Molecular cloning and expression of the human deoxythymidylate kinase gene in yeast

Jin-Yuan Su and Robert A.Sclafani*
Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262, USA

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ABSTRACT

(Deoxy)thymidylate (dTMP) kinase is an enzyme which phosphorylates dTMP to dTDP in the presence of ATP and magnesium. This enzyme is important in cellular DNA synthesis because the synthesis of dTTP, either via the *de novo* pathway or through the exogenous supply of thymidine, requires the activity of this enzyme. It has been suggested that the activities of the enzymes involved in DNA precursor biosynthesis, such as thymidine kinase, thymidylate synthase, thymidylate kinase, and dihydrofolate reductase, are subjected to cell cycle regulation. Here we describe the cloning of a human dTMP kinase cDNA by functional complementation of a yeast dTMP kinase temperature-sensitive mutant at the non-permissive temperature. The nucleotide sequence of the cloned human cDNA is predicted to encode a 24 KD protein that shows considerable homology with the yeast and vaccinia virus dTMP kinase enzymes. The human enzyme activity has been investigated by expressing it in yeast. In this work, we demonstrate that the cloned human cDNA, when expressed in yeast, produces dTMP kinase activity.

INTRODUCTION

The *CDC8* gene of the yeast, *Saccharomyces cerevisiae*, encodes (deoxy)thymidylate kinase (dTMP kinase, EC 2.7.4.9.) which phosphorylates dTMP to dTDP (1,2, and Figure 1) and is required for nuclear and mitochondrial DNA replication in both the mitotic and meiotic cell cycles (3,4). Temperature-sensitive *cdc8* mutants arrest cell growth at the G1/S boundary of the cell cycle at the non-permissive temperature. Furthermore, these mutants are partially defective in meiotic and mitochondrial functions at the permissive temperature (4). The study of yeast dTMP kinase indicates that this enzyme not only provides precursors for DNA synthesis but has a role in regulating the process of DNA replication (1−8).

In eukaryotic cells, the replication of genomic DNA is restricted to the S phase of the cell cycle. The activities of many enzymes involved in DNA replication and precursor synthesis increase as cells enter S phase and decrease after the completion of DNA synthesis. Included among these S phase-specific enzymes are enzymes that are involved in the synthesis of DNA precursors, such as thymidine kinase (9,10,11), thymidylate synthase (12), ribonucleotide diphosphate reductase (13), and dihydrofolate reductase (14,15). In addition, the transcription of the yeast *CDC8* gene has recently been shown to be cell cycle regulated and expressed coordinately with *CDC9* and *CDC21* which encode DNA ligase and thymidylate synthase, respectively (16). Several models have been proposed to explain the coordination between the processes of DNA replication and biosynthesis of nucleotide precursors (for review, see 17). Proteins important for both processes may form multienzyme complexes or functional compartments. We (1) and others (2) have suggested that the yeast dTMP kinase is one of these proteins.

In an effort to investigate the regulation of the human dTMP kinase in both nucleotide biosynthesis and DNA replication, we cloned the human gene. A human cDNA was cloned by complementing a yeast *cdc8* temperature-sensitive mutant at the non-permissive temperature with a human HepG2 cDNA library which was constructed in a yeast expression vector (18). Southern hybridization of total human genomic DNA with this human dTMP kinase cDNA indicates that the human dTMP kinase gene is contained within a 6 kb EcoRI DNA restriction fragment. We have shown that the sequence of the dTMP kinase gene is highly conserved among the enzymes of human, yeast, and vaccinia virus. When the human cDNA is expressed in a yeast strain in which the yeast endogenous dTMP kinase gene is deleted it supports the cell's mitotic growth but not the mitochondrial functions. The human enzyme expressed in yeast cells has lower activity than that of endogenous yeast enzyme in our assay.

MATERIALS AND METHODS

Strains and media

The *E. coli* strain DH5 was used for all cloning procedures. The Human HepG2 cells were allowed to grow until confluent stage in OPTI-MEM I medium (Gibco BRL) with 3.75% fetal calf serum and were kindly provided by Lih-Jen Su (Medical Oncology Division, University of Colorado Health Sciences Center). All yeast strains employed in this study were *Saccharomyces cerevisiae*. These yeast strains included

* To whom correspondence should be addressed

199(*MATα trp1 ura3 leu2 can*^{R} *cdc8-1*), 226(*MATα ade1,2 leu2 ura3 can*^{R} *cyh*^{R} *cdc8-2*), 349(*MATa ade1,2 leu2 trp1 ura3 lys2 cyh2 cdc8-3*) 345(*MATa ade1,2 leu2 trp1 ura3 tyr1 his7 lys2 cyh2 cdc8-4*), 392(*MATα leu2 ura3 trp1 his7 can1 cdc8Δ*::LEU2 (pRC4-TK$^+$=*TK TRP1*)), 396 (*MATα leu2 ura3 trp1 his7 can1 cdc8Δ*::LEU2 (Ycp50-CDC8$^+$)), and JY129 (*MATα leu2 ura3 trp1 his7 can1 cdc8Δ*::LEU2 (p561=*HuTMPK URA3*)). Yeast and bacterial media have been described (1,19). DNA transformations employed the sphaeroplast method (20) except that 1.5% low melting temperature agarose replaced 3% agar in the overlay for regeneration. Mitochondrial defective colonies or petites (rho$^-$) were detected by an inability to grow on YEPG agar with glycerol as carbon source (3,4).

Plasmids

The human HepG2 cDNA library was kindly provided by Chiron Company (Emeryville, CA), which was constructed in the yeast expression vector pAB23BX (18). The vector contains a selectable *URA3* gene of yeast. Plasmid pRC4-TK$^+$ contains a herpes virus thymidine kinase (TK) gene and a selectable yeast *TRP1* gene (1). Plasmid Ycp50-CDC8 contains a yeast wild type *CDC8* gene and a selectable yeast *URA3* gene (8).

Characterization of plasmid DNA and DNA sequencing

Plasmid isolation, DNA restriction enzyme digestions, nucleic acid agarose electrophoresis followed previous procedures (1,19). Dideoxy-DNA sequencing employed the modified T7 DNA polymerase (Sequenase) as described by the manufacturer (US Biochemicals). The p561 1 kb EcoRI insert DNA restriction fragment (Figure 2) cloned into M13mp18 and M13mp19 was used as a template with the universal DNA primer # 1212 (New England Biolabs). DNA sequences were analyzed with the MICROGENIE (Beckman Inst.) software program on a NCR PC8 computer. The nucleic acid sequences are from 3/88 update version 54 from GENBANK.

Genomic Southern blot analysis

Total DNA was prepared from human HepG2 cells following general procedures (21). Genomic Southern blot hybridization followed previous procedures which was carried out under high stringency conditions (1,19). The blots were probed with nick-translated ^{32}P-labelled probes (1,19) to the EcoRI DNA restriction fragment of p561.

dTMP kinase assay

The dTMP kinase assay using crude extracts was performed as described (1). A unit of dTMP kinase activity is defined as the conversion of 1.0 pmol of dTMP to dTDP + dTTP per mg of protein extract per min.

RESULTS

Plasmids prepared from the HepG2 cDNA library were used to transform yeast *cdc8* mutant (strain 199). All the Ura$^+$ transformants selected at 22°C were then replicated to YEPD plates and screened for temperature resistant (Tsm$^+$) colonies at 36°C. Five Tsm$^+$ colonies from 8,000 Ura$^+$ transformants were chosen for further analysis. When these five transformants were streaked out on non-selective plates some of the Ura$^-$colonies that had lost their plasmids also became temperature sensitive (Tsm$^-$). This indicates the temperature resistant property was mediated by the presence of the plasmid.

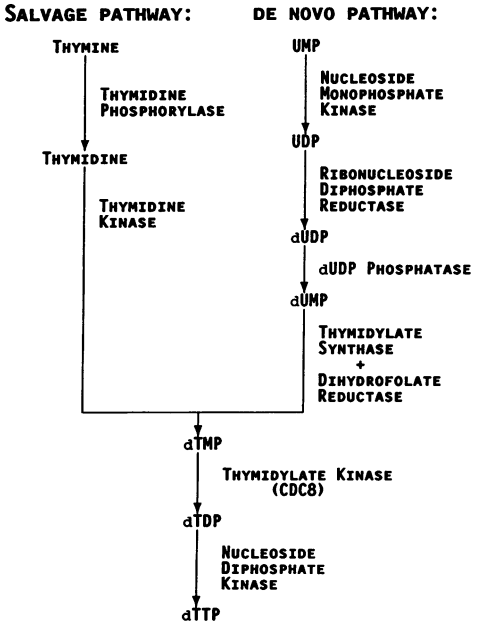

Figure 1. Pathways of salvage and *de novo* thymidylate biosynthesis. The yeast *CDC8* gene which encodes thymidylate kinase is indicated in the figure. It should be noted that the scheme of this figure represents a general thymidylate biosynthesis pathway for all eukaryotic cells. However, like all the fungi, yeast does not have the thymidine kinase (29, 30). Therefore, the salvage pathway does not exist in yeast. UMP, uridine-5′-monophosphate; UDP, uridine-5′-diphosphate; dUDP, 2′-deoxy-uridine-5′-diphosphate; dUMP, 2′-deoxy-uridine-5′-monophosphate; dTMP, 2′-deoxy-thymidine-5′-monophosphate; dTDP, 2′-deoxy-thymidine-5′-diphosphate; dTTP, 2′-deoxy-thymidine-5′-triphosphate.

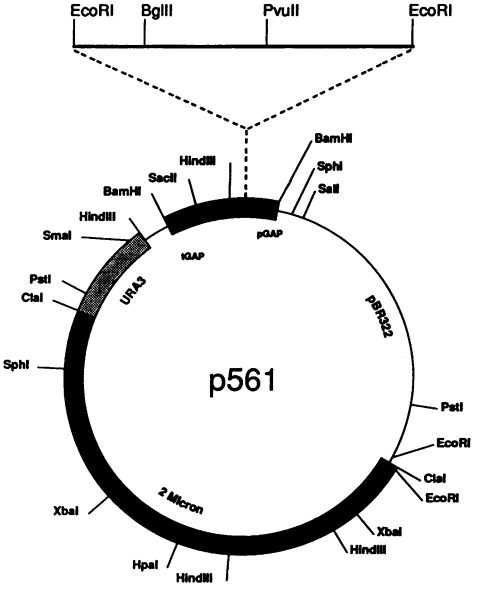

Figure 2. DNA restriction endonuclease map of plasmid p561. Vector pAB23BX (18) is drawn as a circle and the human cDNA insert as a line. The insert is an 1 kb *EcoRI* fragment. The vector and the insert are not drawn to the same scale. The yeast promoter of glyceraldehyde-3-phosphate dehydrogenase (GAP) is on the right side of the insert shown, and the 5′ *EcoRI* site is not encoded by the cDNA. The poly(A) tail at 3′ end and the cloning linker enzyme sites of the insert are not shown.

Plasmids recovered from all five Tsm$^+$ yeast transformants were found to have the same cDNA insert on the vector by using DNA restriction enzyme mapping. One of these plasmids has been designated as p561 (Figure 2). To confirm that the cDNA clone complements yeast *cdc8* mutations, we retransformed p561 into three other different yeast *cdc8* temperature-sensitive mutant alleles (strains 226, 349, and 345). All the yeast *cdc8* alleles were complemented by this p561 clone. However, it did not complement other yeast temperature-sensitive mutations such as *cdc7* (protein kinase, 22) and *cdc21* (thymidylate synthase, 23).

The human dTMP kinase cDNA insert was isolated from p561 as an 1 kb EcoRI DNA restriction fragment for further study. In genomic Southern analysis of human HepG2 DNA, the 1 kb cDNA probe hybridizes to a 6 kb EcoRI DNA restriction fragment (Figure 3). This indicates that the cloned cDNA is a single copy human gene. The sequence of the 1 kb EcoRI DNA restriction fragment is shown in Figure 4. It contains a coding region of 633 nucleotides that encodes approximately a 24 KD protein. This corresponds to the previous identification of the molecular weight of the purified human dTMP kinase (24). When compared to the amino acid sequences of the yeast dTMP kinase and a recently identified vaccinia virus dTMP kinase (25), human dTMP kinase showed high homology with them. The overall identity of amino acid sequences among these enzymes is more than 42% (Figure 5).

In order to assay specifically the human dTMP kinase in yeast cells, the yeast dTMP kinase gene was deleted from cells in which human gene was expressed. Because the yeast *CDC8* gene is essential for yeast growth, we used yeast strain 392 in which the yeast *CDC8* gene has been deleted and replaced by a selective marker, the *LEU2* gene, and dTMP kinase is supplied by a herpes virus TK gene on plasmid pRC4-TK$^+$ TRP1$^+$ (1). Herpes virus TK contains both TK and dTMP kinase activities (26) and thereby, complements the dTMP kinase deficiency. Cells of strain 392 that have lost the herpes virus TK plasmid become Trp$^-$ and can only survive if another dTMP kinase gene is present (1,4). Trp$^-$ cells of strain 392 arose upon transformation either with plasmid Ycp50-CDC8$^+$, as expected, or with plasmid p561. In this manner, isogenic strains 396 and JY129 that contain only the yeast or human dTMP kinase genes, respectively, were constructed. The mitotic growth of strain JY129 was fully supported by the presence of the human dTMP kinase gene at various growth temperatures. However, the mitochondrial function (respiratory competence) of JY129 was unstable. After growth in rich medium at 30°C for 24 hours, 75% of the cells became petites and were respiratory incompetent.

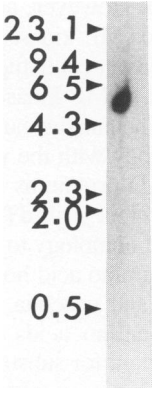

Figure 3. Southern hybridization of the human HepG2 cDNA to genomic DNA. The purified HepG2 genomic DNA was digested with *EcoRI* and analyzed on a 0.7% agarose gel electrophoresis. The Southern blot from the gel was hybridized with 1 kb *EcoRI* fragment of p561 as the probe. The molecular weight of the DNA markers are indicated in kb.

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EcoRI
AATTCGCCAGCGGCGCGGTGGACAGTCATGGCGGCCCGGCGCGGGGCTCTCATAGTGCTGGAGGGCGTGG   70

ACCGCGCCGGGAAGAGCACGCAGAGCCGCAAGCTGGTGGAAGCGCTGTCGCGGGCCACCGCCCGAACT  140

GCTCCGGTTCCCGGAAAGATCAACTGAAATCGGCAAACTTCTGAGTTCCTACTTGCAAAAGAAAAGTGAC  210

GTGGAGGATCACTCGGTGCACCTGCTTTTTTCTGCAAATCGCTGGGAACAAGTGCCGTTAATTAAGGAAA  280

AGTTGAGCCAGGGCGTGACCCTCGTCGTGGACAGATACGCATTTTCTGGTGTGGCCTTCACCGGTGCCAA  350

GGAGAATTTTTCCCTAGACTGGTGTAAACAGCCAGACGTGGGCCTTCCCAAACCCGACCTGGTCCTGTTC  420

CTCCAGTTACAGCTGGCGGATGCTGCCAAGCGGGGAGCGTTTGGCCATGAGCGCTATGAGAACGGGGCTT  490
      PvuII
TCCAGGAGCGGGCGCTCCGGTGTTTCCACCAGCTCATGAAAGACACGACTTTGAACTGGAAGATGGTGGA  560

TGCTTCCAAAAGACTCGAAGCTGTCCATGAGGAACTCCGCGTGCTCTCTGAGGACGCCATCCGCACTGCC  630

ACAGAGAAGCCGCTGGGGGAGCTATGGAAGTGACCCAAGGCTGCCCACTGGAGACGCCTCTCCCTGCAGT  700

CCCCCGAGAGGTGGGAGACTCGCGGAAGGCCCCGTCCCCAGCGGAGTCCAGACCCCACAACTTCAGGAGC  770

TCTTCCCGGCAGCAGAGATCTGCAGGCTGCCTCTTCTGCCCCGGAGCTGGGGTGCACTGGGGACCCCCG  840
          BglII
TGGTGGGGACCTTGGCAGTGTGGACATGAGCAGAGCGATGGAGCAGTCTCCTGCCCTCTCCCCTGTCCTG  910

ATGGCACTCTGTTGTATTTTCTTACTGAAGTTCAGTGATAACTCTGAGCAGTTTCATTGTGATCACTGTA  980

AATGGTAATCAGTTGGAATT                                                    1000
      EcoRI
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Figure 4. DNA sequence of the 1 kb *EcoRI-EcoRI* fragment of p561. The coding region (bases 28−663) of the human dTMP kinase is underlined. The restriction endonuclease enzyme sites corresponding to the insert DNA on p561 in Figure 2 are also indicated. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X54729.

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(1) HUMAN dTMP KINASE          MAARRGALIVLEGVDRAGKSTQSRK  25
(2) YEAST dTMP KINASE          MMGR-GKLILIEGLDRTGKTTQCNI  24
(3) VACCINIA VIRUS dTMP KINASE M-SR-GALIVFEGLDKSGKTTQCMN  23
                                        *    ****

(1) LVEALSRGPPP-ELLRFPERSTEIGKLLSSYL-QKKS-DVEDHSVHLLFSAN  74
(2) LYKKL-Q-PNC-KLLKFPERSTRIGGLINEYLT-DDSFQLSDQAIHLLFSAN  72
(3) IMESI-P-ANTIKYLNFFQRSTVTGKMIDDYLTRKKTYN--DHIVNLLFCAN  71

(1) RWEQVPLIKEKLSQGVTLVVDRYAFSGVAFTGAKE-N-FSLDWCKQPDVGLP 124
(2) RWEIVDKIKKDLLEGKNIVMDRYVYSGVAYSAAKGTNGMDLDWCLQPDVGLL 124
(3) RWEFASFIQEQLEQGITLIVDRYAFSGVAYAAAKGAS-MTLSKSY--ESGLP 120
                 * *******  ***** ****

(1) KPDLVLFLQLQ-LADAAKRGAFGHERYENGAFQERALRCFHQLMKDT-TLNW 174
(2) KPDLTLFLSTQDVDNNAEKSGFGDERYETMKFQEKVKQTFMKLL-DK-EIR- 173
(3) KPDLVIFLESGSKEIN--RNV-GEEIYEDVTFQQKVLQEYKKMI-EEGDIH- 167

(1) KMVDASKRLEAVHEELRVLSE-D-AIRTATEKP-LG-ELWK           211
(2) KGDESITIVDVTNKDIQEVEALIWQIVEPVLSTHIDHDKFSFF        216
(3) WQIISSEFEEDVKKEL--IKNIVIEAIHTVTGP-VGQ-LWM          204
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Figure 5. Protein comparison of human, yeast, and vaccinia virus dTMP kinase sequences. Boxed residues indicate identity between all three dTMP kinase sequences. Residues marked with '*' indicate regions of conservation with the herpesvirus enzymes (28).

Crude extracts were prepared from strains 392, 396 and JY129 for the dTMP kinase assay. As indicated in Table 1, the specific activity of human dTMP kinase in yeast is about 150 enzyme units in the presence of 0.2 mM dTMP as enzyme substrate. This enzyme activity is lower than when either herpes virus TK gene or the endogenous yeast *CDC8* gene was expressed in yeast (Table 1). The effect of various nucleotides on the human dTMP kinase is shown in Table 2. Only the substrate dTMP but not other nucleotides could act as competitors of the enzyme activity. Therefore, the human enzyme activity was specific for dTMP. When the products of the enzyme, dTDP and dTTP, were added at 0.7 mM to the assay, feedback inhibition was observed. dTDP and dTTP inhibited 82% and 70% of the enzyme activity, respectively (Table 2). Similar feedback inhibition results have been demonstrated with the purified human dTMP kinase enzyme (24,27). The dTMP kinase encoded by the cDNA clone is probably the sole enzyme in human cells, because only one type of dTMP kinase exists in both cytoplasmic and mitochondrial fractions from human cells (27).

Table 1. The dTMP kinase activities of three isogenic yeast strains

Yeast	dTMP kinase gene	dTMP kinase activity (specific unit*)
396	$CDC8^+$	840±35
392	HSV-TK	350±20
JY129	HuTMPK	150±20

The construction of the strains used is described in the Text. The enzyme assay mixture contained 50 mM Tris-Hcl buffer (ph 7.6), 10 mM $MgCl_2$, 5 mM ATP, 0.25 mg/ml BSA, 0.2 mM dTMP, *Methyl*-^{3}H-dTMP (0.8 μci; 60 ci/mmol). 10 μg of crude protein extract was added in the reaction mixture. The reaction was incubated at 37°C for 30 min and terminated by adding 20 mM EDTA and boiling for 10 min (1). HSV-TK, herpes virus thymidine kinase gene; HuTMPK, human dTMP kinase gene.

*Enzyme activity was the average number from three independent determinations. The specific activity was expressed as 1 pmol dTDP + dTTP production/ mg of protein extract/min.

Table 2. Specificity of dTMP as the substrate for the human thymidylate kinase assay

Competitor	Thymidylate Kinase Activity (%)
None	100*
dAMP, 0.5mM	103
dCMP, 0.5mM	98
dGMP, 0.5mM	100
dUMP, 0.5mM	96
AMP, 0.5mM	99
UMP, 0.5mM	95
GMP, 0.5mM	100
CMP, 0.5mM	100
dTTP, 0.7mM	30
dTDP, 0.7mM	18

* Activity was expressed as percentage in comparison to control using dTMP as the substrate. The protein extract from strain JY129 was used as the enzyme source in this assay. The condition of the enzyme assay was the same as in Table 1. The concentration of each nucleotide added to the reaction in addition to 0.2 mM dTMP in the control is indicated. When an excess amount of dTMP (0.5mM) was added in the reaction, it reduced the incorporation of ^{3}H-dTMP into dTDP and dTTP proportionally.

DISCUSSION

Functional complementation of yeast (*Saccharomyces cerevisiae*) mutants has been used recently to clone three human purine biosynthesis genes (18). In this study, we demonstrate the cloning of a human dTMP kinase cDNA by a similar approach. A human cDNA library constructed in a yeast expression vector was introduced into yeast *cdc8* (dTMP kinase) temperature sensitive mutants. The human dTMP kinase cDNA was cloned by its ability to complement the temperature sensitive phenotype. The human origin of the cloned cDNA was confirmed because it hybridized to human genomic DNA (Figure 3). The sequences of the cloned cDNA contained a coding region of 633 nucleotides that would produce a protein of 24 KD. The molecular weight of this putative protein is in agreement with a previously identified human dTMP kinase (24). However, another study has showed that the purified human dTMP kinase has a molecular weight estimated by glycerol gradient centrifugation to be 50 KD (27). We postulate that human dTMP kinase forms a dimer *in vivo* with two equal subunits. The putative human dTMP kinase shares a 42% amino acid homology with the yeast enzyme, which has been shown to be a 24 KD protein as well (2). Recently it has been reported that the vaccinia virus dTMP kinase gene encodes a protein with a significant homology to yeast dTMP kinase (25). There is more than 42% amino acid homology among the three enzymes (human, yeast, and vaccinia virus) as seen in Figure 5. Several stretches of amino acids that define domains of homology could be important for substrate binding and catalytic functional domains. Among these we have found that a glutamate residue (Glu^{75} of the yeast enzyme), which is highly conserved in all three dTMP kinases (Figure 5), is important for the function of the enzyme because the yeast *cdc8-1* temperature-sensitive mutation has a lysine at this position (Su, J.-Y. and Sclafani, R.A., unpublished results). The proposed ATP-binding domain and nucleotide/nucleoside binding sites of the yeast and herpes virus enzymes (28) are also conserved in the human enzyme (Figure 5). Very likely, all cellular and viral enzymes evolved from a common ancestor (25, 28). However, the herpesvirus enzymes, which show little or no homology to many of the conserved regions of these three dTMP kinases, may have diverged in evolution to acquire TK activity, as proposed (25, 28).

When expressed in yeast cells, the human dTMP kinase completely supports the yeast mitotic growth in the absence of yeast endogenous dTMP kinase. However, these strains have a mitochondrial defect. A large number (75%) of yeast cells with the human enzyme as the sole dTMP kinase became respiratory defective in a 24 hour growth period. This correlates with our previous observation that in yeast different levels of dTMP kinase activity are required for mitotic and mitochondrial DNA replication (4). Because yeast mitochondria have neither a dTMP kinase gene nor any dTMP kinase activity (2), they depend on the exogenous supply of DNA precusors from the nuclear encoded (*CDC8*) gene. We have used isogenic yeast strains, which only differ in the dTMP kinase gene (yeast, human, or herpes virus) on the plasmid, to assay the dTMP kinase activity (Table 1). While the yeast strains containing either herpes virus TK gene (strain 392) or wild type yeast *CDC8* gene (strain 396) did not show any mitochondrial defect, the yeast strain with human dTMP kinase gene (strain JY129) became respiratory defective at a high rate. The lower level of human enzyme activity expressed in yeast cells compared to yeast and herpes virus enzymes activity in similar extracts may explain why mitochondria do not have enough dTTP for DNA replication.

Since the genetic regulatory elements including the promoters of these three dTMP kinase genes expressed in yeast cells are different, the difference of the enzyme activities among them could be at the transcriptional level. We speculate that a yeast cell may preferentially supply the DNA precursors to the nuclear DNA and that a level of dTMP kinase activity *in vitro* between 200–350 units may be required for yeast to support both nuclear and mitochondrial DNA replication efficiently.

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