Characterization of Distinct Nuclear and Mitochondrial Forms of Human Deoxyuridine Triphosphate Nucleotidohydrolase*

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Deoxyuridine triphosphate nucleotidohydrolase (dUTPase; EC 3.6.1.23) was purified from HeLa cells by immunoaffinity chromatography. Based on SDS-polyacrylamide gel electrophoresis, two distinct forms of dUTPase were evident in the purified preparation. These proteins were further characterized by a combination of NH$_2$-terminal protein sequencing, mass spectrometry, and mass spectrometry-based protein sequencing. These analyses indicate that the two forms of dUTPase are largely identical, differing only in a short region of their amino-terminal sequences. Despite the structural difference, both forms of dUTPase exhibited identical binding characteristics for dUTP.

Each form of dUTPase has a distinct cellular localization. Cellular fractionation and isopycnic density centrifugation indicate that the lower molecular weight form of dUTPase (DUT-N) is associated with the nucleus, while the higher molecular weight species (DUT-M) fractionates with the mitochondria. The DUT-N isoform is approximately 30-fold more abundant in HeLa cells than DUT-M as determined by densitometry.

The NH$_2$-terminal protein sequence of both DUT-N and DUT-M did not match previous reports of the predicted amino-terminal sequence for human dUTPase (Mclntosh, E. M., Ager, D. D., Gadsden, M. H., and Haynes, R. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8020–8024; Strahler, J. R., Zhu X., Hora, N., Wang, Y. K., Andrews, P. C., Roseman, N. A., Neel, J. V., Turk, L., and Hanash, S. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4991–4995). A cDNA corresponding to the DUT-N isoform was isolated utilizing an oligonucleotide probe based on the determined NH$_2$-terminal sequence. The cDNA contains a 164-amino acid open reading frame, encoding a protein of M$_r$ 17,748. The DUT-N cDNA sequence matches the previously cloned cDNAs with the exception of a few discrepancies in the 5' end. Our data indicate a 69-base pair addition to the 5' end of the previously reported open reading frame.

Deoxyuridine triphosphate nucleotidohydrolase (dUTPase)$^1$ is a ubiquitous enzyme that functions in the hydrolysis of dUTP to dUMP and pyrophosphate. This reaction is thought to occur primarily to limit pools of intracellular dUTP in order to prevent significant dUMP incorporation into DNA during replication and repair (3). A second role of dUTPase is to provide substrate (dUMP) for the de novo synthesis of thymidylate. The effects of a compromised dUTPase activity have been well documented in prokaryotes (4). Mutations in Escherichia coli dUTPase, which lower enzyme activity to 5% of wild type levels, cause an increase in the intracellular dUTP pools. The result of elevated dUTP pools is an increased incorporation of dUMP into DNA. Uracil-DNA glycosylase initiates the base excision repair pathway in a reiterative, self-defeating repair process, which results in removal and reincorporation of dUMP. This ultimately leads to DNA fragmentation and cell death (4).

The consequences of a reduced dUTPase function in eu- karyotes are not as well documented because of a lack of mutants. A dUTPase null mutant in the yeast Saccharomyces cerevisiae was shown to be inviable (5), a result similar to what is observed in the bacterial system. In the mammalian system, indirect evidence has shown that anti-folate analogs and other inhibitors of de novo thymidylate biosynthesis cause an increase in the ratio of dUTP to dTTP resulting in DNA fragmentation and cell death (6–8). Recently, Canman and co-workers (9, 10) demonstrated that, in certain human tumor cell lines, increased levels of dUTPase are responsible for an increase in resistance to the cancer chemotherapeutic agent fluorodeoxyuridine (FuDR), a thymidine synthase inhibitor. Together, these studies provide substantial evidence suggesting that dUTPase, the chief regulator of dUTP pools, mediates a critical step in FuDR toxicity.

In addition to prokaryotes and eukaryotes, a number of viruses are known to encode a dUTPase function. A diverse group of viruses including herpesviruses (11–14), poxviruses (15), and retroviruses (16, 17) encode a viral dUTPase activity. A specific subset of the lentivirus group encodes dUTPase as part of the pol gene product in addition to the reverse transcriptase, inte- grase, and protease functions (16). In contrast, the human immunodeficiency virus types 1 and 2 (HIV1 and HIV2) do not contain a virus-encoded dUTPase function (17) and may rely on the dUTPase of the host cell. The question of whether dUTPase

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The abbreviations used are: dUTPase, deoxyuridine triphosphate nucleotidohydrolase; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); FuDR, fluorodeoxyuridine; HIV, human immunodeficiency virus; MS, mass spectrometry; MALDI-MS, matrix-assisted laser desorption mass spectrometry; MALDI-MS/MS, tandem mass spectrometry.

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is essential for viral replication has been addressed in both herpesvirus and retrovirus groups (16, 18, 19). Null mutants of viral dUTPases demonstrate that this enzyme is required for successful viral replication in nondividing cells in which the cellular levels of dUTPase are exceptionally low. In contrast, virus-encoded dUTPase is not required for replication in actively growing cultured cells where dUTPase levels are high (16, 18). It has been postulated that virus-encoded dUTPase expands the tropism of certain viruses by allowing viral replication in nondividing cell types with low cellular dUTPase activity (16).

Our laboratory has undertaken a detailed biochemical characterization of the dUTPase enzyme function in human cells. In this report, evidence is presented identifying and characterizing two distinct forms of dUTPase that exist in humans. Cellular fractionation experiments suggest that the more abundant, lower mass form of dUTPase (DUT-N) localizes in the nucleus, while the higher mass form (DUT-M) is associated with the mitochondria. We also present the full-length cDNA encoding the DUT-N isofrom.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**HeLa S3 cells (CCl 2.2) were purchased from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum purchased from Life Technologies, Inc. Sf21 cells for baculovirus expression of dUTPase were obtained from Clontech Laboratories, Inc. and maintained in Grace’s insect cell media supplemented with yeastolate, lactalbumin hydrolysate, and 10% fetal bovine serum (Life Technologies, Inc.).

**Purification of dUTPase—**Purification of dUTPase from HeLa S3 and Sf21 cells was performed using a modification of the method developed by Caradonna and Adamkiewicz (12). Briefly, cellular extracts were partially purified by streptomycin sulfate fractionation, ammonium sulfate fractionation, and DEAE-cellulose chromatography as described previously (12). This partially purified fraction was then subjected to immunooaffinity chromatography. dUTPase-specific monoclonal antibodies described by Dignam et al. (24). The resulting nuclear fractions (1 ml) were collected, neutralized by the addition of 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, and 150 mM NaCl and then incubated with the antibody-Sepharose overnight at 4°C with gentle agitation. The matrix was applied to a column and washed with 10 bed volumes of wash buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, and 0.5 M NaCl. dUTPase was eluted with 25 ml of 100 mM glycine, pH 2.5. Fractions (1 ml) were collected, neutralized by the addition of 100 mM Tris-HCl, pH 8.0, and assayed for dUTPase activity. Peak fractions were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5, and 10% glycerol. Purified protein was fractionated by 15% SDS-PAGE and silver-stained according to the procedures of Merrill et al. (21).

**Cellular Fractionation—**Isolation of Mitochondria and Nuclei from HeLa Cells—Cellular fractionation of HeLa cells and purification of mitochondria by sucrose gradient sedimentation was performed according to the procedures described by Rickwood et al. (22). The resulting mitochondrial protein extract was used for subsequent immunoblot analysis or purification of mitochondria-associated dUTPase by the method described above.

**Enzyme Assays—**dUTPase activity was measured using the procedure described by Caradonna and Adamkiewicz (12). Isolation and Sequencing of cDNA Clones—A human T cell cDNA library in λ gt10 was purchased from Clontech. The library was screened using a synthetic oligonucleotide probe based on the amino acid sequence of DUT-N (determined in this report) and the human dUTPase cDNA sequence reported in the EMBL/GenBank Data Libraries (1): 5′-AAGAGACACCCGCTTACCCCATGTA-3′. The library screening protocol was based on standard procedures as described by Sambrook, et al. (25). Two cDNA isolates (1.1 and 0.9 kb) were cloned into the EcoRI site of pGEM-3Z (Promega). These clones were sequenced using the Sequenase deoxyxynucleotide chain termination kit (U.S. Biochemical Corp.) according to the manufacturer’s recommendations. Bal-31 exonuclease was used to generate a series of deletion clones in order to sequence the entire cDNA. The sequence was determined from both strands.

**Expression of Recombinant dUTPase—**The coding region of the DUT-N gene was subcloned into the baculovirus expression vector pBacPAK8 (Clonetech) and dUTPase overproduced in Sf21 insect cells as per the manufacturer’s recommendations. The resulting recombinant protein was purified by the method described above and shown to be functional by enzyme assay (K_m = 2.5 µM).

**Antibodies—**dUTPase-specific monoclonal antibodies were generated as described (20). These antibodies are widely used for immunoprecipitation and immunooaffinity chromatography, not for immunoblot analysis. dUTPase-specific polyclonal antibodies, useful for immunoblot analysis, were raised against recombinant DUT-N protein (expressed in the baculovirus system) according to procedures outlined by Harlow and Lane (26). The antibodies were then immunooaffinity-purified utilizing recombinant dUTPase bound to Sepharose. The resulting immunopurified dUTPase-specific polyclonal antibody was used at a dilution of 1:100.

**Western Blot Analysis of dUTPase—**Protein was fractionated by 15% SDS-PAGE and transferred to nitrocellulose according to Towbin et al. (27). Western blot analysis was performed according to standard protocols, and the protein bands were visualized with the ECL chemiluminescence Western blotting detection system (Amersham Corp.).
mg/ml 10:10:1.1 EtOH/CH₃CN/H₂O), applied to the stainless steel target, and allowed to air-dry prior to insertion into the mass spectrometer. Spectra were obtained in the linear mode and are the sum of 20–50 laser shots.

Structural information may be discerned from MALDI-MS by analysis of metastable ions that decompose in the field-free drift region of the time-of-flight analyzer (29–33). The (M + H)⁺ ions presumably become activated in the ion source through multiple collisions with matrix and analyte ions but do not decompose until they are in the first field-free drift region after having been fully accelerated. These so-called metastable ions decompose, producing fragment ions that have essentially the same velocity as the parent ion but have energies proportional to the ratio of the fragment-to-parent ion mass (29–33). These product ions may be analyzed in the reflecting mode of operation by stepping down the reflecting voltage to bring the lower mass products into energy focus at the reflecting detector. A resolution of >2000 (full-width half-maximum definition) has been achieved for mass-selected product ions produced by MALDI-MS (28, 47). This resolution is sufficient to determine monoisotopic mass up to at least m/z 1800 in the product ion analysis mode, and it greatly reduces uncertainty in the mass assignment and structural interpretation of fragment peaks. Metastable ion mass spectra (also referred to as postsource decay spectra) were acquired in eight consecutive, overlapping mass scale segments, each representing a 25% mass change from the previous segment. The segments were combined and externally mass-calibrated (versus a metastable ion spectrum of a model peptide such as renin tetradecapeptide or substance P, residues 2–11) by the data system. A Bradbury-Nielsen ion gate was used for precursor ion selection (28, 34, 35, 47). The resolution of precursor ion selection is in excess of 100 (28, 47).

RESULTS

Identification of Multiple Forms of Human dUTPase—Fractionation by SDS-PAGE and silver staining of purified dUTPase from HeLa S3 cells reveals two closely migrating protein species, which immunopurify using a monoclonal antibody against human dUTPase (Fig. 1, lane 1). The lower molecular weight species (designated DUT-N) is at least 30-fold more abundant than the higher molecular weight species (designated DUT-M) as determined by densitometry (data not shown). To verify the identity of the two forms, immunoblot staining polyclonal antibodies were generated using a recombinant form of the DUT-N protein expressed in the baculovirus system (see "Experimental Procedures") and demonstrated the exclusive mitochondrial association of the higher molecular weight species of dUTPase (DUT-N) as determined by densitometry (data not shown). To further establish the identity of the two forms, immunoblot staining by SDS-PAGE and silver staining of purified dUTPase was utilized to determine the overlapping regions of reported cDNA sequences encoding human dUTPase (1, 2). The predicted translational start site indicated by these authors does not correspond to the native NH₂-terminal sequence of the major form of dUTPase in HeLa cells, determined in this study. In an effort to resolve this discrepancy, we identified and characterized several independent dUTPase cDNAs.

Isolation and Sequence Analysis of dUTPase-specific cDNAs—An oligonucleotide probe designed from the amino-terminal protein sequence of DUT-N was used to screen a human T cell (Jurkat) λgt10 cDNA library. Out of 43 positive clones identified, 10 were chosen for plaque purification, and those with the largest inserts (1.1 and 0.9 kb) were subcloned for sequence analysis. The nucleotide sequence and open reading frame of the 1.1-kb dUTPase cDNA is presented in Fig. 2. The nucleotide sequence of the 0.9- and 1.1-kb isolates were identical in their overlapping regions. The dUTPase open reading frame corresponds to a 164-amino acid open reading frame beginning at position 30. The position of the polyadenylation signal in the 3'-untranslated region is unknown.

a deduced amino acid sequence encoded in the 5'-untranslated region of reported cDNA sequences encoding human dUTPase (1, 2). The predicted translational start site indicated by these authors does not correspond to the native NH₂-terminal sequence of the major form of dUTPase in HeLa cells, determined in this study. In an effort to resolve this discrepancy, we identified and characterized several independent dUTPase cDNAs.

Identification of Distinct Nuclear and Mitochondrial Forms of dUTPase—In an effort to clone and characterize the human dUTPase coding region, approximately 10 μg of the more abundant, lower mass dUTPase protein (DUT-N) was purified by immunofinity chromatography and subjected to NH₂-terminal microsequencing. Sequence information was obtained for the first 25 NH₂-terminal residues (Fig. 2, underlined). The amino-terminal methionine was absent from the native protein, indicating a posttranslational removal of this residue. A search of the Protein Identification Resource sequence data base revealed a match between a portion of this protein sequence and
approximately two-thirds of each protein was mapped in these experiments. Coverage is not expected to be complete for several reasons. First, not all peptides are extracted or recovered from the gel with equal efficiency. Second, not all peptides are ionized with equal efficiency in the MALDI-MS experiment, and the choice of matrix can have a significant effect on the specific components of a mixture that are detected and their apparent relative ratios. (36). Third, many of the peptides that were not detected are relatively small and would, if present, have molecular ions in the region dominated by the intense background from the liquid matrix used. Finally, supression can occur in complex mixtures such that only the most easily ionized and most abundant peptides are detected.

With the exception of the signal at m/z 1776, all of the major peptide-derived signals fit the sequences shown (the signals at m/z 1300 and 2183 correspond to monooxidized forms of the peptides of M+1, 1284 and 2167, respectively, each of which contains a Met residue that presumably has partially converted to Met-sulfoxide). The 1776 peptide is unique to the mitochondrial form of the protein. Furthermore, based on the observation that it was also present in tryptic digests that had not been reduced and alkylated prior to MS analysis (data not shown), it could not contain Cys. Absence of Cys suggests that this peptide cannot be a simple modification of the NH2-terminal peptides of DUT-N.

The new technique of metastable ion analysis in MALDI-MS (MALDI-MS/MS) was used to provide the sequence of this peptide, and to confirm the sequence of several of the other peptides observed in the MALDI-MS data. The parent ion of the 1776 peptide was selected from the mixture shown in Fig. 5 for further MS analysis using a Bradbury-Nielsen ion-gating device (34, 35) in the Fisons VG MALDI mass spectrometer (28, 47). Fragment ions are produced from the highly activated, metastable peptide ions as they undergo decomposition (sometimes referred to as “postsource decay”) during flight in the field-free portion of the time-of-flight instrument. The fragment ions formed have energies in proportion to their masses, and may be analyzed in the reflectron portion of the reflecting time-of-flight instrument by purposefully bringing to focus at the final detector ions of energy lower than that of the parent (29-33).

The MALDI-MS/MS spectra of the peptides of (M + H)+ = 1776 and (M + H)+ = 2066 are shown in Fig. 6. The dominant fragment ions observed in these spectra correspond to Yn ions (H-(NH-CHR-CO)n-OH + H) and internal acyl ions denoted by single-letter codes. The internal acyl ions are formed by two amide bond cleavages, the first occurring NH2-terminal to Pro, and the second involving any residue COOH-terminal to the Pro (e.g. PET, Fig. 6, HN_CHR=CO-NH-CR10=CO-NH_CR11=CO-OH, where R9 = Pro, R10 = Glu, R11 = Thr, and yn indicates the cyclization of R9 to the NH). The subsequence for the peptide of (M + H)+ = 2066, the sequence of residues 2-15 are defined by the fragment ions observed (Fig. 5).

The subsequence for the peptide of M+1 = 1776 determined by MALDI-MS/MS overlaps with the Edman data for the NH2-terminus of DUT-M, indicating that the residues 22 and 23 missing in the Edman data correspond to glycine and proline, respectively (Fig. 6). Based on the Edman and MS data, a M, of 1775.9 is predicted for the tryptic peptide AGGSAPG-PETPAISPSKR that would overlap with the determined sequence of DUT-N. This predicted M, corresponds very closely to that observed in the MALDI-MS data (Figs. 4 and 6). In addition, other major signals observed in the MALDI-MS/MS data can be assigned to internal acyl ions for the partial sequences PETPAIS and PGPE and to the Y10 ion for PSKR (Fig. 5).

Thus, the MS data define the region of greatest uncertainty in the Edman data and establish the junction of the isoforms. Together these data indicate that the nuclear associated DUT-N and the mitochondrial associated DUT-M variants...
Kinetic Analysis of Nuclear and Mitochondrial Variants of dUTPase—In order to determine if the different amino termini resulted in a variation in enzymatic activity, nuclear and mitochondrial dUTPase were purified separately, and 

\[ K_m \]

values were determined for each (see "Experimental Procedures"). Despite the structural differences, both forms of dUTPase exhibit identical 

\[ K_m \]

values of 2.5 \( \mu \)M for dUTP.

DISCUSSION

The dUTPase function has been shown to be important in DNA replication (3–5) and is highly conserved throughout evolution (14). Our laboratory is investigating the basic biochemical and regulatory aspects of human dUTPase. We have previously described HeLa-derived dUTPase as a 22.5-kDa phosphoprotein with a 

\[ K_m \]

value for dUTP of 2.5 \( \mu \)M and a requirement for Mg\(^{2+}\) (12, 20).

To further characterize the human enzyme, we set out to isolate a dUTPase-specific cDNA. Evidence presented in this report demonstrates that the cDNA sequence described corresponds to the major form of the dUTPase protein from HeLa cells (DUT-N). cDNA and amino-terminal protein sequence analysis indicates that the open reading frame of the DUT-N isoform of dUTPase contains 24 more amino-terminal residues than previously reported (1, 2). The NH\(_2\)-terminal methionine is removed in the mature DUT-N protein. Utilizing the methods described in this report, there is no evidence suggesting the existence of an expressed form of dUTPase in HeLa cells corresponding to the predicted translation start site reported by McIntosh, et al. (1) or Strahler, et al. (2).

Nuclear and Mitochondrial Forms of dUTPase—In this study, we demonstrate that multiple forms of dUTPase exist within human cells. Cellular fractionation of HeLa cells and Western blot analysis suggest that the smaller molecular weight species of dUTPase (DUT-N) is associated with the nucleus and is at least 30-fold more abundant than a larger molecular weight species (DUT-M), which is apparently associated with the mitochondria.
Nuclear dUTPase, human

| A | S | Y | T | G | A | A | N | G | W | G | E | L | P | A | G | G | S | A | P | P | E | R | K | P | A | S | P | E | S | K | E | G | H | L | Y | T | P | A | P | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1676.7 | 1687.7 |

Mitochondrial dUTPase, human

| A | S | T | Y | G | A | A | N | G | W | G | E | L | P | A | G | G | S | A | P | P | E | R | K | P | A | S | P | E | S | K | E | G | H | L | Y | T | P | A | P | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1776.6 | 1676.7 |

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Fig. 6. Comparison of the structural differences between the nuclear and mitochondrial forms of dUTPase. Comparison of the sequences of nuclear (top) and mitochondrial (bottom) human dUTPase. Solid underlines indicate tryptic peptides whose (M + H)^+ ions were observed in the MALDI-MS data (see Fig. 5). The calculated, monoisotopic molecular weights of the peptides are shown. Dashed underlines indicate the Edman sequencing data obtained on the intact proteins; the gap in the sequence data for the mitochondrial form is indicated by the absence of dashed underline. The sequence within the box was obtained by MALDI-MS/MS of the tryptic peptide of molecular weight 1776 (see Fig. 5 and text). Residue 10 in the sequence of the nuclear form is a phosphoserine (pS; see accompanying article (48) for discussion).

Western blot analysis of partially purified cytosolic extract (Fig. 3, lane 2) demonstrates the presence of the mitochondrial associated dUTPase. This analysis also reveals another previously undetected dUTPase species of slightly greater molecular weight than the mitochondrial associated form. We speculate that this protein may represent a precursor form of mitochondrial dUTPase. Many proteins residing in mitochondria are encoded by nuclear genes. These proteins are typically translated as precursor proteins containing an extended amino-terminal leader region containing amphiphilic amino acids (37). Upon transfer into the mitochondria, the signal sequence is proteolytically removed by a signal peptidase. It is feasible that the immunoreactive, larger mass protein species present in cytosolic extract represents an unprocessed precursor form of mitochondrial dUTPase. This also suggests that the DUT-M protein identified in this study corresponds to the fully processed mitochondrial form. Future delineation of the unprocessed mitochondrial dUTPase protein as well as cloning of a full-length mitochondrial dUTPase cDNA may reveal further attributes of a mitochondrially targeted protein.

Analysis of the DUT-N and DUT-M protein species by mass spectrometry indicates that the two forms of dUTPase are largely identical except for a short region at their amino termini. The fact that the nuclear and mitochondrial forms are so similar in amino acid sequence raises the possibility that they are the result of alternative splicing or differential transcription from separate promoters within the same gene. There are several examples of proteins that are partitioned or distributed to different intracellular compartments through the use of alternative splicing (for review, see Smith et al. (38)). The actin-filament-severing protein gelsolin is expressed as a plasma and a cytoplasmic protein. The two proteins are identical except for 25 amino-terminal residues and are expressed by different promoters within the same gene. In addition, they undergo differential alternative splicing of 5’ exons to generate distinct amino termini (39). It is possible that expression of the nuclear and mitochondrial forms of dUTPase is regulated through the use of an analogous alternative splicing mechanism. The data presented in this report are consistent with this model. Northern blot analysis of HeLa poly(A) mRNA reveals two messages of 1.1 and 2.3 kb, respectively. The more abundant 1.1-kb message appears to correspond in size to the nuclear dUTPase. It is possible that the 2.3-kb mRNA species may correspond to the larger mitochondrial dUTPase. It will be of interest to determine the genomic organization of the two dUTPase isoforms as well as to uncover the mechanisms of expression.

Kinetic Analysis of the Nuclear and Mitochondrial Forms of dUTPase—Determination of the $K_m$ values for the nuclear and mitochondrial forms of human dUTPase reveal that they are both equivalent 2.5 $\mu M$. This is in close agreement with previous determinations for the purified HeLa enzyme (12). The fact that these two forms of dUTPase have identical $K_m$ values is consistent with known structural information. McGeoch (14) first noted that there are five regions of high amino acid conservation that are common to all known dUTPases. Each of these five regions is present in both species of human dUTPase. In addition, the crystal structure of the E. coli dUTPase enzyme has been determined (40). The determined structure indicates that many of these conserved domains border upon a cleft, thought to be the active site. Site-directed mutagenesis of many of the most highly conserved amino acids compromises or inactivates dUTP hydrolyzing function, further illustrating the importance of the conserved regions to the catalytic activity of dUTPase. The differences between the nuclear and mitochondrial forms are restricted to the amino-terminal domain, which is a nonconserved region of the protein. This implies that the amino-terminal domain of dUTPase is not an essential component of the active site. Further evidence supporting this theory is observed in a recombinant form of dUTPase that lacks the first 22 amino-terminal residues of the DUT-N protein. This truncated recombinant protein was reported to be catalytically active with a $K_m$ for dUTP of 2.5 $\mu M$ (41), further suggesting that the amino-terminal region of human dUTPase is not a critical component of the active site.

Replication and Repair of the Mitochondrial Genome—Although there have been extensive studies of the mode and rate of mtDNA replication, little is known about the enzymology and biochemistry of mtDNA replication and repair functions. Several enzymes involved in mtDNA replication from a variety of sources have been identified (reviewed by Clayton (42)). Information about DNA repair of the mitochondrial genome is also limited, although recently a few DNA repair enzymes specific to the mitochondria have been identified including photolyase (43) and uracil-DNA glycosylase (44). It seems likely that the majority of the enzymatic functions necessary for efficient replication and repair of the nuclear genome are also required for mtDNA replication. The existence of a mitochondrially targeted variant of dUTPase suggests that this enzyme function may govern dUTP levels at specific locations in the cell, perhaps in close proximity to replicating DNA.

dUTPase as a Target for Drug Development—Although there has been no report of direct inhibition of dUTPase in human cells, evidence from viral, bacterial, and yeast systems strongly

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2 R. D. Ladner and S. J. Caradonna, unpublished observation.
suggest that the dUTPase function is vital for efficient mammalian DNA replication. Indirect evidence of the essential nature of this enzyme function in humans is exhibited by the action of certain chemotherapeutic agents that inhibit de novo thymidylate metabolism (7, 8, 10). It appears that elevation of dUTP pools and subsequent imbalance of the cellular dUTP:dTTP ratio is the lethal mechanism by which chemotherapeutic agents such as FUdR function. Thymidylate metabolism is a pathway that has long been a target for effective and widely utilized chemotherapeutic agents (FUdR, methotrexate, etc.). Inhibition of dUTPase in conjunction with traditional thymidylate synthase inhibitors (such as FUdR) would likely accelerate the dUTP/dTTP pool imbalance and aid in the cytotoxic effect. Moreover, inhibition of the dUTPase enzyme would be selective for aggressively replicating tissues. Data from this laboratory demonstrate a strong correlation between nuclear dUTPase protein levels and the proliferation status of tissues.2

In addition to potential cancer chemotherapy, inhibition of human dUTPase may also hold promise as an antiviral therapy as well. There has been evolutionary pressure to conserve the dUTPase function in many viral genomes, and loss of the viral encoded enzyme lowers replication efficiency in certain viruses (16, 18, 19). It has been postulated that viruses that do not encode an enzyme function in humans is exhibited by the human dUTPase enzyme encoded dUTPase function (such as HIV) must rely entirely on (16, 18, 19). It has been postulated that viruses that do not encode a dUTPase function (such as HIV) must rely entirely on the host enzyme (20). Thus, the human dUTPase enzyme should also be considered as a potential candidate in the search for new anti-HIV targets.

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