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Expression of Deoxynucleotide Carrier Is Not Associated with the Mitochondrial DNA Depletion Caused by Anti-HIV Dideoxynucleoside Analogs and Mitochondrial dNTP Uptake

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ABSTRACT

Our previous studies suggested that the dNTP/dNDP transporter systems that exist in mitochondria for transporting dNTP/dNDP from the cytoplasm to the mitochondria for mitochondrial DNA (mtDNA) synthesis play a critical role in delayed cytotoxicity of anti-human immunodeficiency virus (HIV) dideoxynucleoside analogs in mitochondria. A protein, termed mitochondrial deoxynucleotide carrier (DNC), based on its ability to transport dNTPs in reconstituted proteoliposomes, was recently isolated. Lacking cellular information to substantiate DNC's involvement in the delayed cytotoxicity of dideoxynucleoside analogs, we expressed DNC and reconstituted it into proteoliposomes. The K_m values for dNTPs uptake by reconstituted DNC were in the millimolar range, which is a thousandfold higher than that of the physiological level. Furthermore, we found that overexpressing DNC (wt and G177A-mutated DNC) in RKO cells did not sensitize the cells to the mtDNA depletion caused by β-D-2′,3′-dideoxycytidine (ddC), 2′,3′-didehydro-2′,3′-dideoxythymidine, and 2′,3′-dideoxyinosine or affect the mtDNA recovery rate after ddC treatment. Mitochondria isolated from DNC-overexpressing cells did not significantly differ from that isolated from RKO cells in terms of the rate of uptake or the incorporation of dTTP into mitochondria DNA. Down-regulation of DNC expression by small interfering RNA was also ineffective in changing the action of dideoxynucleoside analogs on the mtDNA depletion and the rate of dTTP uptake into isolated mitochondria. Down-regulation of both DNC and thymidine kinase-2 also did not cause mtDNA depletion. We conclude that DNC does not play an important role in the delayed cytotoxicity (mtDNA depletion) of anti-HIV dideoxynucleoside analogs and dNTPs uptake into mitochondria.

At clinical dosage, anti-human immunodeficiency virus dideoxynucleoside analogs such as zidovudine (AZT), d4T, ddC, and ddI could cause delayed type toxicity such as myopathy, cardiomyopathy, peripheral neuropathy, lipodystrophy, and lactic acidosis in patients (White, 2001). Based on cell culture studies, we postulated that the delayed toxicity could be caused by the action of those compounds in depleting cellular mtDNA through their incorporation at the terminal of mtDNA (Chen and Cheng, 1989; Chen et al., 1991).

To have dNTPs for mtDNA synthesis, dNTPs must be either imported from the cytoplasm through a carrier or synthesized by salvaging deoxynucleosides within the mitochondria (Elpeleg et al., 2002). The existence of a mechanism for mitochondria dNTP uptake was suggested by DNA synthesis experiments using isolated mitochondria using dNTPs that could be used for synthesizing mtDNA (Parsons and Simpson, 1973; Chen and Cheng, 1992; Enriquez et al., 1994).

For ddC to deplete mtDNA, because ddC cannot be phosphorylated to ddCTP in mitochondria, it will require the transport of ddCTP from the cytoplasm to the mitochondria (Chen and Cheng, 1992). ddC is unable to deplete mtDNA in cytoplasmic dCyd kinase-deficient CEM cells (Chen and Cheng, 1992). The presence of a mitochondria-associated dNTP transport system was further demonstrated by using a proteoliposome system reconstituted with partially purified mitochondrial proteins (Bridges et al., 1999). The dCTP transport activity in proteoliposomes was time-dependent and could be activated by Ca^{2+}. The K_m value of dCTP in the presence of Ca^{2+} was shown to be 3 μM, within physiological range. dCDP but not dCMP or dCyd could inhibit the transport activity. Other deoxynucleoside triphosphates could also

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ABBREVIATIONS: d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; ddC, β-D-2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; mtDNA, mitochondrial deoxyribonucleic acid; DNC, deoxynucleotide carrier; siRNA, small interfering RNA; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; wt, wild-type; ANOVA, analysis of variance; RT-PCR, reverse transcription-polymerase chain reaction; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

inhibit the uptake of dCTP with a potency the order of dGTP = dATP > TTP.

A protein from the gene SLC25A19 has been termed a deoxynucleotide carrier (DNC, 36 kDa), and it was recently suggested to be a mitochondria transporter located on the inner membrane (Dolce et al., 2001). Functional analysis showed that DNC in a reconstituted proteoliposome system could transport dNTPs. It was postulated to be associated with the delayed cytotoxicity of dideoxynucleoside analogs. Correlations with a mutation of this protein [Gly177-to-Ala (G177A)] and Amish lethal microcephaly were found (Rosenberg et al., 2002). Functional analysis indicated that the G177A-mutated DNC protein in proteoliposome could not take up dATP. They proposed that mitochondrial deoxynucleotide transport may be essential for the prenatal brain growth.

Cellular information to support whether DNC is a key transporter of dNTP uptake into mitochondria and associated with mtDNA depletion caused by dideoxynucleoside analogs is not available. In this report, we studied the behavior of DNC in vitro. Our results suggest that the DNC is not associated with the delayed cytotoxicity caused by dideoxynucleoside analogs and not important in dNTPs uptake into mitochondria. The term "deoxynucleotide carrier" should be reconsidered.

Materials and Methods

Expression of DNC. Human DNC with C-terminal His-tag expression was constructed as reported previously (Dolce et al., 2001). DNC protein was expressed in *Escherichia coli* BL21(DE3) and was purified as described previously (Dolce et al., 2001). The dNTP transport activities of DNC were also assayed as described previously (Dolce et al., 2001).

Reconstitution of DNC. Liposomes were prepared by the size extrusion (MacDonald et al., 1991). Desired amounts of cholesterol or cardiolipin were added to egg yolk or soybean phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in chloroform. Mixtures were dried in vacuum overnight. The dried lipid was dissolved in buffer (20 mM PIPES, pH 6.8, 1 mM EDTA) to a final concentration of 10% (w/v) by intensely vortexing. The mixture was frozen (liquid nitrogen) and thawed (37°C water bath) three times and passed through a 100-nm polycarbonate membrane filter 13 times. Proteoliposomes were generated by the detergent removal method (Palmieri et al., 1995). Then, 826 μl of 20 mM PIPES, pH 6.8, 1 mM EDTA was mixed with 180 μl of 10% Triton X-114, 16 μg of DNC, and 220 μl of 10% liposome. After vortexing, the detergent was removed by passing through the same column packed with 2 ml of Amberlite XAD 1 × 200 13 times. The amount of DNC protein incorporated into liposomes varied between 15 and 25% of the protein added to the reconstitution mixture. [α-^{32}P]dATP, dCTP, and dTTP, 200 μM to 4 mM (100 Ci/mmol), were used to study uptake activity of DNC at 37°C. Dowex ion exchange columns (Sigma-Aldrich, St. Louis, MO) (1 × 3.5 cm) were used to remove the external radioactivity not transported into proteoliposomes (Bridges et al., 1999).

Cell Culture, Transfection, and Cloning of Stable Transfectants. DNC was cloned into the RVYtet vector, a tet-off system (kindly provided by Dr. David Ward, Yale University, New Haven, CT). Site-directed mutagenesis (QuikChange XL; Stratagene, La Jolla, CA) was used to change the nucleotide at 530 from G into C (amino acid at 177 from G to A). Retrovirus was generated by cotransfection of RVYtet-DNC and PVSVG into GP2–293 cells using the standard calcium phosphate method. After 48 h, medium containing retrovirus was used to transfect RKO cells. The transfectants were selected by growing cells in culture medium containing 250 μg/ml hygromycin B and 1 μg/ml doxycycline. Expression of DNC was induced by removing doxycycline from culture medium. Immunofluorescent microscopy was used to check the clones expressing DNC. Clones with homologous expression of DNC were isolated and expanded.

siRNA Transfection. RKO cells (2 × 10^5) grown in RPMI 1640 medium supplemented with 10% fetal bovine serum were plated into six-well plates, 4 h before transfection (day 0). Cationic lipid complexes were prepared by incubating 200 nmol/ml siRNA (DNC sense sequence, 5′-CCUCCAAAACCUGCUUUGUtt-3′; control sequence, 5′-GUUCCUCCAACUUCUAGCAUUtt-3′, synthesized from Dharmacon, Lafayette, CO) with 6 μl of Oligofectamine (Invitrogen, Carlsbad, CA) in 200 μl of Opti-MEM (Invitrogen) for 15 min. The complexes were added to the cells to a final volume of 0.8 ml. After incubation for 4 h, 0.5 ml of RPMI 1640 medium supplemented with 30% fetal bovine serum was added to each well. The transfection was repeated the next day (day 1). On day 2, cells from each well were reseeded in the absence of siRNA, based on the experimental requirements. The thymidine kinase-2 (TK2) siRNA was purchased from Ambion (Austin, TX).

Generation of Monoclonal Antibody and Western Blotting. The purified recombinant DNC protein (50 μg), mixed with Freund's complete adjuvant (Sigma-Aldrich), was used as the antigen to immunize a 4-week-old BALB/c mouse. The mouse was immunized twice more at 2-week intervals with a mixture of DNC protein and Freund's incomplete adjuvant. The mouse was then boosted by injection of DNC protein (25 μg) intravenously. After 4 days, the mouse was killed, and the spleen cells were fused with Sp2/0-Ag14 cells using polyethylene glycol 1000. Hypoxanthine (100 μM)/aminopterin (4 nM)/thymidine (16 μM) was used to select hybridoma cells. Cloning was performed using the limiting dilution. Standard indirect enzyme-linked immunosorbent assay was used to screen for the hybridoma clones producing antibody against DNC. Monoclonal antibody production was demonstrated by Western blotting with total lysate of *E. coli* containing recombinant human DNC protein. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The protein was then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) with a Miniprotein II transferring apparatus (Bio-Rad). The membranes were blocked and probed in PBS-Tween buffer (1× PBS buffer, 0.2% Tween 20) containing 5% nonfat milk. The immunoreactive bands were visualized by enhanced chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Boston, MA), and densitometry scanning was performed with the personal densitometer (Amersham Biosciences Inc., Piscataway, NJ).

Confocal Microscopy. In brief, 10^5 cells were seeded onto 22 × 22-mm glass coverslips in 35-mm culture dishes and incubated overnight. Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized by 0.5% Triton X-100 in PBS. To block nonspecific binding, 1% bovine serum albumin in PBS was used. DNC protein was targeted by DNC monoclonal antibody (C5–1-2) at 1:100 dilution followed by FITC-conjugated anti-mouse IgG at 1:100 dilution. Mitochondria were counterstained with 500 nM Mito-tracker (Molecular Probes, Eugene, OR). Cells were sealed in anti-fade reagent (Molecular Probes). Confocal micrographs were scanned by a laser scan confocal microscope LSM 510 (Carl Zeiss, Thornwood, NY).

Determination of mtDNA Content. Aliquots of cells cultured in the presence or absence of drugs were harvested by centrifugation (800*g*) and were washed twice with PBS. Cell pellets were resuspended in 100 μl of 10 mM Tris-HCl, pH 7.5, and were subjected to three freeze-thaw cycles. The cell lysates were incubated with RNaseA (10 μg/ml) at 37°C for 1 h. The samples were then treated with 100 μg/ml proteinase K at 55°C for 3 h. After incubation, an equal volume of 20× standard saline citrate (1× standard saline citrate is 0.15 M NaCl plus 0.015 M sodium citrate) was added to each sample. The lysates were spotted onto Hybond paper by using Miliford II slot blot apparatus (Schleicher & Schuell, Keene, NH). mtDNA quantification was based on total cell number and was detected with an mtDNA-specific probe. Cellular DNA, as an internal control, was probed by an *Alu*DNA probe, as described previously (Chen et al., 1991). The intensities of the autoradiographic bands were quantified by a scanning densitometer.

Cell Growth Assay. Exponentially growing cells were plated in a 24-well plate (1×10^4 cells/well). After desired time, cells were fixed and stained for 2 h with 0.5% methylene blue in 50% ethanol, followed by washing with tap water to remove unbound dye. Plates were air dried and then cells were dissolved in 1% sarkosyl by shaking at room temperature for 3 h. Cell growth was quantitated based on the amount of methylene blue adsorbed by the cells as measured by a spectrophotometer (Molecular Devices, Sunnyvale, CA) at 595 nm. All experiments were performed in triplicate wells and were repeated at least three times.

Lactic Acid Determination. Lactic acid production in cell culture medium on day 4 was determined by using a lactic acid determination kit based on colorimetric development in an enzymatic reaction: lactic acid and NAD are converted to pyruvic acid and NADH (absorbance, at 360 nm) by lactate dehydrogenase (Sigma-Aldrich).

Mitochondria Potential Determination. After being seeded on coverslips for 1 day, cells were incubated with 1 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide in serum-free medium for 30 min at 37°C (in live cells, this dye exists either as a green-fluorescent monomer at depolarized membrane potentials or as an orange-fluorescent J-aggregate at hyperpolarized membrane potentials). After washing with PBS, the living cells were illuminated at 488 nm, and the emission was collected at 515 nm (green) and 580 nm (red) by using flow cytometry (BD Biosciences, Franklin Lakes, NJ).

Reactive Oxygen Species. The level of reactive oxygen species in cells was measured using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (Molecular Probes). In brief, cells seeded overnight were incubated with 1 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester for 30 min at 37°C. Pancreatin was used to form single cell suspensions that were subjected to flow cytometry. Cells were illuminated at 488 nm, and the emission was collected at 515 nm.

Mitochondria Calcium. The level of mitochondria calcium was determined using rhod-FF-AM (a lower-affinity Ca^{2+} indicator that accumulates primarily in mitochondria). In brief, cells seeded overnight were incubated with 1 μM rhod-FF-AM for 30 min at 37°C. Pancreatin was used to form single cell suspensions that were subjected to flow cytometry. Cells were illuminated at 540 nm, and the emission was collected at 580 nm.

dNTP Uptake and DNA Synthesis in Isolated Mitochondria. Mitochondria of RKO cell lines were isolated by the "two-step" procedure described by Bogenhagen and Clayton (1974). The reactions were performed using the methods described by Chen and Cheng (1992). dATP, dCTP, dGTP (each 3 μM) and 1 μM [α-^{32}P]dTTPs (50 Ci/mmol; Amersham Biosciences Inc.) were used in the assay. Mitochondria (100 μg of protein per reaction for DNA synthesis, 25 μg of protein per reaction for dNTP uptake assay) in a total volume of 0.2 ml were used for each assay. The reactions were carried out at 37°C for 2 min for the uptake assay and 2 h for the DNA synthesis assay. For the DNA synthesis assay, ice-cold 15% trichloracetic acid was used to precipitate acid-insoluble fractions from the reaction. The acid-insoluble pellets were washed three times with 10% trichloracetic acid. The pellets were dissolved in 200 μl of 0.5% sarkosyl in 0.1 M sodium phosphate buffer at pH 7.4.

Results

Dependence of dCTP Uptake on Cardiolipin and Cholesterol Content in Proteoliposomes. It was reported that cardiolipin could promote DNC activity (Dolce et al., 2001). We compared whether cardiolipin could promote dCTP uptake activity of DNC in the liposome prepared with phosphatidylcholine purified from either egg yolk or soybean.

Without cardiolipin, both phosphatidylcholine sources resulted in similar dCTP uptake activity. However, cardiolipin (plateau at 15%) could stimulate dCTP uptake selectively from the liposome prepared from phosphatidylcholine purified from egg yolk but not soybean (Fig. 1).

Many reports had suggested that cholesterol could promote the activity of membrane transporters. We tested whether this phenomenon was applicable to DNC. We found that

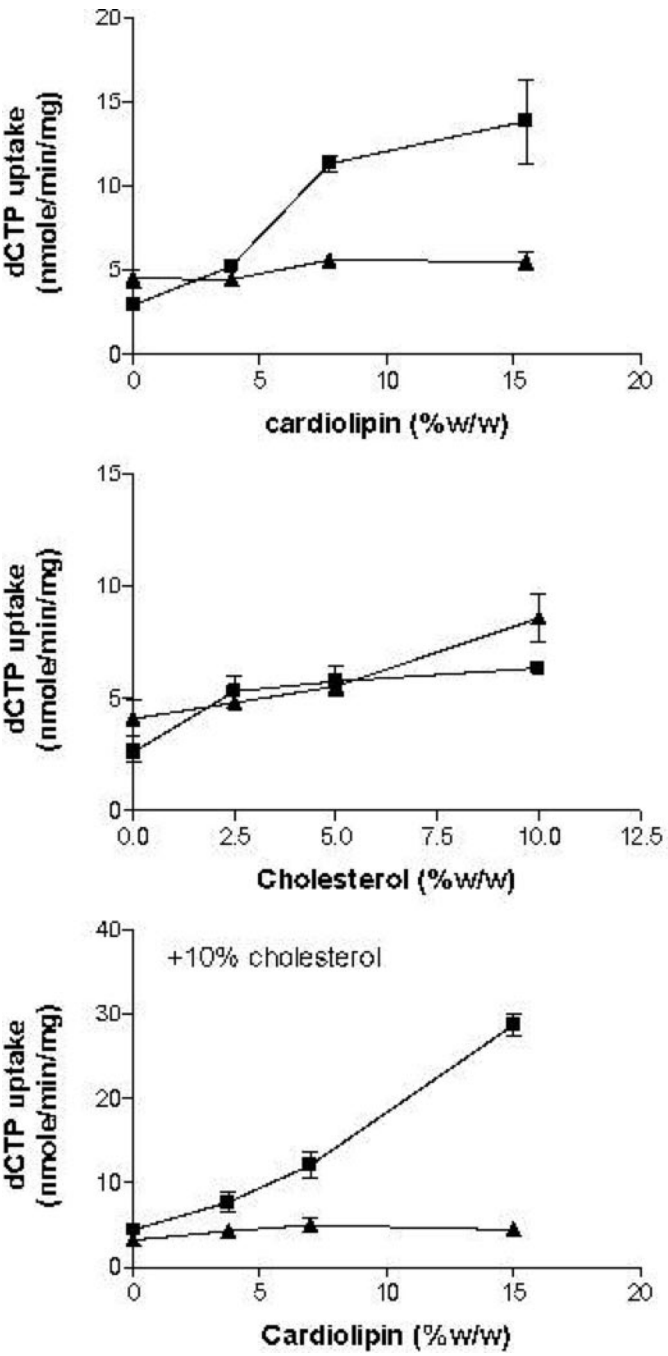

Fig. 1. Dependence of the dCTP uptake on the cardiolipin and cholesterol content in DNC-proteoliposomes formed by using phosphatidylcholine purified from egg yolk (■) or soybean (▲). Uptake was initiated by the addition of 1 mM [α-^{32}P]dCTP for 2 min. The rest of the procedures were the same as described under *Materials and Methods*.

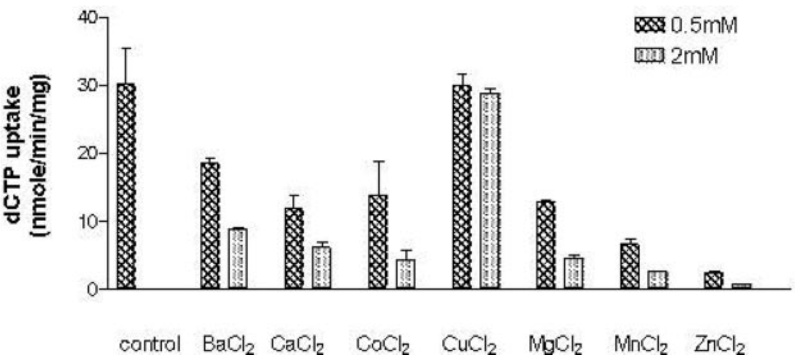

Fig. 2. Effect of divalent metal ions on dCTP uptake activity of DNC.

dCTP uptake activity of DNC could be promoted by cholesterol in either type of phosphatidylcholine. The combined effect of cardiolipin and cholesterol was also studied. Cholesterol could enhance the stimulating activity of cardiolipin at 15% but not at lower concentrations when egg yolk phosphatidylcholine was used (Fig. 1).

Effect of Metal Divalent Ions on dCTP Uptake Activity of DNC. Because our previously described dCTP transporter required calcium to function optimally (Bridges et al., 1999), we tested the effect of different divalent metal ions on the dCTP uptake activity of DNC. We found that none of the divalent metal ions could stimulate the DNC activity. In fact, 2 mM calcium chloride inhibited 70% of the activity of DNC (Fig. 2).

Kinetic Properties of DNC. K_m and V_{max} values of DNC were determined by using an optimized cardiolipin (15%) concentration with or without 10% cholesterol. DNC had higher K_m and V_{max} values for dCTP than those for dATP and dTTP. Internal ADP had little effect on the K_m values but decreased the V_{max} values (Table 1).

The addition of cholesterol increased the V_{max} values by 3-fold for dCTP, 5-fold for dATP, and 1.5-fold for dTTP (Table 1). Cholesterol increased K_m values for dATP but had less impact on that of dCTP and dTTP. When considering relative efficiency (V_{max}/K_m), addition of cholesterol had more impact on dCTP than dATP and dTTP.

Presence and Localization of DNC Protein in Cells. The intrinsic levels of DNC protein in RKO cells could not be detected by our monoclonal antibody. Stable cell lines established by infection with RVYtet-DNC's retrovirus and hygromycin B selection show low levels of DNC protein expression when growing in medium containing 3 μg/ml doxycycline (without affecting the growth of cells). This may be caused by leakage of the tet-off system. DNC protein was highly expressed 72 h after removing doxycycline from the medium (Fig. 3A). The size of DNC protein (36 kDa) expressed from DNC cells or DNC-G177A cells was the same as the DNC expressed from *E. coli*. This indicated that integral DNC protein was imported into the mitochondria (also see confocal pictures) with no apparent postmodification of DNC.

When DNC protein was induced in DNC cells or DNC-G177A cells, bright green fluorescence from anti-mouse-FITC, which recognizes the DNC monoclonal antibody, can be overlapped with the red fluorescence from Mito-tracker, which stains specifically mitochondria (Fig. 3B). Moreover, the green fluorescence from the mitochondria could be blocked by premixing purified DNC protein with the monoclonal antibody-DNC (data not shown). Mutation of G177A did not affect the localization of DNC in mitochondria. Furthermore, the density and shape of the mitochondria of cells expressing mutated G117A DNC did not change significantly compared with that of the cells expressing wild-type DNC.

Effect of DNC Protein Expression on Cell Growth and Mitochondria Functions. DNC protein or mutated G177A DNC expression did not affect the rate of RKO cell growth. We studied several cellular parameters that may be influenced by the functional status of mitochondria, including lactic acid production, mitochondria potential, cellular ATP/ADP level, reactive oxygen species level, glutathione level, and mitochondria calcium. All of the above-mentioned parameters were not affected significantly by the expression of wild-type or G177A mutated DNC (data not shown).

Effect of Overexpression of DNC on the mtDNA Depletion and the Lactic Acid Production Caused by Dideoxynucleoside Analogs (ddC, d4T, and ddI). The ability to cause the mtDNA depletion and induced lactic acid production in RKO cells was ddC > ddI > d4T.

Results indicate that the mtDNA depletion and induced lactic production caused by ddC and ddI were not affected by

TABLE 1

Comparison of kinetic data of reconstituted proteoliposome with or without cholesterol

K_m values were derived from a Lineweaver-Burk plot. V_{max} values were calculated using the Michaelis-Menton equation. Values are presented as mean ± S.D. from at least three independent experiments.

	PC + 15% CL			PC + 15% CL + 10% Cholesterol		
	K_m	V_{max}	Efficiency V_{max}/K_m	K_m	V_{max}	Efficiency V_{max}/K_m
	mM	*nmol/min/mg*		*mM*	*nmol/min/mg*	
dCTP	3.2 ± 0.6	45 ± 5.9	14	5.8 ± 1.1	124 ± 4.9	21
dCTP/iADP	2.1 ± 0.4	18 ± 3.6	8.6	3.0 ± 0.7	44.4 ± 18	14.7
dATP	1.1 ± 0.3	11 ± 1.2	10	3.8 ± 1.0	61 ± 16	16
dATP/iADP	1.7 ± 0.4	7 ± 0.5	4.1	2.7 ± 0.7	12.1 ± 1.6	4.5
dTTP	0.8 ± 0.2	4.9 ± 0.8	6.1	1 ± 0.3	7.5 ± 1.5	7.5
dTTP/iADP	0.6 ± 0.2	3.2 ± 0.6	5.3	0.6 ± 0.2	4.5 ± 1	7.5

CL, cardiolipin; iADP, 10 mM internal ADP; PC, egg yolk phosphatidylcholine.

the overexpression of wt DNC and G177A mutated DNC (two-way ANOVA, $P > 0.05$) (Fig. 4).

A high concentration of d4T (100 μM) caused less mtDNA depletion in induced RKO cells that contained a higher level of DNC ($P < 0.05$) than for control or G177A-mutated DNC (Fig. 4A). However, induced lactic acid production was similar at 100 μM d4T (Fig. 4B). After longer treatment (6 days instead of 4 days), the cells with a lower dose (6 and 25 μM) of d4T did not increase the difference of the inhibitory effect

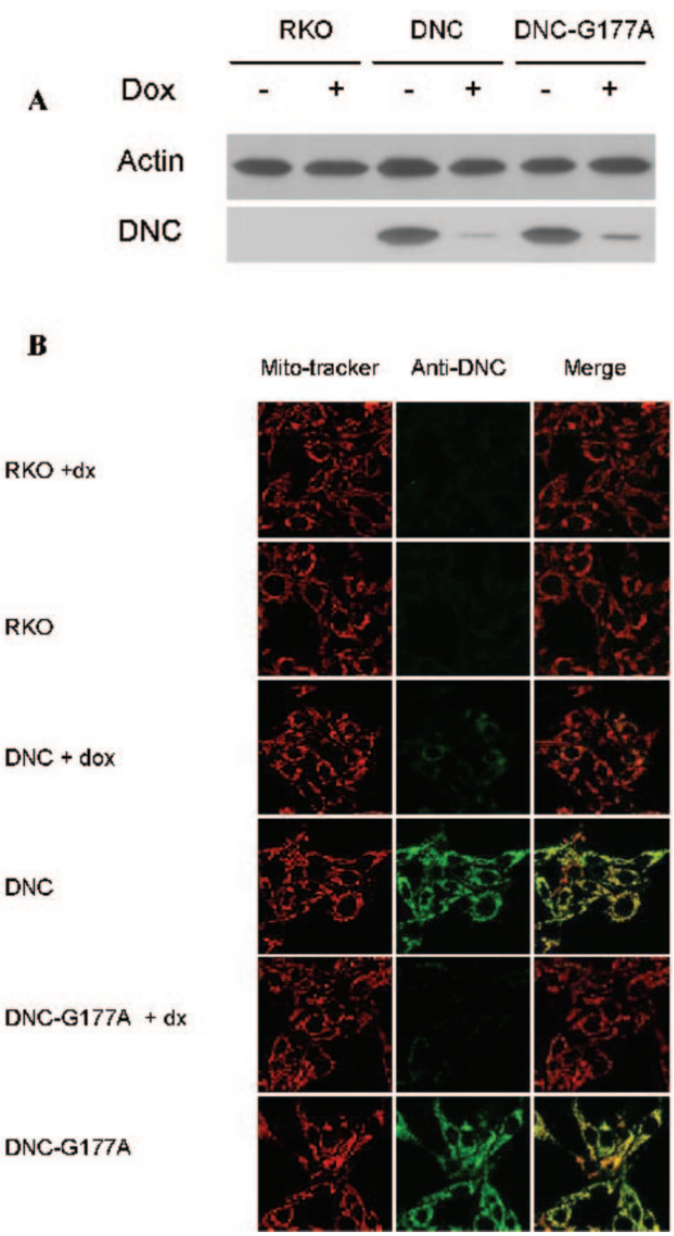

Fig. 3. A, expression of DNC protein in different cell lines. Cell lysates from different DNC inducible cell lines grown with or without 3 μg/ml doxycycline for 72 h were prepared as described under *Materials and Methods*. The cell lysates were analyzed by Western blotting. Top bands were detected by anti-actin monoclonal antibody and used as internal control; bottom bands were detected by anti-DNC monoclonal antibody. B, subcellular localization of DNC protein of RKO-DNC cell lines (400× magnification). Cells with or without 3 μg/ml doxycycline for 72 h were fixed, immunofluorescently stained first with anti-DNC monoclonal antibody and second with anti-mouse IgG-FITC antibody, and mitochondria were counterstained with Mito-tracker as described under *Materials and Methods*. Green indicates DNC; red indicates mitochondria.

of mtDNA between DNC induced and noninduced cells (data not shown). Results from the high dose (100 μM) of d4T and longer treatment (6 days) were not considered because the cell growth inhibition was more than 40% compared with less than 10% cell growth inhibition with the low dose of d4T (data not shown).

Effect of Overexpressing DNC on the Ability of mtDNA to Recover from ddC Treatment. Cell lines with or without overexpressing DNC were treated by ddC at 20 μM for 4 days. mtDNA was measured at different times after reseeding the cells. Results showed that ddC had similar potency in cell lines causing mtDNA depletion down to about 10% of control mtDNA. After removal of the extracellular ddC, the action of intracellular ddC continued to deplete the mtDNA to about 5% of control at 24 h (Fig. 5). After 24 h, the mtDNA began to increase up to ~40% of control at 75 h after ddC removal (Fig. 5). All conditions exhibited similar cell growth rates (data not shown). The rate of mtDNA recovery was not affected by the overexpression of DNC (two-way ANOVA, $P > 0.05$).

Effect of Overexpressing DNC on dTTP Uptake and Incorporation into Isolated Mitochondria. We studied whether overexpressing DNC in mitochondria could alter the rate of dNTP uptake and DNA synthesis in mitochondria. Results showed that the rates of dTTP uptake in mitochondria isolated from wt DNC-induced and noninduced RKO cells were similar (Fig. 6A). The dTTP uptake activity in isolated mitochondria can be inhibited by d4TTP in a dose-related manner. However, there was no difference in the inhibitory effect of d4TTP on dTTP uptake in mitochondria isolated from wt DNC-induced and noninduced RKO cells. In addition, the rates of mtDNA synthesis were similar among RKO control, RKO DNC-induced, and noninduced cells (Fig. 6B). Overexpressing DNC did not change the inhibitory effect of d4TTP on the mtDNA synthesis.

Effect of Down-Regulated Expression of DNC on mtDNA Depletion and the Lactic Acid Production Caused by Dideoxynucleoside Analogs (ddC, d4T, and ddI). We studied whether down-regulating the expression of DNC could cause any difference in mtDNA depletion caused by different dideoxynucleoside analogs. Because our antibody was not sensitive enough to detect the endogenous level of DNC protein in RKO cells, we used DNC-overexpressing cells as a positive control for demonstrating that siRNA could suppress the expression of DNC protein. Western blotting results indicated that DNC could be suppressed by siRNA DNC by almost 90% but not by siRNA control sequence (Fig. 7A). RT-PCR results showed that the mRNA of DNC in RKO and DNC-overexpressing cells could be down-regulated by siRNA DNC (Fig. 7B). The immunofluorescence results agreed with the results of Western blotting (Fig. 7C). Confocal photographs showed that the green florescence emitted from anti-DNC antibodies was reduced by almost 90% in the case of DNC-overexpressing cells treated with siRNA DNC but was not reduced by siRNA control sequence. Treatment of siRNA on RKO cells did not affect cytochrome *c* protein expression (confocal pictures not shown). It is conceivable that the endogenous DNC protein expression could be suppressed substantially.

The treated siRNA cells were tested for depletion of mtDNA and the lactic acid production caused by dideoxynucleoside analogs (ddC, d4T, and ddI). Results indi-

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cated that the mtDNA depletion and induced lactic acid production (data not shown) caused by ddC, d4T, and ddI were not affected by the treatment of siRNA DNC, which could down-regulate DNC protein effectively. (Fig. 8A)

Effect of Down-Regulating the Expression of DNC on dTTP Uptake into Isolated Mitochondria. We also studied whether down-regulating DNC in mitochondria could alter the rate of dTTP uptake in isolated mitochondria. Results showed that the rates of dTTP uptake are similar in mitochondria isolated from RKO and DNC-overexpressing cells treated with control sequence siRNA or target sequence siRNA (Fig. 8B).

Effect of Down-Regulating the Expression of DNC and TK2 on the Mitochondrial DNA Content. We studied whether down-regulating DNC and TK2 could cause mitochondria DNA damage. Results indicated that using siRNA to down-regulate one or both of DNC and TK2 (Fig. 9A) did not affect the mitochondrial DNA content (Fig. 9B) nor the action of dideoxynucleoside analogs in RKO cells.

Discussion

DNC was suggested to play a key role in dNTP uptake into mitochondria. Our studies indicated this was probably not the

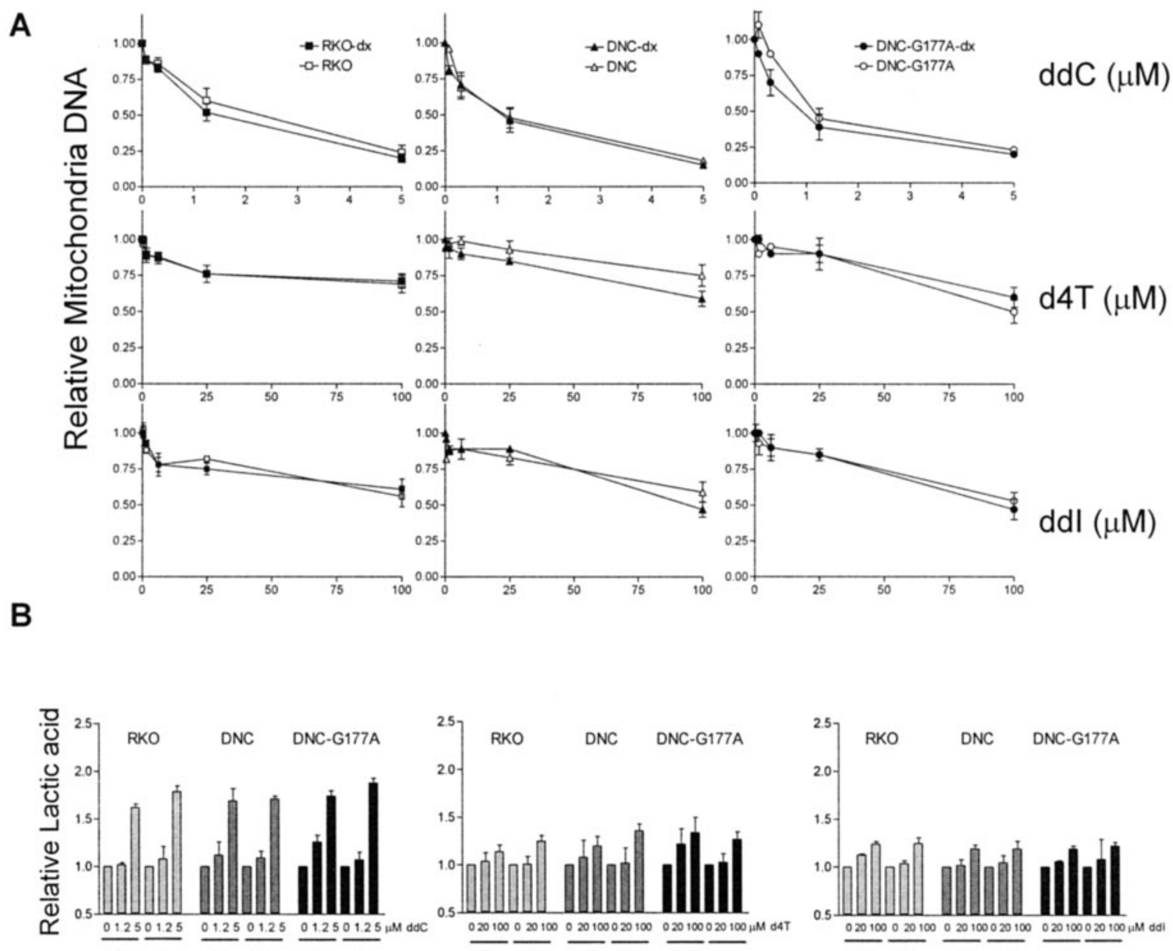

Fig. 4. Inhibitory effect of dideoxynucleoside analogs ddC, d4T, and ddI on the mtDNA content (A) and the lactic acid production (B) of RKO cells. After doxycycline was removed from culture medium for 72 h for DNC induction, cells were treated by dideoxynucleoside analogs for 4 days. Two-way ANOVA analysis or Student's *t* test were used to determine whether there was any significant difference between the results. The rest of the procedures were the same as described under *Materials and Methods*.

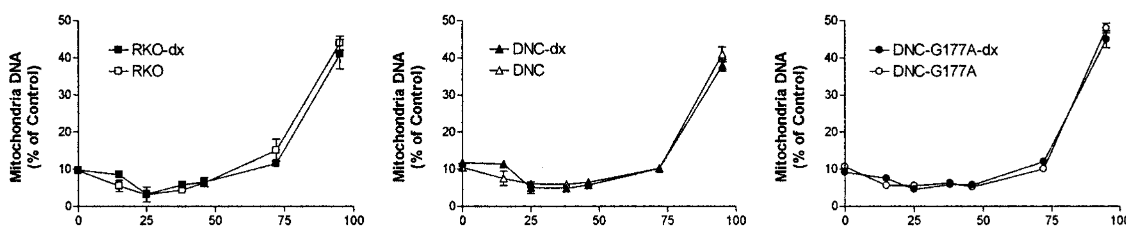

Fig. 5. Recovery of mtDNA of RKO cells. Cells were pretreated with ddC at 20 μM for 4 days. The content of mtDNA was measured at different time points after cells were reseeded in ddC-free medium. The rest of the procedures were the same as described under *Materials and Methods*.

case, and the DNC properties were different from our previously described transport system. First, the activity of reconstituted DNC was cardiolipin-dependent, optimized at 15% (w/w), by using phosphatidylcholine isolated from egg yolk but not from soybean. Our dCTP transport system was not cardiolipin-dependent and behaved better in liposomes prepared by phosphatidylcholine isolated from soybean. Second, calcium inhibited the activity of DNC, whereas calcium stimulated our dCTP transport system by 8-fold. Third, the K_m values (600 μM–5.6 mM) of dNTPs uptake were a hundred- to a thousandfold higher than the K_m (3 μM) value of our dCTP transport system. Indeed, the K_m value of ddCTP and dCTP uptake in isolated mitochondria was about 1.82 and 0.82 μM, respectively (Rossi et al., 1999). DNC may have a very low efficiency for the uptake of dNTPs into mitochondria.

The kinetic results of DNC reported by Dolce et al. (2001) also did not support the idea that DNC could function as a transporter of dNTPs or dNDPs in mitochondria, because the K_m or K_i values of the nucleotides, except ADP, were well above the physiological concentration. The K_i value of ADP is 32 μM, which is only 2-fold higher than the K_i value of dADP (14 μM) when competing with dATP with a K_m value of 100 μM. However, the intracellular concentration of ADP is a hundred- to a thousandfold higher than that of dADP or dNTP. Therefore, it is unlikely that DNC functions as the transporter of dNTP or ddNTP in mitochondria.

The results obtained from the reconstitution experiments in vitro may not reflect the behavior of the protein in cells. This could be caused by nonoptimal renaturing conditions in the reconstitution experiments, the requirement for post-translational modification of proteins, or the lack of other proteins necessary for action. We further studied the relationship between the expression levels of DNC and the action of anti-HIV dideoxynucleoside analogs on the mtDNA depletion inside the cells.

According to our previous reports, ddCTP is formed in the cytoplasm and is transported into mitochondria. If DNC can transport ddCTP into the mitochondria and the incorporation of ddCTP as a chain terminator can cause DNA depletion, the overexpression of DNC is likely to increase the uptake of ddCTP into mitochondria and the depletion of mtDNA. Increased expression of wt DNC protein, however, could not sensitize RKO cells or HepG2 cells (data not shown) to mtDNA depletion caused by ddC, d4T, and ddI. Increased lactic acid production is regarded as the product of compensatory glycolysis that results from inhibition of mitochondria oxidative phosphorylation caused by the above dideoxynucleoside analogs. Our results

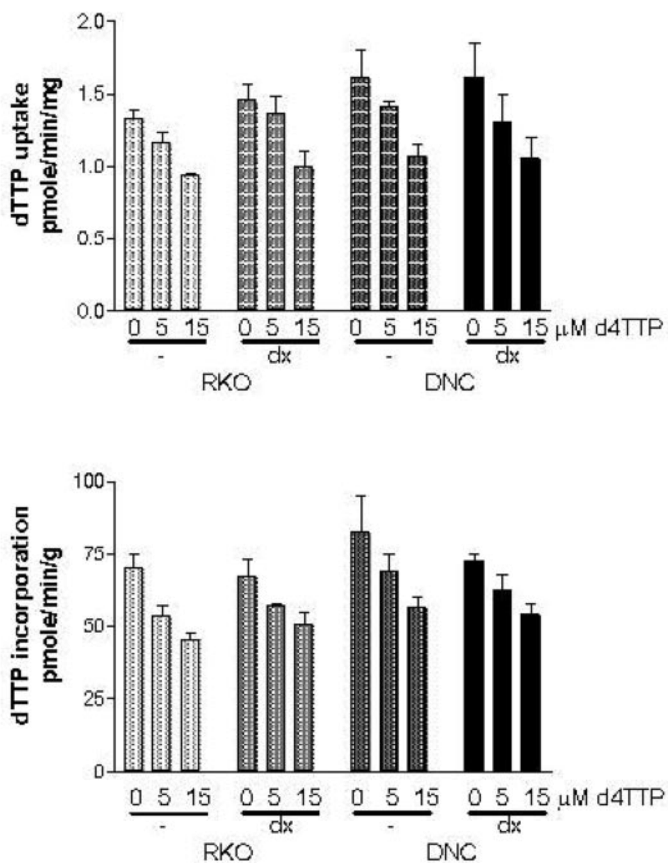

Fig. 6. The uptake (top) and incorporation (bottom) of dTTP into isolated mitochondria from RKO cells. The rest of the procedures were the same as described under *Materials and Methods*.

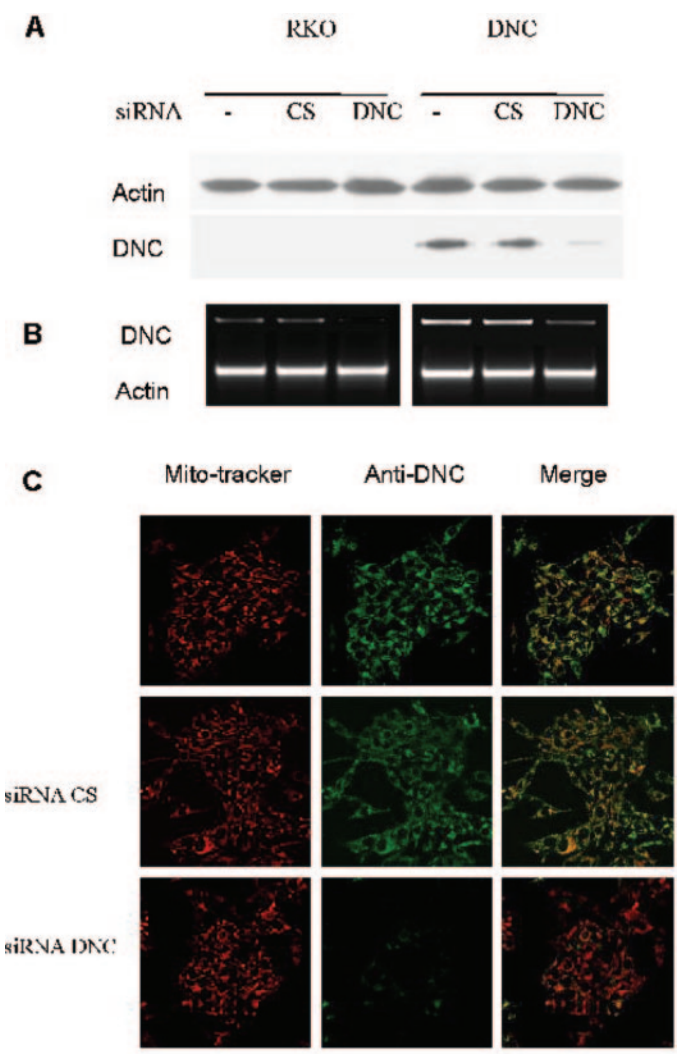

Fig. 7. siRNA-mediated down-regulation of DNC in RKO cells. A, immunoblot shows the DNC content in the cells, and actin has been used as an internal control. B, RT-PCR shows the RNA expression of DNC in the cells, and actin has been used as an internal control. C, immunofluorescence of siRNA-mediated down-regulation of DNC in the DNC expressed RKO cells (400× magnification). The rest of the procedures were the same as described under *Materials and Methods*.

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indicated that lactic acid production was not affected by the overexpression of DNC.

We also studied whether the overexpression of DNC protein could affect the ability of mtDNA to recover from depletion caused by ddC. The lack of recovery in the first 75 h after ddC removal could be caused by the uptake of the remaining cytoplasmic ddCTP or the time required to remove ddC from terminated mtDNA. If dNTPs or dNDPs uptake was the rate-limiting step in mtDNA replication and overexpression of DNC could help the uptake of dNTPs or dNDPs into mitochondria for the repair processes to happen, the recovery rate of mtDNA should be faster. Our results demonstrated that overexpression of DNC did not affect the dynamics of mtDNA content after removing the extracellular ddC. The remaining ddC inside the cells continued to cause similar mtDNA depletion under both noninduced and DNC-induced conditions. The time and rate of mtDNA rebound were also similar under all conditions.

When we examined whether the dTTP uptake in mitochondria had been changed by overexpression of DNC, we found that the induction of DNC did not increase the dTTP uptake or DNA synthesis in isolated mitochondria. Moreover, the inhibitory effect of d4TTP on the dTTP uptake and DNA synthesis in isolated mitochondria was not affected by the overexpression of DNC.

At this point, we could not discount the effect of DNC on transport completely, because of the possibility that the intrinsic level of DNC protein could already be well above the rate-limiting level. We therefore down-regulated the DNC expression level by using siRNA. Results showed that cells with the down-regulated DNC cells did not become more resistant to these dideoxynucleoside analogs. The results on studies of the over- and underexpression in tandem suggested that DNC could not be the transporter associated with the depletion of the mtDNA caused by these dideoxynucleoside analogs, because even the knockdown DNC cells did not have mtDNA damage. The ratio of mtDNA and genomic DNA in overexpressed DNC and down-regulated DNC cells was similar. However, we cannot rule out the possibility of DNC being involved in transporting dNTPs or dNDPs. The salvage pathway that is carried out by deoxyguanosine kinase, TK2, dNMP kinase, and dNDP kinase can supply enough dNTPs for mtDNA synthesis (Mandel et al., 2001; Saada et al., 2001). Therefore, we isolated mitochondria from down-regulated DNC (siRNA-treated) cells to study dNTPs uptake into mitochondria. We found that dTTP uptake in isolated mitochondria were not affected by down-regulating the DNC expression. Furthermore, we used siRNA to lower both DNC and TK2 expression. TK2 has been shown to play a key role in phosphorylating dC and dT (Wang et al., 1999). If we shut down TK2, mtDNA synthesis will depend solely on the importing of dNTP or dNDP from the cytoplasm. Our results indicated that mtDNA content and the action of dideoxynucleoside analogs on mtDNA depletion was not affected by down-regulation of DNC and TK2 expression (Fig.

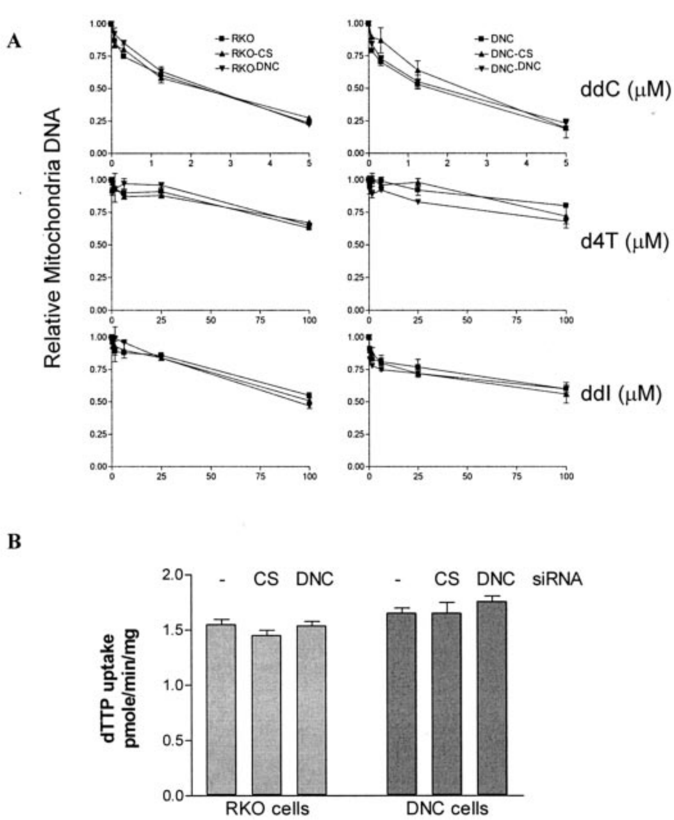

Fig. 8. Effects of down-regulation of DNC. A, inhibitory effect of dideoxynucleoside analogs ddC, d4T, and ddI on the mtDNA content of RKO cells pretreated with siRNA. After doxycycline was removed from culture medium for 72 h for DNC induction, pretreated-siRNA cells were treated with dideoxynucleoside analogs for 4 days. Two-way ANOVA analysis was used to determine whether there was any significant difference between the results. B, uptake of dTTP into isolated mitochondria from siRNA-pretreated RKO and DNC-overexpressing cells. The rest of the procedures were the same as described under *Materials and Methods*.

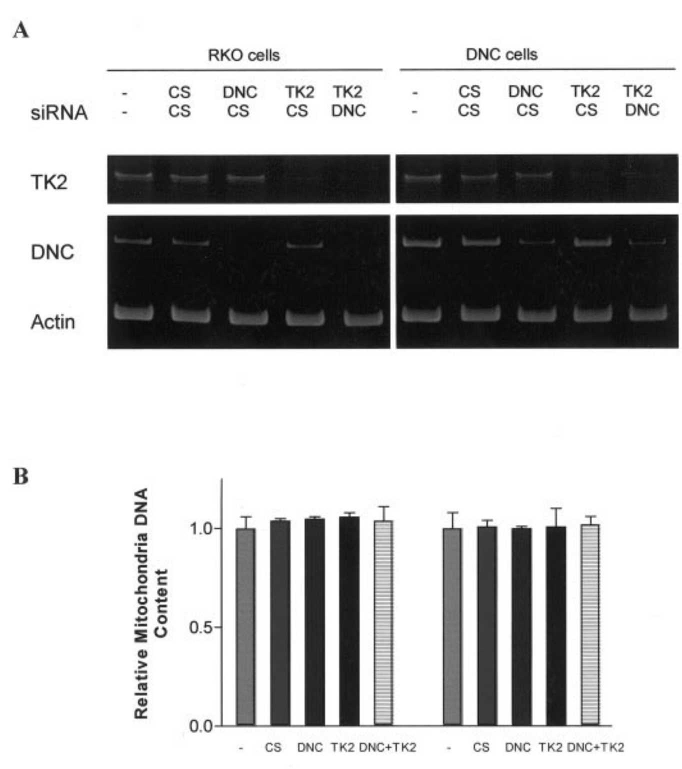

Fig. 9. Effect of down-regulation of DNC and TK2 on mitochondria DNA content. A, RT-PCR shows for the expression of DNC and TK2 in RKO cells and DNC cells after siRNA treatment. B, relative mitochondria DNA in RKO cells and DNC cells (4 days) after siRNA treatment. The rest of the procedures were the same as described under *Materials and Methods*.

9), again suggesting that DNC could not be the key transporter for dNTP or dNDP uptake into mitochondria.

In summary, our findings do not support the postulate that DNC could be associated with the mtDNA depletion caused by the dideoxynucleoside analogs ddC, d4T, and ddI or that DNC can act as a dNTP transporter into mitochondria. The mitochondrial transporter(s) for deoxynucleoside triphosphate and dideoxynucleoside triphosphate analogs remains to be discovered. Based on the evidence that DNC does not seem to affect the mitochondria potential, mitochondrial Ca^{2+} storage, reactive oxygen species of cells, and cellular growth, its function warrants further clarification.

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