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Quantitation of Mitochondrial DNA in Human Lymphoblasts by a Competitive Polymerase Chain Reaction Method: Application to the Study of Inhibitors of Mitochondrial DNA Content

HAO ZHANG, DAVID A. COONEY, AJAY SREENATH, QIMIN ZHAN, RIAD AGBARIA, EMILY E. STOWE, ALBERT J. FORNACE, JR., and DAVID G. JOHNS

Laboratory of Medicinal Chemistry (H.Z., D.A.C., A.S., R.A., E.E.S., D.G.J.) and Laboratory of Molecular Pharmacology (Q.Z., A.J.F.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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SUMMARY

With increasing awareness of the mitochondrial toxicity associated with certain 2′,3′-dideoxynucleosides used in anti-human immunodeficiency virus therapy, procedures for quantitative analyses of drug effects on mitochondrial DNA (mtDNA) have assumed enhanced importance. For this reason we have developed a method to measure the copy numbers of mtDNA in cultured MOLT-4 cells. First a hybrid competitive DNA template was synthesized by conventional polymerase chain reaction (PCR), using two custom-synthesized 40-mer composite primers incorporating mitochondrial displacement loop sequences linked by a non-mitochondrial cDNA template (a 76-base pair sequence from the *tat/rev* region of human immunodeficiency virus cDNA). For the competitive assay, increasing known copy numbers of the hybrid competitive template were added as an internal control to samples containing total cellular DNA. With this approach, two competitive PCR products were generated, 1) a mitochondrial displacement loop-derived fragment (182 base pairs) and 2) a competitive DNA template-derived fragment (156 base pairs). Absolute quantitation was achieved by radiometric comparison of the relative amounts of the two products. To test the versatility of this method, varying amounts of competitive template (6.6×10^4 to 6.6×10^9 copies) were used with a fixed quantity of total cellular DNA taken from cells cultured for 9 days in the presence or absence of selected pyrimidine and purine dideoxynucleosides. The results showed that the copy number of cellular mtDNA is 823 ± 71 copies/cell in MOLT-4 cells. Little selective depletion of mtDNA, compared with total cellular DNA, was seen with the purine dideoxynucleosides examined; however, when the cells were exposed to the pyrimidine dideoxynucleoside 2′,3′-dideoxycytidine (50 nM) for 9 days, mtDNA content was specifically depleted, although total cellular DNA decreased by only 10%. Thus, in addition to the presently used methods of assessing mitochondrial impairment, i.e., Southern blot analysis and electron microscopy, the competitive PCR method provides a third and convenient assay, with particular applicability to determination of mtDNA in very small numbers of cells.

Several factors limit the application of conventional PCR to gene quantitation. Firstly, with conventional PCR the absolute quantity of PCR product does not necessarily bear a linear relationship to the abundance of the target DNA, because the amplified DNA, although it increases exponentially with cycle number, eventually reaches a plateau (1). Secondly, the kinetics of each amplification reaction depend not only upon the initial amount of target DNA but also upon the length of the products, the selectivity of priming sequences, unknown inhibitors in the test samples, and variations in the concentrations of reaction components (2). Recently, CPCR has emerged as a quantitative adaptation of the PCR method for gene quantitation (3). A competitor nucleic acid, having primer sequences identical to those of the target gene but differing from the target DNA by modifications such as a small intron or deletion or mutated internal restriction enzyme site, is mixed with sample DNA before amplification. Thus, known numbers of copies of a synthetic modified internal standard are introduced into the PCR mixture with an unknown sample, and the internal standard is coamplified with the same primers as the target gene. The competitor PCR product is separated from the product derived from the sequence of the target gene by electrophoresis and is distinguished by either size, restriction endonuclease cleavage, or specific hybridization. Absolute gene quantitation is obtained by radiometric or densitometric comparison of the relative amounts of the two products (3).

We have developed a quantitative CPCR method to accurately measure the copy numbers of mtDNA in cultured human T lymphoblastic cells and have applied the method to study the mitochondrial toxicity of prototype dideoxynucleosides of

ABBREVIATIONS: PCR, polymerase chain reaction; CPCR, competitive polymerase chain reaction; D-loop, displacement loop; mtDNA, mitochondrial DNA; ddCyd, 2′,3′-dideoxycytidine; ddIno, 2′,3′-dideoxyinosine; 2′-β-F-ddAdo, 2′-β-fluoro-2′,3′-dideoxyadenosine; HIV, human immunodeficiency virus; bp, base pairs.

TABLE 1

Oligonucleotide primers

Primers mt06 and mt187 were used for amplification of the D-loop region of the heavy strand of mtDNA. Primers Mimic 06 and Mimic 187 were used for competitive DNA template synthesis.

Primer	5′–3′ Sequence	Complementary site
mt06	CAGGTCTATCACCCTATTAA	Light chain 6–25
mt187	CGCCTGTAATATTGAACGTA	Heavy chain 168–187
Mimic 06	CAGGTCTATCACCCTATTAA-GCCTTAGGCATCTCCTATGG	Light chain 6–25 *rev/tat* 5953–5972
Mimic 187	CGCCTGTAATATTGAACGTA-GGTTGCATTACATGCACTAC	Heavy chain 168–187 *rev/tat* 6049–6068

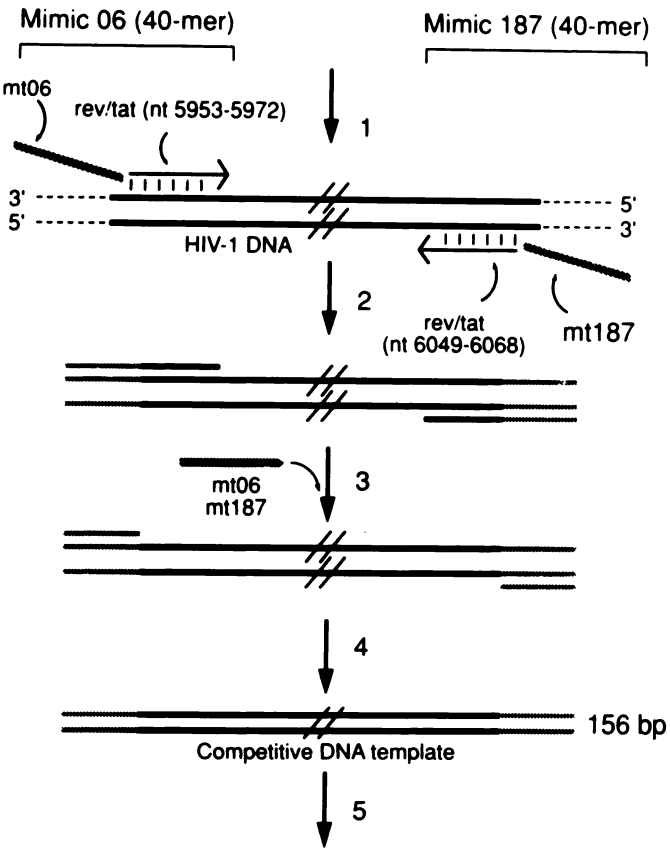

Fig. 1. Schematic diagram illustrating the synthesis of the competitive DNA template for CPCR. A set of primers (Mimic 06 and Mimic 187) were synthesized as described in Materials and Methods. Amplification with these primers results in a PCR product (156 bp) that contains a 116-bp sequence identical to a fragment of the HIV *rev/tat* region and that, as a competitive DNA template, can bind selectively to primer mt06 and primer mt187 (step 1, annealing; step 2, PCR amplification with Mimic 06 and Mimic 187; step 3, amplification with mt06 and mt187; step 4, purification of competitive DNA template; step 5, calculation of copy numbers of competitive DNA). Copy number of the competitive DNA template is calculated from the following equation: copy number = [product (g)/molecular weight of competitive template (g/mol)] × 6.02 × 10^{23}.

clinical interest, i.e., a known mitochondrial toxic agent, namely the pyrimidine dideoxynucleoside ddCyd (4–6), and two purine dideoxynucleosides, ddIno (7) and 2′-β-F-ddAdo (8). In general, however, this method is adaptable to any investigation in which quantitation of mtDNA is required. A preliminary account of some of these studies has appeared (9).

Materials and Methods

Chemicals and reagents. [γ-^{35}S]dATP (1000–1500 Ci/mmol, 12.5 mCi/ml) was purchased from DuPont-NEN (Boston, MA). AmpliTaq DNA polymerase, Stoffel fragment, GeneAmp PCR core reagents, the AmpliTaq cycle sequencing kit, the GeneAmp PCR system 9600, agarose for the separation of GeneAmp PCR products, and other reagents for the PCR assay were purchased from Perkin-Elmer Cetus (Norwalk, CT). Proteinase K and the DNA isolation system were purchased from BRL (Gaithersburg, MD). Bromphenol blue and RNase A was obtained from Sigma Chemical Co. (St. Louis, MO). ddCyd, ddIno, and 2′-β-F-ddAdo were provided by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Primers were custom-synthesized by Lofstrand Laboratories (Gaithersburg, MD). HIV-1 cDNA (PHXB2), which contains 9.718 kilobases of HIV-1 cDNA sequence (10), was kindly provided by Dr. Franco Lori, National Cancer Institute.

Cells. MOLT-4 cells (American Type Culture Collection) were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated (56° for 60 min) fetal calf serum, 44 μg/ml gentamicin, and 4 mM L-glutamine, at 37° in a humidified atmosphere of 95% air/5% CO_2. To determine the effect of ddCyd, ddIno, or 2′-β-F-ddAdo on cell growth, the cells (2×10^5 cells/ml) were treated with various concentration of drug. Every other day, cells were rediluted into fresh culture medium containing freshly prepared drug. Cell numbers were determined by means of a Coulter counter. The cells grew exponentially until the cell doubling time was affected by the drug. The estimation of doubling time was based on the increase in cell number determined at 2-day intervals.

Extraction of total cellular DNA from MOLT-4 cells. Cells were washed twice with phosphate-buffered saline. The cells ($1–5 \times 10^7$) were suspended in 0.5 ml of lysis buffer (0.32 M sucrose, 0.01 M Tris·HCl, pH 7.8, 0.005 M $MgCl_2$, 1%, w/v, Triton X-100) and stored at −70° until use. Five volumes of saline-EDTA (0.075 M NaCl, 24 mM EDTA, 0.5% sodium dodecyl sulfate, 1 mg/ml proteinase K) were added. The cell lysate was incubated at 37° for 16 hr and deproteinized by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:25:1, v/v) and then with chloroform/isoamyl alcohol (25:1, v/v). DNA was precipitated with 0.2 volume of 5 M NaCl and 2 volumes of ethanol at −70° for 1 hr and was rinsed with 70% ethanol. The precipitated DNA was then recovered in 10 mM Tris·HCl, 0.1 mM EDTA, pH 8.0. The sample was treated with DNase-free RNase at 37° for 3 hr and extracted with phenol; the DNA was precipitated overnight at −20° with 3 volumes of cold ethanol. The precipitate was then dissolved in 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and the total DNA concentration was measured as described by Chen and Cheng (4).

Primers. For conventional PCR, two primers were synthesized, i.e., the sense primer (mt06), containing a sequence derived from the mitochondrial D-loop region, and the antisense primer (mt187), containing the sequence derived from the complementary mitochondrial D-loop strand. For competitive DNA template synthesis, two 40-mer primers were constructed. One primer (Mimic 06) contained the mt06 20-mer sequence, with another 20-mer sequence identical to the nucleotide 5953–5972 fragment of the *tat/rev* region of the HIV-1 cDNA

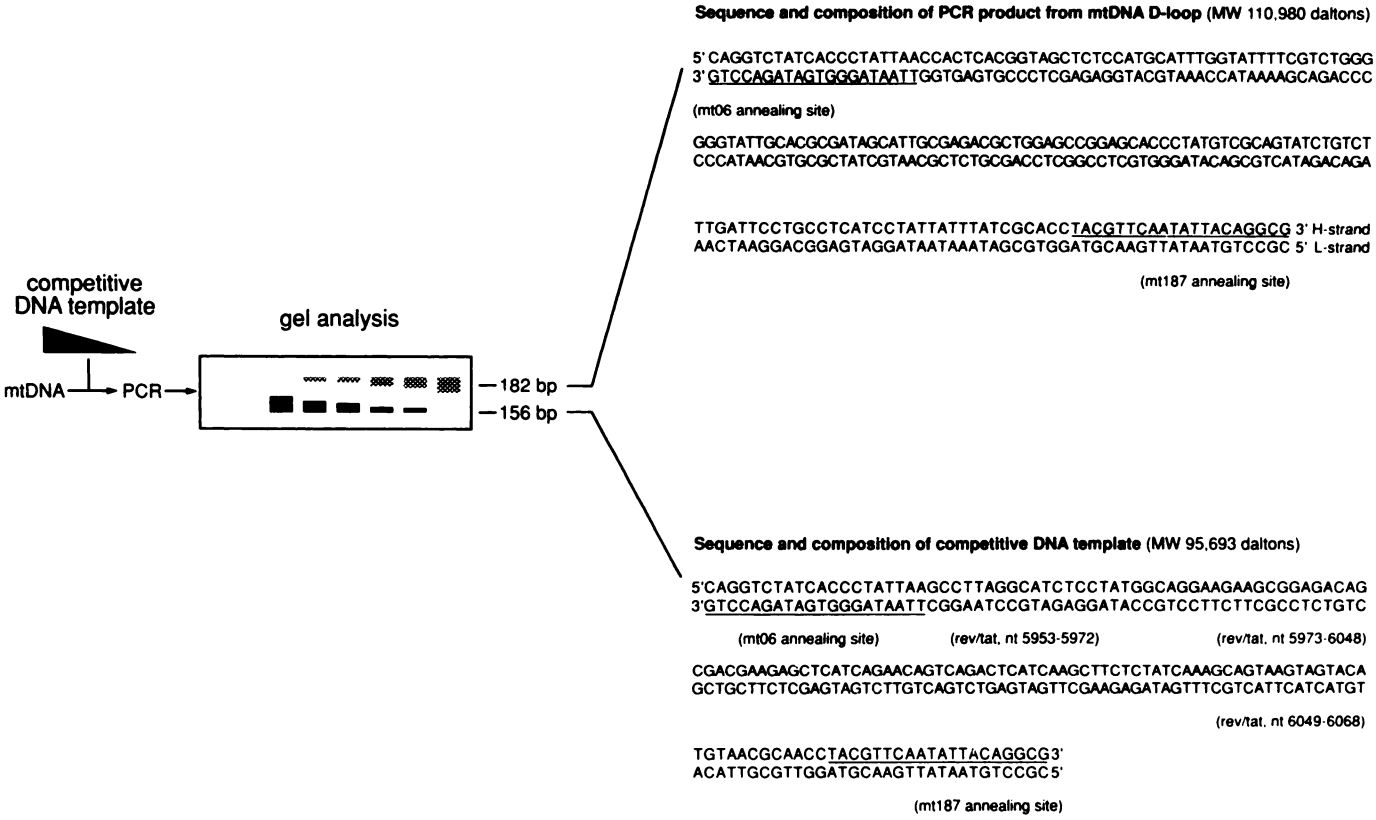

Fig. 2. Schematic diagram illustrating the procedure for mtDNA quantitation by CPCR. As shown, varying amounts of competitive DNA template were coamplified with fixed quantities of total cellular DNA, i.e., known numbers of copies of the competitive DNA template were introduced into DNA samples containing unknown numbers of mtDNA copies and were amplified with primers mt06 and mt187. As shown, two products were generated, one derived from mtDNA (182 bp) and the other derived from the competitive DNA template (156 bp). Sequences and composition of these two PCR products are shown. Competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of the signal for the competitive template-derived product to the signal for the mitochondrial D-loop sequence-derived product (corrected for the 95.7:110.0-kDa molar ratio) versus the logarithm of the copy number of the added competitive template. The copy numbers of mitochondrial D-loop regions in the reaction mixtures were then calculated as described in Materials and Methods.

strand (11)[1]; the other primer (Mimic 187) contained the mt187 20-mer sequence, with an additional 20-mer sequence complementary to the nucleotide 6049–6068 fragments of the *tat/rev* region of the HIV-1 cDNA strand. The complementary sites and sequences (5′ to 3′) of these primers are listed in Table 1.

Synthesis of competitive DNA template. The reaction mixture (100 μl) contained 2 μl of HIV cDNA (PHXB2)[1] (100 ng), 1 μM concentrations of primers Mimic 06 and Mimic 187, 1.56 mM $MgCl_2$, 10 μM concentrations of each of the four deoxynucleoside triphosphates, 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.3, 0.01% gelatin), and 1.5 units of AmpliTaq DNA polymerase, Stoffel fragment. Thirty PCR cycles were performed (1 min at 95°, 1 min at 65°, and 1 min at 72°). Ten microliters of the PCR mixture were then amplified in the presence of 1 μM concentrations of primers mt06 and mt187, under the same conditions. The reaction products were analyzed by electrophoresis on 2% agarose gels and purified by gel chromatography and ultrafiltration (12); the copy number of the competitive DNA template was calculated from the following equation: copy number of competitive DNA template = [competitive template (g)/molecular weight of competitive template (g/mol)] × 6.02 × 10^{23}. Using this method, microgram amounts (10^{12} to 10^{13} copies) of competitive DNA template could be readily obtained. A schematic diagram illustrating the preparation of the competitive template is shown in Fig. 1.

CPCR. The reaction mixture (100 μl) contained 2 μl of total cellular DNA (25.5–255 ng, equivalent to 850–8500 cells), with or without the addition of known numbers of copies of competitive DNA template (6.6 × 10^4 to 6.6 × 10^9 copies), with 1 μM concentrations of primers, 1.56 mM $MgCl_2$, 10 μM concentrations of each of the four deoxynucleoside triphosphates, 10 μCi of [^{35}S]dATP, 10 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.3, 0.01% gelatin), and 1.5 units of AmpliTaq DNA polymerase, Stoffel fragment. Reactions omitting either total cellular DNA or competitive DNA template were run as negative controls. Thirty PCR cycles were performed (1 min at 95°, 1 min at 65°, and 1 min at 72°). The reaction products were analyzed by 2% agarose gel electrophoresis. Gels were stained with ethidium bromide for visualization under UV light. The gels were then dried, and the radioactivity of the mitochondrial and competitive template product bands was measured with a Betascope (Betagen) (13). Alternatively, the product bands were cut out and radioactivity was measured by liquid scintillation counting (14). Competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of the signal for the competitive template-derived product to the signal for the mitochondrial D-loop sequence-derived product (corrected for the 95.7:110.0-kDa molar ratio) versus the logarithm of the copy number of added competitive template; copy numbers of mitochondrial D-loop regions in a given DNA sample were calculated as decribed previously. The complete sequences of the competitive DNA template and the mtDNA product are shown in Fig. 2. Cellular mtDNA was calculated and expressed as copy number per cell.

Analysis of PCR products. Agarose gel electrophoresis was performed using 2% agarose, 1× TAE buffer (0.4 M Tris acetate, 0.02 M disodium EDTA), and 0.5 μg/ml ethidium bromide; samples were mixed with 0.25 volume of loading buffer (40% sucrose, 0.1 M EDTA, 0.25%

[1] HIV DNA sequences were used in this study because of their ready availability in this laboratory. Any appropriate cDNA sequences, however, including a deletion-modified or intron-modified mtDNA sequence, could be used for the preparation of the competitive template.

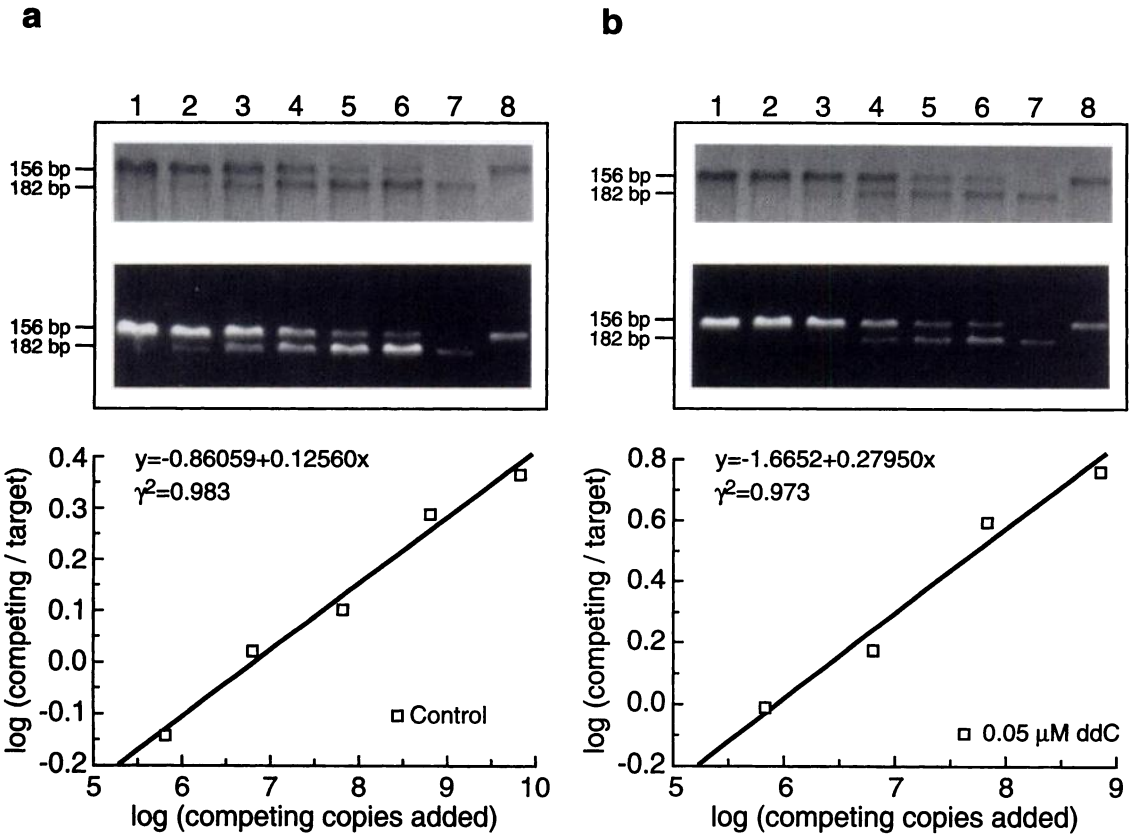

Fig. 3. a, Quantitation of mtDNA in MOLT-4 cells by CPCR. *Upper*, known numbers of copies of the competitive DNA template were introduced into the DNA samples derived from MOLT-4 cells and were amplified with primers mt06 and mt187. As indicated, two products were generated, one derived from the mtDNA (182 bp) and the other derived from the competitive DNA template (156 bp). *Lanes 1-6*, coamplification of varying amounts of competitive DNA template (6.6×10^9, 6.6×10^8, 6.6×10^7, 6.6×10^6, 6.6×10^5, and 6.6×10^4 copies, respectively) with 255 ng of total cellular DNA; *lane 7*, cellular DNA alone (control); *lane 8*, competitive DNA template alone. *Lower*, competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of the signal for the competitive template-derived product to the signal for the mitochondrial D-loop sequence-derived product (corrected for molar ratio) versus the logarithm of the copy number of the added competitive template, and the copy numbers of mitochondrial D-loop regions in the reactions were then calculated as described in Materials and Methods. b, Inhibition of mtDNA synthesis by ddCyd, as determined by CPCR. *Upper*, known numbers of copies of competitive DNA template were introduced into the DNA samples derived from MOLT-4 cells exposed to ddCyd and were amplified with primers mt06 and mt187. The two PCR products are shown in Fig. 2. *Lanes 1-6*, coamplification of varying amounts of competitive DNA template (6.6×10^9, 6.6×10^8, 6.6×10^7, 6.6×10^6, 6.6×10^5, and 6.6×10^4 copies, respectively) with 255 ng of cellular DNA (50 nM ddCyd); *lane 7*, cellular DNA alone; *lane 8*, competitive DNA template alone. *Lower*, competition equivalence points were determined by interpolation on plots of the logarithm of the calculated ratio of the signal for the competitive template-derived product to the signal for the mitochondrial D-loop sequence-derived product (corrected for molar ratio) versus the logarithm of the copy number of added competitive template, and the copy numbers of mitochondrial D-Loop regions in the reactions were calculated as described in Materials and Methods.

bromphenol blue) before electrophoresis for 2 hr at 4 V/cm. Gels were photographed using a Reprostar UV transilluminator (300 nm; CAMAG) and Polaroid type 57 instant film.

Southern blot analysis of mtDNA and chromosomal DNA. For Southern blot analysis, 10 μg of purified cellular DNA from ddCyd-treated or ddIno-treated MOLT-4 cells were digested with *Bam*HI and electrophoresed on 0.7% agarose gels. DNA in the gel was transferred to nitrocellulose paper (4). The mtDNA D-loop fragment (182 bp), generated by PCR using primers mt06 and mt187, was labeled with ^{32}P as described previously (13). This fragment was used as the probe for mtDNA quantitation. A human β-actin probe was used for chromosomal DNA normalization.

Results

Quantitation of mtDNA in MOLT-4 cells. Known numbers of copies of the 156-bp competitive DNA template were introduced into the DNA samples derived from MOLT-4 cells and were amplified with primers mt06 and mt187, as described in Materials and Methods. The amount of total cellular DNA utilized in individual experiments ranged from 25.5 to 255 ng, although most studies were carried out with 25.5–50 ng of cellular DNA. A typical experiment is depicted in Fig. 3a; amounts of competitive DNA template varying from 6.6×10^4 to 6.6×10^9 copies were coamplified with a fixed quantity of total cellular DNA (255 ng). Linearity was found to be maintained over at least 5 orders of magnitude when the logarithmic competing copy numbers were plotted versus the logarithmic ratio of the number of 156-bp products derived from the competitive template to the number of 182-bp products derived from the mitochondrial target sequence. In repeated studies, triplicate analyses of total cellular DNA samples from three separate experiments yielded mean mtDNA copy numbers of 823 ± 71/MOLT-4 cell.

Differential effects of dideoxynucleotides on mtDNA content and on cell growth. We next measured the copy numbers of mtDNA in MOLT-4 cells exposed to ddCyd, a known inhibitor of mtDNA content (4–6). Inhibition of growth

TABLE 2

Effects of dideoxynucleosides on cell growth, mtDNA copy number, and total cellular DNA content

Data are the means ± standard deviations of two experiments, each performed in duplicate (four determinations). Cells (initially 2 × 10^5 cells/ml) were treated with various concentrations of dideoxynucleoside. Every other day, cells were rediluted into fresh culture medium with fresh drug. Cell doubling time was estimated based on the increase in cell number determined at 2-day intervals. Cellular copy numbers of mtDNA and total cellular DNA content on day 9 of dideoxynucleoside treatment were determined as described in Materials and Methods.

Dideoxynucleoside	Concentration	Doubling time	Cellular DNA	mtDNA/total DNA	mtDNA
	μM	*hr*	*pg/cell*	*copies/pg*	*copies/cell*
ddCyd	0	24.5 ± 3.0	30 ± 4	24.0 ± 2.2	720
	0.05	34.5 ± 3.5	27 ± 4	4.7 ± 0.5	127
	0.10	40.6 ± 4.4	22 ± 3	3.7 ± 0.4	81
	0.20	43.4 ± 4.6	16 ± 2	0.12 ± 0.02	2
	0.40	53.5 ± 5.2	14 ± 2	<0.01	<1
ddIno	0	23.1 ± 1.1	34.4	27 ± 2	918
	5	23.2 ± 1.5	36 ± 3	24 ± 2	864
	25	22.7 ± 1.4	44 ± 6	22 ± 2	968
	50	29.3 ± 3.1	30 ± 4	14 ± 1	420
	250	58.1 ± 6.8	19 ± 1	2.0 ± 0.3	38
2′-β-F-ddAdo	0	23.0 ± 2.0	35 ± 3	23.5 ± 3.0	823
	5	22.5 ± 2.5	32 ± 3	27.5 ± 3.4	880
	25	22.9 ± 2.4	37 ± 3	22.6 ± 3.1	836
	50	27.3 ± 3.3	39 ± 4	20.2 ± 2.7	780
	250	37.4 ± 4.2	31 ± 4	17.5 ± 2.5	542

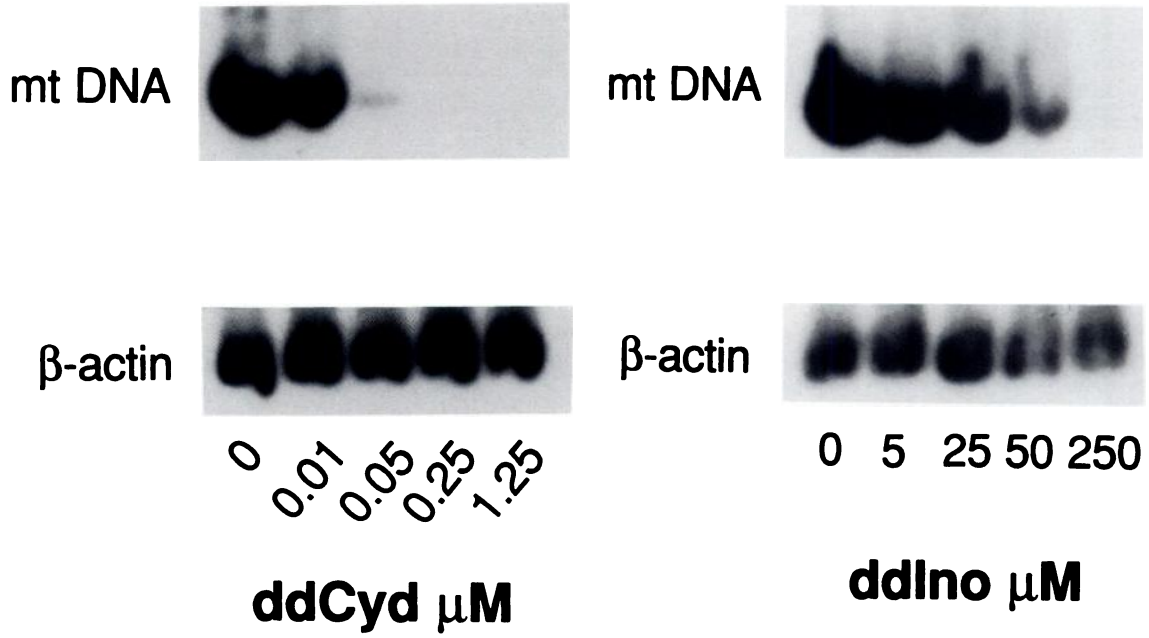

Fig. 4. *Left*, Southern blots showing inhibition by ddCyd of mtDNA in MOLT-4 cells. MOLT-4 cells (2 × 10^5 cells/ml at time 0) were exposed to a range of ddCyd concentrations (0.01–1.25 μM), with redilution in freshly prepared medium with drug every 2 days. At 9 days, cellular DNA (10 μg) was extracted, purified, digested with *Bam*HI, electrophoresed through 0.8% agarose gels, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled 182-bp mtDNA probe, as described in Materials and Methods. *Lane 1*, control; *lane 2*, 0.01 μM ddCyd; *lane 3*, 0.05 μM ddCyd; *lane 4*, 0.25 μM ddCyd; *lane 5*, 1.25 μM ddCyd. *Upper*, mtDNA. *Lower*, same nitrocellulose paper as used for the mtDNA determination, reprobed with a β-actin cDNA probe after removal of the mtDNA probe. *Right*, inhibition by ddIno of mtDNA in MOLT-4 cells. Procedures were the same as for *left*, except that the range of ddIno concentrations examined was 5–250 μM and the total cellular DNA extracted was 15 μg.

of MOLT-4 cells by ddCyd was first examined. When the cells were treated for 9 days with ddCyd over a wide range of concentrations, the doubling times of the cells exposed to the highest ddCyd level (400 nM) increased from 24 hr to 53 hr. However, ddCyd at much lower levels (50 nM) preferentially depleted mtDNA; at this concentration, mtDNA was found to decrease from 720 to 127 copies/cell, whereas total cellular DNA decreased by only 10% (from 30 to 27 pg/cell) (Fig. 3b; Table 2).

The pronounced differences in the effects of ddCyd between the drug-induced decrease in mtDNA content and the other changes observed, such as increased cell doubling time and decreased total DNA, were not a general property of dideoxynucleosides; for example, when the purine dideoxynucleosides ddIno and 2′-β-F-ddAdo were examined under identical conditions, no differential or selective toxicity was demonstrable. With the latter compounds, significant but roughly parallel changes in mtDNA and cell doubling time were not seen until 50 μM and 250 μM, respectively (Table 2).

Southern blot analysis. For comparison with results obtained with ddCyd by CPCR, Southern blot analysis of mtDNA content from 10 μg of purified cellular DNA was carried out in parallel with the CPCR studies, as described in Materials and Methods; to this end, MOLT-4 cells were exposed for 9 days to

a ddCyd concentration range of 0.01–1.25 μM. A human β-actin DNA probe was used for the monitoring of total genomic DNA. Results are shown in Fig. 4, *left*; the severe depletion of mtDNA upon ddCyd exposure was essentially identical to that reported by Chen and co-workers (4, 6) and similar to that seen in the present study with the CPCR method (Fig. 3b; Table 2). For comparison with results obtained with ddIno by CPCR, Southern blot analysis of mtDNA content from 15 μg of purified cellular DNA after ddIno treatment was carried out in parallel with the CPCR studies. MOLT-4 cells were exposed for 9 days to a ddIno concentration range of 5–250 μM. A human β-actin DNA probe was used for the monitoring of total genomic DNA. Results are shown in Fig. 4, *right*; the dose-dependent depletion of mtDNA upon ddIno exposure was similar to that reported by Chen *et al.* (6) and to that seen in the present study with the CPCR method (Table 2).

Discussion

Biosynthesis of mtDNA in mammalian cells can be studied by Southern hybridization (15), by incorporation of isotopically labeled precursors (16), or morphologically, by electron microscopic organelle counts (17). Applications of some of these methods, however, are limited by their qualitative or semiquantitative nature (3).

For pharmacological applications, the potential value of accurate and convenient methods for quantitating mtDNA has become apparent in recent years in studies of the toxicity of nucleoside analogue anti-HIV and anti-hepatitis B virus agents, particularly the pyrimidine nucleoside analogue ddCyd. With this agent, early clinical trials revealed that the dose-limiting toxicity in human subjects appeared to be a painful peripheral neuropathy, an unusual complication that had not been detected during extensive preclinical toxicology studies (18). Subsequent biochemical studies, performed mainly by Chen and Cheng (4), established that this agent has the property of severely depleting mtDNA content, apparently because of the potency of its 5′-triphosphate as an inhibitor of DNA polymerase γ, the enzyme primarily responsible for the synthesis of mtDNA.

Additional studies by those investigators have established that other dideoxynucleosides, in particular other pyrimidine dideoxynucleosides such as 2′,3′-dideoxycytidine-2′,3′-ene and 2′,3′-dideoxythymidine-2′,3′-ene, also manifest significant ability to deplete mtDNA (6). It is apparent, therefore, that a rapid and convenient method of evaluating mtDNA content would be a useful addition to preclinical safety evaluations of potential new antiviral and antitumor agents, particularly agents of the nucleoside analogue class.

To quantitatively measure the content of mtDNA in cultured MOLT-4 cells, we developed a CPCR method. In this method, the primer-targeting sequence was selected from the D-loop structure of mtDNA. Several aspects were considered in the development of the CPCR method. Firstly, in mammalian cells, mtDNA replication begins with synthesis of daughter heavy strands and the majority of mtDNA molecules contain a D-loop structure (15, 19, 20). Secondly, these newly synthesized heavy strands are metabolically unstable, with a mean lifetime of approximately 90 min (20), reflecting the fact that mtDNA replication is a rapidly ongoing process. Thirdly, it is likely that these strands arise from the same initiation events that yield fully replicated daughter mtDNA (21). Finally, we found that, similarly to unidentified reading frame 4 of the light chain of mtDNA, there were multiple copies of the D-loop region of mtDNA in cultured human T lymphoblasts when the D-loop region was coamplified with a chromosomal β-globin gene by conventional PCR (data not shown).

To explore the application of CPCR in studies of mtDNA synthesis inhibitors, we used members of both classes of anti-HIV 2′,3′-dideoxynucleosides, i.e., the pyrimidine nucleoside analogue ddCyd and the purine dideoxynucleoside analogues ddIno and 2′-β-F-ddAdo. Consistent with the finding of Cheng and co-workers using Southern blot methodology, we find by CPCR that ddCyd is highly potent in specifically reducing mtDNA content, whereas ddIno is much less active in this respect (6). There appear to be two major advantages to the CPCR method, however, in determining mtDNA content. Firstly, the CPCR method is more amenable to quantitative assessment of mtDNA. Secondly, this method is more adaptable to very small sample sizes; for example, in the present study the range of total cellular DNA routinely used for mtDNA quantitation was only 25–50 ng, whereas for Southern blot methodology 10 μg of total DNA are often required for optimal assay conditions. The CPCR method would therefore be of practical usefulness in determination of the mtDNA content in very small cell samples, for example in repeated sampling of blood or bone marrow during preclinical and phase I/II studies of new antiviral and antitumor agents with possible mitochondrial toxicity.

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Send reprint requests to: David G. Johns, National Cancer Institute, NIH, Building 37, Room 5B22, Bethesda, MD 20892-4255.