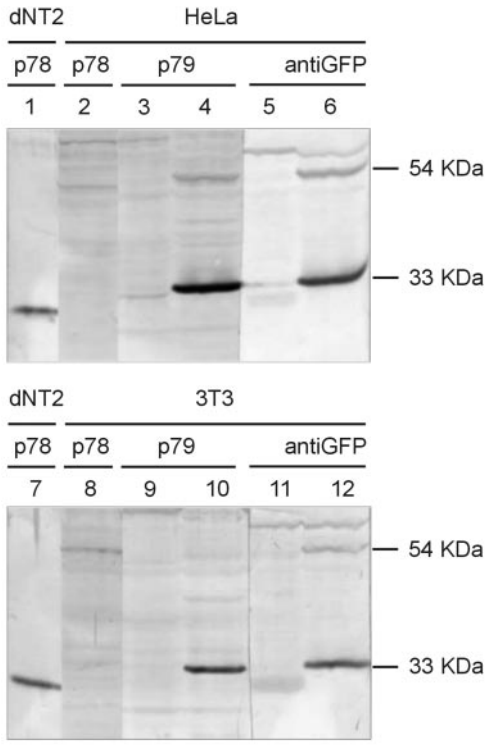

FIG. 2. **Formation of a truncated fusion protein in cells transfected with pc3dNT2-GFP.** Two stable clones of HeLa and 3T3 TK$^-$ cells transfected with the vector were extracted and analyzed by Western blotting with antibodies against dNT2 or GFP. Rows 1 and 7 contain 5 ng of recombinant dNT2 stained with p78 as a marker. Rows 2–6 contain 25 μg of HeLa extract, rows 8–12 contain 25 μg of 3T3 TK$^-$ extract. Rows 3, 5, 9, and 11 are controls from non-transfected cells, the remaining rows are extracts from the transfected clones. The different antibodies are indicated in the figure.

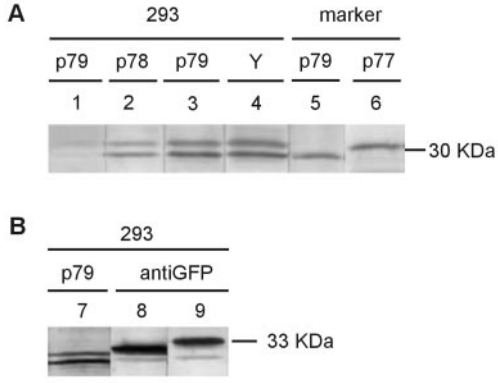

FIG. 3. **Overproduction of dNT2 after transfection with pIRES-dNT2.** Western blots of an extract prepared from 293 cells 72 h after transfection with pIRES-dNT2. *Panel A* (9% polyacrylamide): *lane 1*, control extract before transfection; *lanes 2–4*, extract 72 h after transfection, stained with peptide antibodies p78, p79, or polyclonal antidNT2 (*Y*); *lane 5*, dNT2-marker without leader sequence stained with p79; *lane 6*, dNT2-marker with leader sequence stained with p77. *Panel B* (12% polyacrylamide): *lanes 7* and *8*, extract prepared 72 h after transfection stained with p79 and anti-GFP respectively; *lane 9*, extract from clone 293-R 72 h after induction with ponasterone A.

a second ribosomal binding site separates the cDNAs for dNT2 and GFP. In transiently transfected cells dNT2 and GFP were synthesized separately, with GFP lacking a mitochondrial import signal. Now dNT2 activity increased more than 100-fold, and the cells showed a bright green fluorescence in the cytoplasm. Western blotting with all antibodies against dNT2 except p77 gave two signals of equal strength at about 30 and 31 kDa (Fig. 3*A*). Included are also two markers for dNT2, corresponding to the recombinant enzyme with or without the leader sequence. An extract from an *E. coli* strain overproducing dNT2 with the leader sequence provided the marker for the former. The positions of the two bands from the transfected 293 cells correspond exactly to those of the two dNT2-markers indicating the presence of dNT2 with and without the leader sequence in the transfected cells. p77, the peptide antibody directed against the leader sequence, reacted with the appropriate marker but not with the 31-kDa band proposed to contain the leader sequence (not shown). We believe that this is explained by the low sensitivity of the antibody.

A second Western blot of the same extract, electrophoresed on a higher cross-linked gel, was developed with an antibody against GFP (Fig. 3*B*). One additional lane was developed with p79 to show the positions of the two bands from *panel A*. The GFP antibody did not stain these two bands but gives instead a signal slightly above them, in the position of GFP. Also included for comparison is a parallel run of GFP linked to the severely truncated dNT2 (the 33-kDa species of Fig. 2). This band is located slightly above GFP, reflecting its slightly larger molecular mass.

Fig. 3 suggests that transfection of 293 cells with pIRES-dNT2 resulted in the separate overproduction of dNT2 and GFP, with GFP located in the cytoplasm and dNT2 largely in mitochondria (see below). Only half of the dNT2 molecules lost the mitochondrial presequence. The results also suggest that the severe truncation of dNT2 shown in Fig. 2 was not caused by overproduction of the enzyme but depended on its linkage to GFP in the fusion protein. The higher enzyme activity in cells transfected with pIRES-dNT2 than in cells transfected with plasmids coding for the fusion protein can be attributed to enzyme inactivation by truncation in the latter case.

Time Course of Enzyme Induction—In further work we used an inducible 293 clone (293-4A) stably transfected with a vector (pdNT2) coding for dNT2 but not for GFP. 48 h after addition of 4 μM ponasterone A, dNT2 activity was at least 10-fold higher in extracts from this clone than in corresponding extracts from 293-R. This degree of overproduction halved the growth rate of the cells. Detailed time curves of the overproduction in the two cell lines during induction with 1 or 4 μM ponasterone A are shown in Fig. 4. The inducer was present during a total of 72 h and then removed, with cell growth continuing for a further 48 or 72 h. At various time points we analyzed cell extracts by enzyme assays and Western blotting.

Fig. 4*A* shows the results for clone 293-R. A moderate increase of enzyme activity occurred, more pronounced at 4 than at 1 μM ponasterone A, with plateau values after 24 h. After removal of the inducer, enzyme activity decreased rather slowly. On Western blots, signals for both the complete fusion protein (54 kDa) and its truncated form (33 kDa) increased and decreased in parallel with enzyme activity.

Fig. 4*B* shows the corresponding experiment with clone 293-4A. Plateau values were reached after 48 h at both inducer concentrations, resulting in a specific activity of the extract of 0.35 units/mg of protein at 4 μM ponasterone A. Removal of inducer again resulted in loss of activity. Western blots show two bands for dNT2 corresponding to an enzyme with and without leader sequence. Both increased during induction and are of similar intensity after 72 h, but the faster moving band was more pronounced at early times. After removal of the inducer the intensity of both bands decreased, with the slower band disappearing more rapidly.

Intracellular Localization of Overproduced Enzymes—After fractionation of 293-4A cells induced for 72 h with 1 μM ponasterone A the mitochondrial and nuclear fractions showed the highest specific activity of dNT2, but also the cytosol contained

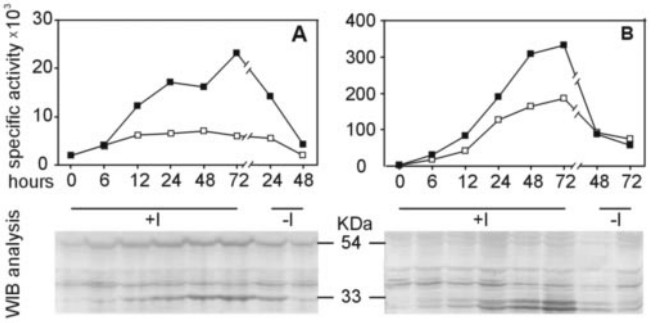

FIG. 4. **Time curve of dNT2 induction by ponasterone A of 293-R cells (*panel A*) or 293-4A cells (*panel B*).** The cultures were induced with either 1 or 4 μM ponasterone A for 72 h after which time fresh medium without inducer was added, and incubation was continued for an additional 48 or 72 h. At the indicated times samples were removed, and cell extracts were analyzed for dNT2 activity and by Western blotting with the polyclonal antibody against dNT2. Western blots are given only for the experiment with 4 μM ponasterone A.

activity (Fig. 5). In fact, total dNT2 activity was almost equally distributed beween the cytosol and mitochondria + nuclei. Over the next 48 h of growth in the absence of inducer, dNT2 activity disappeared rapidly from the cytosol but rather slowly from mitochondria. After 48 h the total amount of activity in the cytosol amounted to only 10% of the activity present in mitochondria + nuclei (Fig. 5). Western blots gave a similar picture, with both overproduced proteins disappearing earlier from the cytosol.

Analyses of extracts from cell fractions of clone 293-R overproducing the fusion protein after a 48-h induction with 4 μM ponasterone A gave results similar to those of Fig. 5 (data not shown). Both the complete and truncated fusion proteins were mainly present in mitochondria but a substantial fraction was also found in the cytosol.

mRNA Decay after Induction of dNT2—After removal of ponasterone A, enzyme activity and the two immune-reactive dNT2 bands, including the unprocessed form of the enzyme, disappeared slowly. Did this depend on a slow decay of the proteins or was the inducible promoter turned off only slowly with continued synthesis of mRNA and protein? On Northern blots (Fig. 6) the large increase in dNT2-mRNA after 72 h induction all but disappeared already 6 h after removal of ponasterone A. At this time dNT2 activity had decreased by only 15%. Clearly the persistence of enzyme activity is due to a slow degradation of the protein and not to continued synthesis of mRNA. Note also that the ratio between enzyme activity and amount of RNA remains identical at 0.4 and 1 μM ponasterone A.

Overproduction of dNT2 Does Not Result in an Increased Excretion of Pyrimidine Deoxyribonucleosides—We described earlier why nucleoside excretion is a sensor of intracellular substrate cycles and nucleotide metabolism (8). Here we investigate the effects of dNT2 overproduction on pyrimidine nucleoside excretion in an isotope chase experiment outlined in "Experimental Procedures." We now only compare in control and overproducing cells the excretion of deoxycytidine, thymidine, and deoxyuridine (Fig. 7), calculated as the percentage of total synthesis of each deoxynucleoside. For comparison we also show in Fig. 7 corresponding data from an earlier experiment with 293 cells overproducing dNT1 (8). In that case deoxyuridine excretion increased 15-fold during induction. Increases in thymidine and deoxycytidine were smaller but statistically significant with p values (Student's t test) of 0.05 or less. In the present case, overproduction of the mitochondrial dNT2 did not change the excretion of thymidine and slightly decreased the excretion of deoxycytidine. Deoxyuridine excretion was too small to be measured also in the induced cells. These results were obtained in parallel duplicate experiments with less than 10% difference between the values for each deoxynucleoside. Thus overproduction of the mitochondrial dNT2 did not increase the excretion of pyrimidine deoxyribonucleosides.

As in the earlier experiments, we found here that induction by ponasterone A increased the excretion of the pyrimidine ribonucleosides uridine and cytidine (data not shown). A simi-

72 hours ponasterone A 1 μM

	Whole cells	cytosol	mito-chondria	nuclei
Spec.activity x 10^3	172	184	314	321
Total activity (units x 10^3)	146	221	141	77
WIB analysis				

72 hours ponasterone A 1 μM + 48 hours without inducer

	Whole cells	cytosol	mito-chondria	nuclei
Spec.activity x 10^3	104	26	200	196
Total activity (units x 10^3)	70	25	112	118
WIB analysis				

FIG. 5. **Distribution of dNT2 in cell fractions from clone 293-4A after induction with ponasterone A.** Cell fractions were prepared 72 h after induction with 1 μM ponasterone A, extracted, and analyzed by dNT2 assays and Western blotting (*WIB*). Specific activity is units/mg of protein; total activity is specific activity $\times$ total amount of protein in extract. Cell fractions were prepared from a total of 30×10^6 cells, whole cell extract was from 10×10^6 cells.

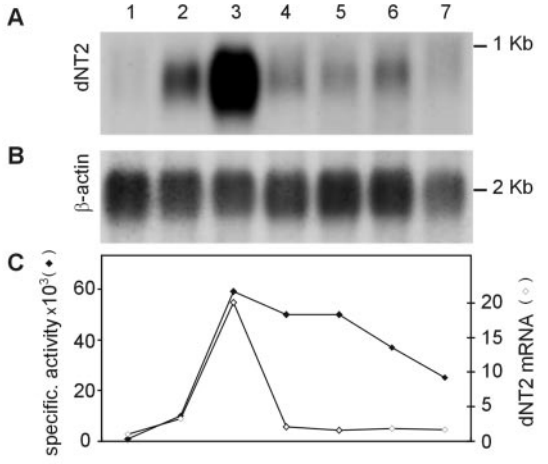

FIG. 6. **Decay of dNT2-mRNA after removal of ponasterone A from 293-4A cells.** The cells were first induced with 0.4 or 1 μM ponasterone A for 72 h after which time ponasterone A was removed and incubation continued for 48 h. *Panel A* shows Northern blots of dNT2-mRNA after 72 h of induction and during the chase period (*1*, non-induced control; *2*, induction for 72 h with 0.4 μM ponasterone A; *3*, induction for 72 h with 1 μM ponasterone A). After removal of ponasterone A, only RNA from cells induced with 1 μM ponasterone A is shown (*4*, 6 h after removal; *5,* after 12 h; *6*, after 24 h; *7*, after 48 h). *Panel B* shows corresponding Northern blots for actin-mRNA. *Panel C* shows the specific dNT2 activities (units·min^{-1}·mg protein^{-1}) (♦) as well as the relative amounts of dNT2-mRNA (◇) in arbitrary units, calculated from the density of the dNT2-mRNA with a correction for the RNA-load, determined from the actin-mRNA.

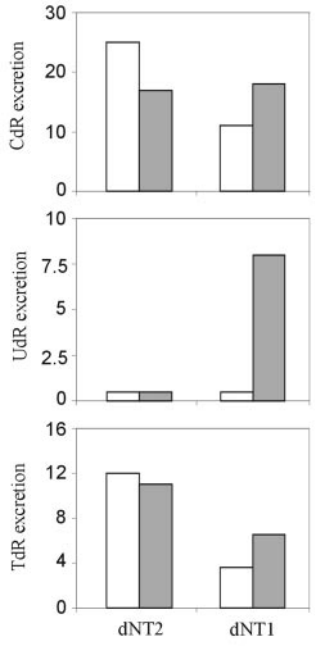

FIG. 7. **Effect of dNT2 overproduction on excretion of pyrimidine deoxyribonucleosides by 293-R cells.** Overproducing and control cells were labeled with [5,6- ^{3}H]uridine, followed by a 2-h chase without isotope. During the chase we determined the excretion of labeled deoxycytidine, deoxyuridine, and thymidine into the medium and calculated the absolute amounts of deoxynucleosides from the determined specific radioactivity of the corresponding dNTPs. The *left* part of the figure shows the excretion of each deoxynucleoside in this experiment as percentage of total deoxynucleotide synthesis (the sum of incorporation into DNA and excretion into the medium). The *right* part of the figure shows for comparison corresponding results from an earlier experiment with 293 cells overproducing dNT1 instead of dNT2. *Open bars*, non-induced controls; *shaded bars*, overproducing cells.

lar increase was, however, found in a control experiment with ponasterone A-treated 293-2-100 cells that are stably transfected with a vector coding for the heterodimeric ponasterone A receptor but not for dNT1 or dNT2 (not shown). Clearly overproduction of uridine and cytidine is not linked to overproduction of either deoxyribonucleotidase but results from the presence of activated ponasterone A receptor.

DISCUSSION

Earlier experiments with recombinant dNT2 provided evidence for the mitochondrial localization of the enzyme (7). We now establish that the recombinant and the native enzyme have similar properties. They behave identically during purification, show the same substrate specificity and the same inhibition by nucleotide analogs (Tables I and II). After fractionation of human HeLa cells in culture the bulk of dNT2 activity was recovered in mitochondria and in a nuclear fraction that was heavily contaminated by mitochondria. In contrast, the related dNT1 was located in the cytosol (Table III). These results show convincingly that dNT2 is a mitochondrial enzyme and that results obtained with the recombinant protein reflect the properties of the native enzyme. The occurrence of a separate deoxyribonucleotidase in mitochondria suggests a highly compartmentalized metabolism of deoxyribonucleotides. Additional evidence comes from the existence of TK2 (9, 10) and a separate mitochondrial dUTPase (27).

In earlier work with other 5′-nucleotidases we investigated cultured cells overproducing one nucleotidase at a time to determine the effects of enzyme overproduction on nucleotide metabolism (8). Those experiments suggested the involvement of two cytosolic enzymes via nucleoside/nucleotide substrate cycles: cytosolic High-K_m nucleotidase (cN II) participates in the regulation of IMP and GMP pools, dNT1 in the regulation of dUMP and dTMP pools. The overproducing cell lines used in those experiments were stably transfected with constructs containing each nucleotidase coding sequence under the control of an inducible promoter. Nucleotide metabolism and DNA synthesis could thus be compared before and after induction.

For a similar approach with dNT2 we now constructed cell lines that can be induced to overproduce this enzyme. Clone 293-R produced an enzymatically active, inducible fluorescent fusion protein in which dNT2 was linked via its C terminus to GFP. Under induction with ponasterone A this cell line overproduced dNT2 activity ~10-fold, but contained, in addition to the expected complete fusion protein, a truncated form in which a short C-terminal fragment of dNT2 was linked to GFP (Fig. 4*A*). This form was actually more abundant than the complete fusion protein. It lacks enzyme activity as the catalytic site of dNT2 is located in the N terminus (6, 28). The same phenomenon occurred in clones of HeLa and 3T3 cells that constitutively overproduced the dNT2-GFP fusion protein (Fig. 2). Fusion of proteins to GFP is a common method for their intracellular identification and localization. Our results provide a caveat against an uncritical use of this approach.

This complication did not arise in cells that overproduced dNT2 separate from GFP (Fig. 3) or lacked the GFP-coding cDNA completely (Fig. 4B). The activity of dNT2 in these cells increased several hundredfold with the solubilized dNT2 representing up to 0.1% of the total protein in cellular extracts. The cells contained approximately equal amounts of dNT2 with and without the mitochondrial leader sequence. After cell fractionation we recovered both forms of dNT2 in the mitochondrial and nuclear fractions as well as the cytosol (Fig. 5*A*).

The presence of dNT2 in the nuclear fraction is in all probability an artifact caused by contaminating mitochondria. As shown in Fig. 1 the nuclear fraction also contained large amounts of mitochondrial marker enzymes. Further purification of the nuclei removed these enzymes and dNT2 in parallel, but we were never able to remove mitochondria completely. We saw no reason to purify the nuclear fraction routinely as this would involve considerable losses and would make it difficult to calculate the distribution of dNT2 between mitochondria and cytosol (Fig. 5). In our calculations we assumed that dNT2 of the nuclear fraction represents mitochondrial contamination.

The finding of a large amount of dNT2 in the cytosol of overproducing cells is more difficult to explain. At the peak of overproduction only 50% of the enzyme is found in mitochondria, the rest is in the cytosol (Fig. 5). dNT2 is a mitochondrial matrix enzyme, and the more than 100-fold overproduction of the enzyme may have saturated the matrix space and/or the mitochondrial import mechanism resulting in accumulation of the enzyme in the cytosol. In agreement with this we found that after removal of the inducer dNT2 disappeared much more rapidly from the cytosol than from the mitochondria (Fig. 5). This is, however, not the complete explanation as the isolated cytosol also contained some dNT2 without the leader sequence, which is removed *after* import of the complete protein into mitochondria (29, 30). The large overproduction of dNT2 may affect the integrity of the mitochondrial membranes and cause release of soluble mitochondrial proteins into the cytosol either in the living cells or during the fractionation procedure. Also the presence of some citrate synthase activity, cytochrome *c,* and HSP60 in the cytosol (Fig. 1) suggests disruption of the mitochondrial membranes. However a comparison of the amount of citrate synthase activity (Table I) and dNT2 activity (Fig. 5) shows a preferential accumulation of the latter in the cytoplasm. Both enzymes are located in the mitochondrial matrix. We suggest that

several factors may contribute to our finding of dNT2 activity in the cytosol of overproducing cells. In further experiments this phenomenon can be minimized by choosing cells several days after withdrawal of the inducer when most of the cytosolic activity has disappeared (Fig. 5).

After removal of the inducer the dNT2-mRNA all but disappeared from the cells within a few hours (Fig. 6), in contrast to the slow disappearance of the protein. This experiment demonstrates that after removal of the inducer dNT2 transcription stops rapidly and no further overproduction of dNT2 takes place. Disappearance of dNT2 is caused by protein degradation but occurs only slowly.

The complexity of these results must be considered in future experiments in which the effects of enzyme overproduction on the mitochondrial deoxyribonucleotide metabolism are investigated. We have here described one experiment in which we examined the effects of a moderate overproduction of dNT2 on the total pyrimidine deoxyribonucleotide metabolism by the methodology used earlier to demonstrate the importance of the cytosolic dNT1 for the maintenance of pyrimidine deoxyribonucleotide pools (8). Mitochondrial pools only represent a small fraction of the total pools and the experiment therefore addresses the question whether overproduction affects the metabolism of cytosolic deoxyribonucleotides. We found no effect of dNT2 overproduction even though a fraction of the overproduced enzyme was detected in the cytosol. The relevance of this result becomes apparent from an inspection of Fig. 7 that compares two identical experiments involving overproduction of either mitochondrial dNT2 or cytosolic dNT1, with a striking specificity of the effects for the cytosolic 5′-nucleotidase. In future experiments we plan to address specifically thymidine nucleotides in the mitochondrial compartment.

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