Developmental Regulation of dUTPase in *Drosophila melanogaster**

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dUTPase prevents uracil incorporation into DNA by strict regulation of the cellular dUTP:dTTP ratio. Lack of the enzyme initiates thymineless cell death, prompting studies on enzyme regulation. We investigated expression pattern and localization of *Drosophila* dUTPase. Similarly to human, two isoforms of the fly enzyme were identified at both mRNA and protein levels. During larval stages, a drastic decrease of dUTPase expression was demonstrated at the protein level. In contrast, dUTPase mRNAs display constitutive character throughout development. A putative nuclear localization signal was identified in one of the two isoforms. However, immunohistochemistry of ovaries and embryos did not show a clear correlation between the presence of this signal and subcellular localization of the protein, suggesting that the latter may be perturbed by additional factors. Results are in agreement with a multilevel regulation of dUTPase in the *Drosophila* proteome, possibly involving several interacting protein partners of the enzyme. Using independent approaches, the existence of such macromolecular partners was verified.

Faithful conservation and transmission of genetic information are crucial for living organisms. The enzyme dUTPase prevents uracil incorporation into DNA by hydrolysis of dUTP into dUMP and inorganic pyrophosphate and provides a unique and essential preventive DNA repair function. Lack of dUTPase leads to uracil-substituted DNA that perturbs base excision repair, resulting in DNA fragmentation and thymineless apoptosis of the cell. The physiological role of the enzyme argues for regulation of dUTPase presence, localization, and/or function, depending on cell status.

This implication was closely investigated in human cells. Two isoforms of the enzyme were identified: a mitochondrial isoform with constitutive expression, and a nuclear isoform with cell cycle-dependent expression (1). Both are transcribed from the same gene using alternative promoters and differ only

in their N-terminal region responsible for adequate localization (2). dUTPase in mitochondria is necessary for integrity of mitochondrial DNA that replicates independently from the cell cycle. In mature lymphocytes, correlation between dUTPase presence and cell mitogenic status suggested enzyme up-regulation in stages associated with DNA synthesis, whereas in immature populations, enzyme levels are constitutive (3). The requirement of actively dividing cells for high dUTPase levels is also confirmed by studies in rat liver regeneration (4) and mitogen-activated T and B cells (5). Overexpression of dUTPase induces resistance to chemotherapeutic agents that target thymidylate biosynthesis (6). This suggests that in addition to inhibition of thymidylate synthase and dihydrofolate reductase, dUTPase targeting as a novel chemotherapeutic strategy may ensure synergistic effects.

Cancer cells are usually characterized by deficiency in most apoptotic pathways and are not easily induced into committing suicide. Programmed cell death pathways still operable after malignant transformations are therefore of high therapeutic value. Thymineless cell death was recently suggested to be independent from p53, a central factor in most apoptotic pathways (7, 8). This finding underlines the importance and possible gains of targeting thymidylate metabolism in tumor cells.

In multicellular organisms, apoptosis plays a key role not only in prevention (and therapy) of malignant transformations but in other developmental processes as well. Apoptosis of T and B cells, triggered by receptor-ligand interactions, is essential in the development of the immune system to prevent autoimmunity as well as to facilitate selection of lymphocytes (9, 10). Down-regulation of dUTPase in anti-IgM antibody-induced B-cell apoptosis was reported recently (11), indicating that the thymineless pathway might also be of importance in cell death during development. Putative accumulation of uracil-containing DNA under thymineless conditions in larval stages of *Drosophila melanogaster* may play a role in programmed cell death necessary for metamorphosis in the pupal stage (12). The role of dUTPase was considered to be central in this process; moreover, a developmentally induced heat-stable dUTPase inhibitor protein was also suggested to exist (13). Endogenous macromolecules specifically antagonizing with factors involved in apoptosis and/or DNA metabolism and repair are undoubtedly of great importance because these may provide a drug design lead molecule already tailored by evolution. Unfortunately, lack of knowledge about the molecular biology, physiology, and cellular status of fly dUTPase impeded a systematic assessment of its regulatory pathways.

To approach this problem, we have cloned the fly enzyme and performed kinetic and structural characterization of the recombinant protein (14). To describe the role of dUTPase in *Dro-*

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sophila that might also reveal important clues about eventual parallel mechanisms in *Homo sapiens*, we decided to proceed with physiological characterization of fly dUTPase.

In the present work, expression, localization, and developmental patterns of *Drosophila* dUTPase are described. Two isoforms of the enzyme were isolated by immunoprecipitation and identified by mass spectrometry. Discrepancy between protein and mRNA levels as well as cellular localization patterns suggested the possibility of regulation by additional factors.

EXPERIMENTAL PROCEDURES

Schneider Line 2 (S2)[1] Cell Procedures—*Drosophila* S2 cells (Invitrogen) were cultured at 22 °C in *Drosophila* SFM (Life Technologies, Inc.) medium. Extracts were prepared from cell pellets washed twice in PBS, resuspended in lysis buffer (10 mM TRIS, pH 7.2, also containing 10% glycerol, 0.15 M NaCl, 1 mM dithiothreitol, and proteinase inhibitor mix (Sigma)), sonicated for 1 min, and centrifuged at 18,000 × g.

Collection of Different Developmental Stages of Drosophila—Oregon-R wild-type flies were kept on cornmeal-yeast food, containing Nipagin as fungicide, at room temperature. Two-h early embryos, 12-h embryos, 36-h first larvae, 60-h second larvae, 96-h third larvae, and 8-day pupae, staged according to Ashburner (15), were collected, washed, and homogenized with a Potter-Elvehjem homogenizer and sonicated in phosphate-buffered saline (PBS). Ovaries were fractionated into immature and more mature populations by trypsinolysis and filtration. A heat-stable fraction of larval extract was prepared by 5-min incubation at 100 °C in a boiling water bath of the crude extract, followed by centrifugation. Protein concentration measurements by Bradford assay indicated that ~5% of the total protein content stays in the solution phase after this heat treatment.

Subcellular Fractionation of Drosophila Embryos—Twelve-h embryos were collected, washed, and homogenized in 50 mM 1,4-piperazinediethanesulfonic acid buffer, pH 7.9, also containing 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was subjected to differential centrifugation at 4 °C, following the procedure described by Igaki *et al.* (16). The first pellet after centrifugation at 700 × g for 10 min contained the nuclear fraction. Supernatant from this step was further centrifuged at 24,000 × g for 10 min to sediment the mitochondrial fraction. The cytoplasmic fraction was obtained after a final centrifugation at 54,000 × g for 1 h. Equal amounts of protein from each fraction were applied on SDS-PAGE and analyzed by Western blotting.

Preparation of Polyclonal Antibody for Drosophila dUTPase—Rabbits were immunized with recombinant full-length *Drosophila* dUTPase (14). Three immunizing shots were given, at time intervals of 2–3 weeks, first in complete free in incomplete Freund's adjuvant or in physiological saline (17–19). Serum was used at a dilution of 1:100,000 in Western blot on nitrocellulose membranes.

Western and Far-Western Blotting—Extracts were run using SDS-PAGE (20) and transferred to nitrocellulose membrane (Sigma). Blots were stained first with Ponceau dye and then developed with the antiserum, followed by staining with secondary antibody (alkaline phosphatase or horseradish peroxidase labeled anti-rabbit IgG, at 1:80,000 and 1:2,500 dilutions, respectively). For visualization, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate or the enhanced chemiluminescence kit of Amersham Biosciences was used. Monoclonal anti-α-tubulin (Sigma) was used as loading control. For far-Western blotting, wet membranes were blocked with 5% bovine serum albumin in Tris buffered saline with 0.1% Tween 20 for 1 h and incubated with 30 μg/ml of recombinant *Drosophila* dUTPase at 4 °C overnight. After extensive washing with PBS, the procedure was continued according to Western blotting protocol.

Immunoprecipitation—dUTPase isoforms were immunoprecipitated from S2 cells with partially purified polyclonal antibody coupled to cyanogen bromide-activated Sepharose (Sigma). Antigen was eluted from the resin by boiling for 5 min in 0.1 M Tris buffer, pH 8.0, also containing 10% SDS (21).

Protein Identification by Mass Spectrometry—Proteins were in-gel digested by trypsin (Promega) after reduction and alkylation. The tryptic digests were analyzed on a Reflex III matrix-assisted laser desorption/ionization, time-of-flight mass spectrometer (MALDI-TOF MS, Bruker, Germany) unfractionated as well as after reversed-phase high-

pressure liquid chromatography fractionation. 2,5-Dihydroxy benzoic acid was used as the matrix. All mass spectra were acquired in positive reflectron mode, with delayed extraction, using external calibration. A database search was performed on a National Center for Biotechnology Information database using ProteinProspector.[2] To obtain sequence information, post source decay (PSD) analysis of selected components was performed.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and DNA Gel Electrophoresis—Total RNA was isolated from different developmental stages of *Drosophila* and S2 cells by TRIzol reagent (Invitrogen/Life Technologies, Inc., Grand Island, NY). Reverse transcription was performed with RNase H(−) (Promega) at 37 °C for 1.5 h. For PCR amplification of dUTPase cDNAs, primers were designed to straddle the putative intronic region of the dUTPase gene (see Fig. 2). DNA gel electrophoresis was performed on 1.5% agarose gels using standard procedures.

Immunofluorescent Microscopy—Ovaries and discs were dissected in *Drosophila* Ringer's and fixed for 1 h at room temperature in 4% paraformaldehyde diluted in 100 mM 1,4-piperazinediethanesulfonic acid buffer, pH 6.9, also containing 1 mM MgCl₂, 1 mM EGTA, 2.5% Tween 20, and an equal volume of heptane. Two-h embryos were collected, dechorionated (protocol 9.2, Ref. 22), fixed for 10–12 min (23), and rehydrated (protocol 9.4, method 1 of Ref. 22). Immunostaining of ovaries, discs, and embryos (protocol 9.7, method 1 of Ref. 22) was followed by 4′,6-diamidino-2-phenylindole incubation (protocol 9.6, Ref. 22). S2 cells were cultured on microscope slides, fixed in 3% bovine serum albumin in PBS for 30 min, permeabilized with 0.1% Triton X-100 in H₂O for 5 min, blocked in 1% bovine serum albumin in PBS for 30 min, and incubated with antibodies and 4′,6-diamidino-2-phenylindole for 30 min and for 10 min, respectively, with PBS washing between steps. Antiserum at 1:10,000 dilution and FITC-conjugated anti-rabbit immunoglobulins (Sigma) at 1:500 dilution were used. Samples mounted in FluorSave Reagent (Calbiochem) were visualized with a Leica DMLS fluorescence microscope and with an Olympus confocal laser scanning microscope.

mRNA in Situ Hybridization—cDNA was prepared by PCR on the dUTPase gene containing the pET-22b expression plasmid (14) using dUTPase-specific primers (forward, 5′-ATGCCATCAACCGATTTCGC-CGACATT; reverse, 5′-TTACGTAGCAACAGGAGCCGGAG-3′). The DIG Nucleic Acid Labeling and Detection Kit (Roche) was used for labeling the cDNA with digoxigenin and *in situ* detection. Ovaries were fixed, and hybridization was performed according to Ephrussi *et al.* (24). Samples were mounted in PBS buffer containing 90% (v/v) glycerol and analyzed with a Leica DMLS microscope.

Surface Plasmon Resonance—Full-length (1–187) and C-terminal-truncated (1–159) recombinant *Drosophila* dUTPases (14) were immobilized in 10 mM 4-morphineethanesulfonic acid buffer on sensor chips by *N*-ethyl-*N*′-(dimethyl-aminopropyl)carbodiimide hydrochloride activation. Chips were analyzed using either 5 μl/min or 20 μl/min flow rate in a Biacore X instrument (Biacore AP, Uppsala, Sweden) with flow buffer (10 mM HEPES, pH 7.4, also containing 0.15 M NaCl, 3 mM EDTA, and 0.005% P20 detergent). Thirty- to 35-μl injections of recombinant purified dUTPase (1–159), serum albumin (both at 0.15 mg/ml), and first instar larval extract (at 0.05–0.28 mg/ml concentrations) were applied on the dUTPase chips.

RESULTS

Two Isoforms of dUTPase Protein in Drosophila—A monospecific antibody was required for dUTPase detection in *Drosophila* samples. Fig. 1A demonstrates that a highly specific polyclonal serum with no cross-reactivity to other cellular proteins was raised against recombinant full-length *Drosophila* dUTPase. In *Drosophila* S2 cell extract (Fig. 1A, *lane 5*), two distinct serum-reactive protein bands (with molecular masses of 23 and 21 kDa) suggested that two dUTPase isoforms might be present. Immunoprecipitated dUTPase proteins (Fig. 1B) were subjected to in-gel trypsinolysis and mass spectrometry (Fig. 1, *C* and *D*). For the two putative isoforms, 64 and 59% of the peptide masses detected in the mass spectra showed clear matches to predicted dUTPase tryptic fragments, providing 72 and 66% coverage (Fig. 2, compare underlines and overlines) of the dUTPase sequence, respectively. Peptide mass fingerprints

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[1] The abbreviations used are: S2, Schneider line 2; PBS, phosphate-buffered saline; PSD, post source decay; RT-PCR, reverse transcription-polymerase chain reaction; NLS, nuclear localization signal.

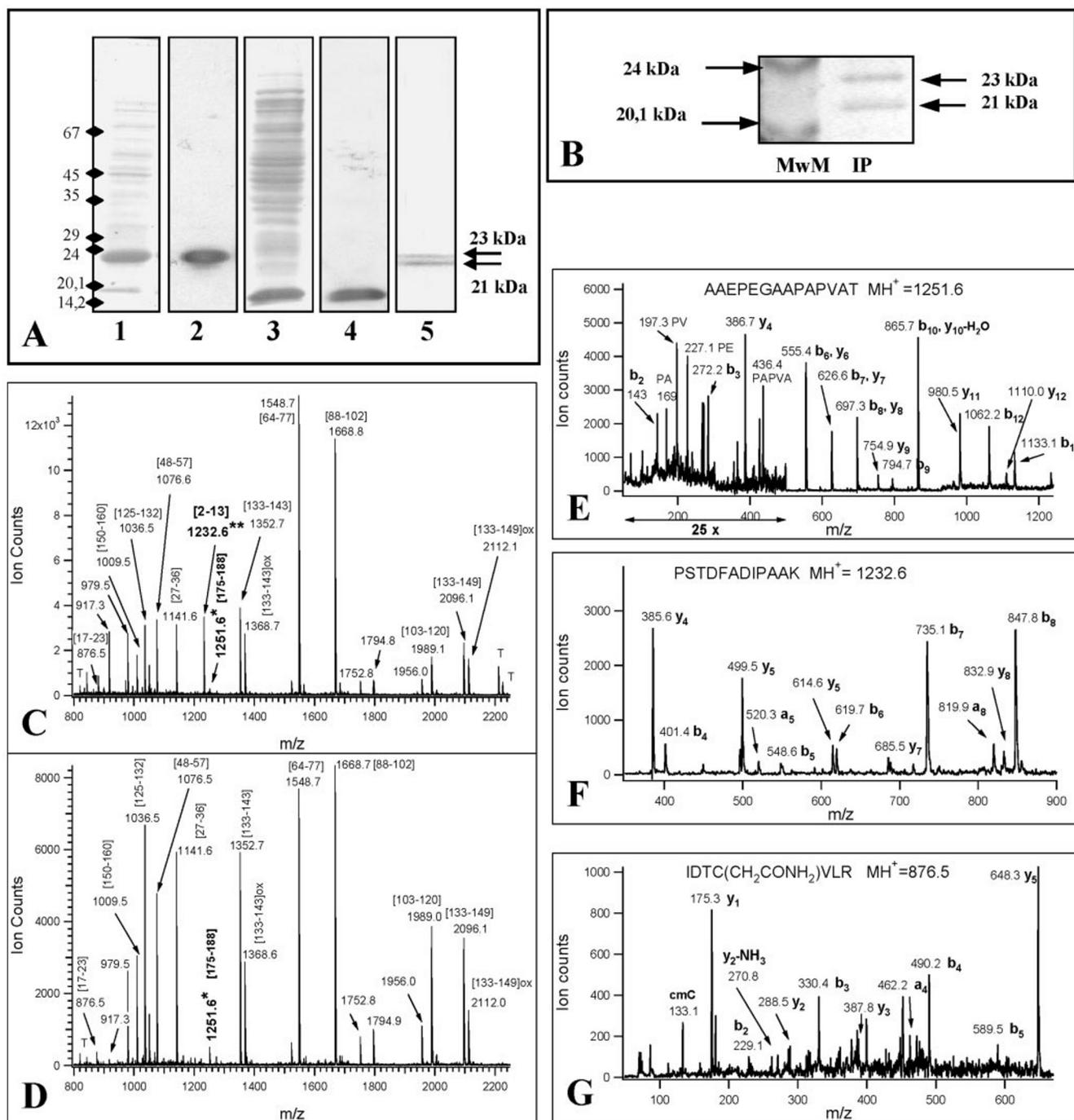[2] Internet address: prospector.ucsf.edu/ucsfhtml4.0/msfit.htm.

FIG. 1. **Identification of Drosophila dUTPase isoforms.** *A*, characterization of the *Drosophila* dUTPase antiserum. *Lanes 1* and *3*, protein stain; *lanes 2* and *4*, Western blot of extracts from *Escherichia coli* cells expressing full-length and truncated (1–159) *Drosophila* dUTPase, respectively (14). *Lane 5*, Western blot of *Drosophila* S2 cell extract. *Marks*, marker positions; *arrows*, dUTPase isoforms. *B*, immunoprecipitation of *Drosophila* dUTPase from S2 cells. *MwM*, markers; *IP*, immunoprecipitate on SDS-PAGE. *Arrows*, estimated molecular masses for the isoforms. *C* and *D*, mass spectra of unfractionated tryptic in-gel digests of 23- and 21-kDa *Drosophila* dUTPase, respectively. Peptides are labeled with masses and corresponding amino acid sequence positions. *Single asterisk*, *Drosophila*-specific C-terminal peptide; *two asterisks*, peptide unique for the 23-kDa species. *E–G*, PSD spectra of the common C-terminal (*m/z* of 1251.6) and the different N-terminal (*m/z* of 1232.6 and 876.5) peptides of the isoforms. Peptide fragment ions are labeled according to the nomenclature (56).

of the two digests are almost identical, excluding a unique peptide ((2–13) with *m/z* 1232.6) in the larger isoform (Fig. 1*C*, *double asterisks*).

Genomic data on *Drosophila* dUTPase predicted a unique C-terminal 28-residue extension (25). A peptide with *m/z* 1251.6 corresponding to the C-terminal tryptic segment of this region was detected in the mass spectra of both investigated protein samples (*single asterisk* in Fig. 1, *C* and *D*). PSD analysis (26) of this peptide from both 23- and 21-kDa samples confirmed the sequence as AAEPEGAAPAPVAT (residues in

one-letter code, Fig. 1*E*). This indicates that the unique Ala-Pro-rich C-terminal segment is present in both putative isoforms. The potential significance of this result will be addressed below.

Having determined that C termini are identical in the two putative isoforms, N termini were investigated. PSD analysis of the peptide with *m/z* 1232.6, corresponding to the segment (2–13) of *Drosophila* dUTPase and present only in the mass spectrum of the 23-kDa isoform, provided independent sequence information (Fig. 1*F*). Interestingly, the determined

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tr-A  -----cgacgctgtgttaaaccgcgttattttcagaccagaattc   40
tr-B  --ttccgacgctgtgttaaaccgcgttattttcagaccagaattc   43

tr-A  tgcaagtaagctgaaaaaagtctctgtactttcgaagcattcctg   85
tr-B  tgcaa----------------------------------------   48

tr-A  taataactcaatttgctccaaatgccatcaaccgatttcgccgac  130
tr-B  ---------------------------------------------
       1                        M  P  S  T  D  F  A  D

tr-A  attccagctgccaagaagatgaagatcgacacgtgcgtcctgcga  175
tr-B  -------ctgccaagaagatg*************************   86
       9        I   P  A  A  K  K  M  K  I  D  T  C  V  L  R

tr-A  ttcgccaaactcaccgagaatgctttggagccggtgaggggatcc  220
tr-B  *********************************************  131
      24    F  A  K  L  T  E  N  A  L  E  P  V  R  G  S

tr-A  gccaaagcggcgggagttgacctgcgcagcgcctacgacgttgtg  265
tr-B  *********************************************  176
      39    A  K  A  A  G  V  D  L  R  S  A  Y  D  V  V

tr-A  gtgccggcacgcggaaaggccattgtcaagaccgatctgcaagtg  310
tr-B  *********************************************  221
      54    V  P  A  R  G  K  A  I  V  K  T  D  L  Q  V

tr-A  caggttccggagggctcctacggacgcgtagccccacgatccggg  355
tr-B  *********************************************  266
      69    Q  V  P  E  G  S  Y  G  R  V  A  P  R  S  G

tr-A  ctggcggtgaagaacttcattgatgtgggcgccggtgtggtggac  400
tr-B  *********************************************  311
      84    L  A  V  K  N  F  I  D  V  G  A  G  V  V  D

tr-A  gaggattatcgcggcaatctcggcgtcgtcctgttcaatcactca  445
tr-B  *********************************************  356
      99    E  D  Y  R  G  N  L  G  V  V  L  F  N  H  S

tr-A  gatgttgatttcgaggtgaagcatggcgaccgcatcgcccagttc  490
tr-B  *********************************************  401
     114    D  V  D  F  E  V  K  H  G  D  R  I  A  Q  F

tr-A  atttgcgagcgtatcttctatccgcaactggtgatggtggacaaa  535
tr-B  *********************************************  446
     129    I  C  E  R  I  F  Y  P  Q  L  V  M  V  D  K

tr-A  ctggaggacaccgagcgcggcgaggcaggattcggttccaccgga  580
tr-B  *********************************************  491
     144    L  E  D  T  E  R  G  E  A  G  F  G  S  T  G

tr-A  gtcaaggatctgccggcagccaaggcgcagaacggcaacggagaa  625
tr-B  *********************************************  536
     159    V  K  D  L  P  A  A  K  A  Q  N  G  N  G  E

tr-A  aaggctgccgaaccggagggagctgctcggctcctgttgctacg  670
tr-B  ************************************          581
     174    K  A  A  E  P  E  G  A  A  P  A  P  V  A  T

tr-A  taaacgaggaactattgccgcattcttaatcccttcatggtcact  715
tr-B  *****************************************      626

tr-A  atcaaagagtttcattctgtatccttaagtgaaatatgttaatgt  760
tr-B  *********************************************  671

tr-A  attggtatattttatctattaaaaccgcaaatctacgaagaaa   804
tr-B  *******************************************  715
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FIG. 2. **mRNA and amino acid sequence of Drosophila dUTPase isoforms.** Alignment of predicted mRNA transcripts (tr-A and tr-B) and the translated protein sequence revealed the position of the putative intron (starting at nucleotide 48 in tr-B). Start codons are in *bold*, common coding regions of the two transcripts are marked with *stars* in tr-B. The 20-bp long segment from the 5′-untranslated region (*5′-UTR*) present in both tr-A and tr-B and the last 22-bp segment of the coding region that was used for primer design in RT-PCR are shown in *dark gray boxes*. Nucleotide sequences are numbered in normal font at the *right end* of each row. In the protein sequence, the N-terminal 14-residue peptide region encoded only in the longer predicted transcript is *italic*. The identified putative NLS is *framed*. Segments denoted by *underlines* or *overlines* correspond to tryptic peptides for which mass spectral peaks were identified in the case of the 23- and 21-kDa isoforms, respectively. *Light gray boxes* indicate the five dUTPase conserved motifs. The *Drosophila*-specific extra C-terminal 28-residue region is in *bold*.

sequence PSTDFADIPAAK argues for lack of the N-terminal methionine residue from the physiological dUTPase 23-kDa species. Similar posttranslational processing has been reported for the nuclear isoform of human dUTPase (1). The absence of this peptide from the tryptic digest of the 21-kDa isoform, even after reversed-phase high-pressure liquid chromatography fractionation, may indicate that the smaller species has a different N terminus. In this digest, the peptide with m/z 876.5, corresponding to residues 17–23 of the predicted dUTPase sequence, could be detected as closest to the N terminus. PSD spectrum of this peptide confirmed its sequence as IDTCVLR (Fig. 1*G*). These data indicate that N termini are in fact different in the two putative isoforms. This difference is in excellent agreement with the observed alteration in molecular masses of intact isoforms, as determined on SDS-PAGE gels (see Fig. 1*B*).

The lack of the indicated N-terminal segment in the shorter isoform does not influence the five conserved dUTPase sequence motifs, necessary for catalytic function. However, alteration of the N terminus results in breakdown of a putative nuclear localization signal (NLS) in the 21-kDa isoform (Fig. 2 and "Discussion").

To exclude the possibility that the shorter isoform is generated by uncontrolled proteolysis in cell extracts, its existence was addressed at the mRNA level. The recent *Drosophila* Genome Release 3.1 reports an intronic region, conforming to the widely accepted GT-AG rule (27), in the single dUTPase gene denoted as CG4584. The resulting two expected mRNA transcripts are termed tr-A and tr-B in Fig. 2. For semiquantitative RT-PCR, forward and reverse primers (Fig. 2, *dark gray boxes*) were designed to straddle the putative intronic region of the dUTPase gene. RT-PCR with various *Drosophila* samples showed two bands at positions expected for the predicted 670- and 578-bp lengths of the amplified region of tr-A and tr-B, respectively (Fig. 3*E*). These results confirmed the existence of two splice variants of dUTPase mRNA in *Drosophila* corresponding to two protein isoforms with different N termini.

Developmental Control of dUTPase Is Different at Protein and mRNA Levels—The two isoforms were followed at both protein and mRNA levels throughout development. Both protein isoforms showed a drastic decrease during larval stages (Fig. 3*A*), in agreement with the previous hypothesis (compare the Introduction of Deutsch (12)). In the pupal stage, the enzyme is again at a higher level. The ratio of the two protein isoforms is about 1:1 and does not change significantly during development (Fig. 3*A*). Adult flies express dUTPase mostly in their ovaries; male flies do not contain the enzyme at detectable levels (Fig. 3*B*). Mature as compared with immature follicles become significantly enriched in the short 21-kDa variant (Fig. 3*C*). This phenomenon parallels a change in subcellular localization of the enzyme (see below).

In addition to developmental regulation of dUTPase expression, enzyme levels were also suggested to change during the cell cycle in human cells (1). To investigate whether this control may also apply for fruit fly, we analyzed dUTPase levels in cycling and resting cells. Extracts of cells in the logarithmic growing stage showed high dUTPase levels, whereas the enzyme in overgrown cell populations was beyond detection limit (Fig. 3*D*).

In striking contrast to the strong developmental control of the dUTPase protein presence, mRNA levels show a constitutive character during fly development (Fig. 3*E*). Relative amounts of transcripts show some alteration in different stages (Fig. 3*E*). The considerable difference between changes of the mRNA and the protein levels during development suggests the existence of a posttranscriptional regulatory mechanism of the dUTPase isoforms in *Drosophila*.
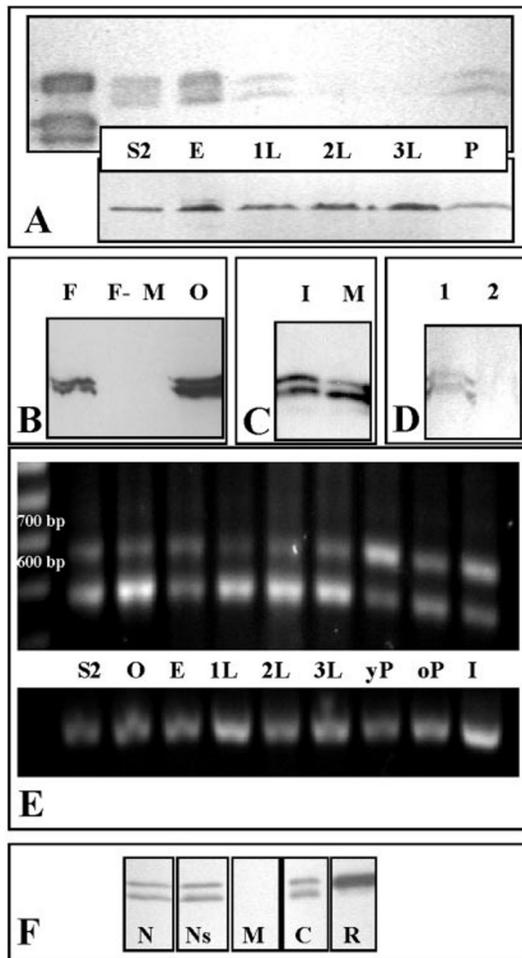
Developmental Regulation of Drosophila dUTPase

FIG. 3. **Developmental control of Drosophila dUTPase isoforms at the protein and mRNA levels.** *A*, Western blot of dUTPase isoforms. *Upper blot*, *leftmost lane*, control sample prepared from purified recombinant full-length and truncated dUTPase species; lanes marked with *S2*, *E*, *1L*, *2L*, *3L*, and *P*, extracts from S2 cells, embryos, first-, second-, and third-stage larvae and pupae, respectively. *Lower blot*, loading control developed with tubulin antibody. *B*, *Drosophila* dUTPase is present mostly in the ovary of adult flies. Extracts of 15 adult female (*F*) or male (*M*) flies, as well as 15 adult female flies, after removal of ovaries (*F-*) and 15 isolated ovaries (*O*) were blotted and probed with anti-dUTPase antiserum. *C*, detection of *Drosophila* dUTPase in immature (*lane I*) and mature (*lane M*) follicles. Individual follicles from 50 ovaries were prepared and Western-probed for dUTPase. *D*, *Drosophila* dUTPase in actively cycling (*lane 1*) and noncycling (*lane 2*) S2 cells. Extracts from an equal amount of S2 cells from logarithmically growing or overgrown cultures were Western-probed for dUTPase. *E*, mRNA levels of dUTPase transcripts. Total mRNA from S2 cells (*S2*), ovaries (*O*), embryos (*E*), first-, second-, and third-stage larvae (*1L*, *2L*, and *3L*), young (7 days), and old (9 days) pupae (*yP* and *oP*) and adult flies (imago, *I*) was prepared for RT-PCR. Base pair numbers above corresponding marker positions are to the *left*. *Upper gel*, RT-PCR with specific primers designed for dUTPase transcripts. *Lower gel*, loading control with primers for the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene. *F*, dUTPase content in different subcellular fractions of *Drosophila* embryos. Proteins of the subcellular fractions were analyzed on Western blots. *N*, fraction enriched in nuclei; *Ns*, supernatant of N; *M*, fraction enriched in mitochondria; *C*, cytoplasmic fraction; *R*, recombinant full-length dUTPase reference.

Subcellular Localization of dUTPase in Different Tissues of Drosophila—Between the two isoforms, the full-length dUTPase contains a putative NLS (PAAKKMKID; Fig. 2 and "Discussion"). However, the shorter isoform does not possess any potential subcellular localization signal. To investigate the presence of the two isoforms in different cellular organelles, subcellular fractionation experiments of *Drosophila* embryos were carried out. Fig. 3*F* demonstrates that: i) the nuclear fraction contains both dUTPase iso-

forms in 1:1 ratio; ii) the mitochondrial fraction does not contain a detectable amount of the enzyme; and iii) the cytoplasmic dUTPase composition is comparable to that found in the fraction sedimenting with the nuclei.

To complement the above experiments performed in homogenized samples with cellular studies, immunohistochemistry was performed (Fig. 4). Follicles passing throughout maturation in the ovary were stained for dUTPase and DNA (Fig. 4*A*). In immature follicles, developing cells showed uniformly nuclear dUTPase staining, and the oocyte could not yet be distinguished (Fig. 4*A*, *asterisk*). During further development, nurse cells and the oocyte were visualized as separate compartments (Fig. 4*A*, *arrow*), and dUTPase stain was more diffuse and less intensive. In more mature follicles (Fig. 4*A*, *cross*), there was a clear distinction between large-nuclei nurse cells and the egg cell with its small nucleus (Fig. 4*A*, *dashed arrow*), which is positioned at one of the poles of the follicle. Within these nurse cells, no dUTPase stain was observed in the nuclei but the enzyme is present exclusively within the cytoplasm at a low intensity, comparable to dUTPase stain observed in the oocyte. Results indicate alteration in dUTPase localization during follicle development. The observed shift of dUTPase stain from nucleus to cytoplasm may be a prelude to protein transport from nurse cells to oocyte, a well-known mechanism for maternal origin of other proteins and mRNAs. *In situ* hybridization confirmed that nurse cells pump dUTPase mRNA into the oocyte (Fig. 5).

Fig. 4*B* shows an early embryonic stage, collected from young flies that were allowed a maximum of 2 h for egg deposition. Morphology of the presented embryo corresponds clearly to the stage where just a limited number (about seven to eight) of nuclear mitotic cycles has yet passed (see Fig. 9.4 in Ref. 22). In this stage, the organism fully depends on maternal protein and mRNA and has not yet started zygotic gene expression (28). The maternal dUTPase pool is visible, mostly associated with the nuclei (Fig. 4*B*). In the late embryonic stages with zygotic expression where gastrulation is already present, dUTPase retains nuclear localization but its distribution becomes heterogenic within the whole embryo (not shown). During larval stages, dUTPase expression is confined to actively proliferating imaginal discs (Fig. 4*C*), corresponding to a decrease of the enzyme level observed on Western blot (Fig. 3*A*). Within the imaginal discs, the enzyme shows mainly nuclear localization (Fig. 4*D*).

The S2 cell line constitutes cells of diverse morphology. In addition to the major fraction of smaller sized, epithelial-like cells (29), some large cells of possible macrophage origin are also observable. dUTPase localization is also heterogeneous (Fig. 4*E*). In most cells, the enzyme is confined to the nucleus; however, cytoplasmic localization is also evident. Results from a quantitative analysis indicate that dUTPase localizes to nuclei in ~85% of S2 cells.

Immunohistochemical results indicate that both cytoplasmic and nuclear localization of dUTPase can be observed in a variety of *Drosophila* tissues. In actively dividing imaginal discs and embryos, localization is mainly confined to the nucleus (Fig. 4, *B–D*). In other larval tissues, the enzyme is either absent (Fig. 4*C*) or shows weak diffuse staining in the cytoplasm (not shown).

Existence of Interacting Macromolecular Partners of Drosophila dUTPase—Results on the developmentally constant 1:1 ratio of the two dUTPase isoforms (Fig. 3, *A, B,* and *D*), among which only one possesses a potential NLS (Fig. 2), are not straightforward to reconcile with the immunohistochemistry data obtained on immature follicles, early embryos, and larvae (Fig. 4, *A–D*), where the protein seems to be present
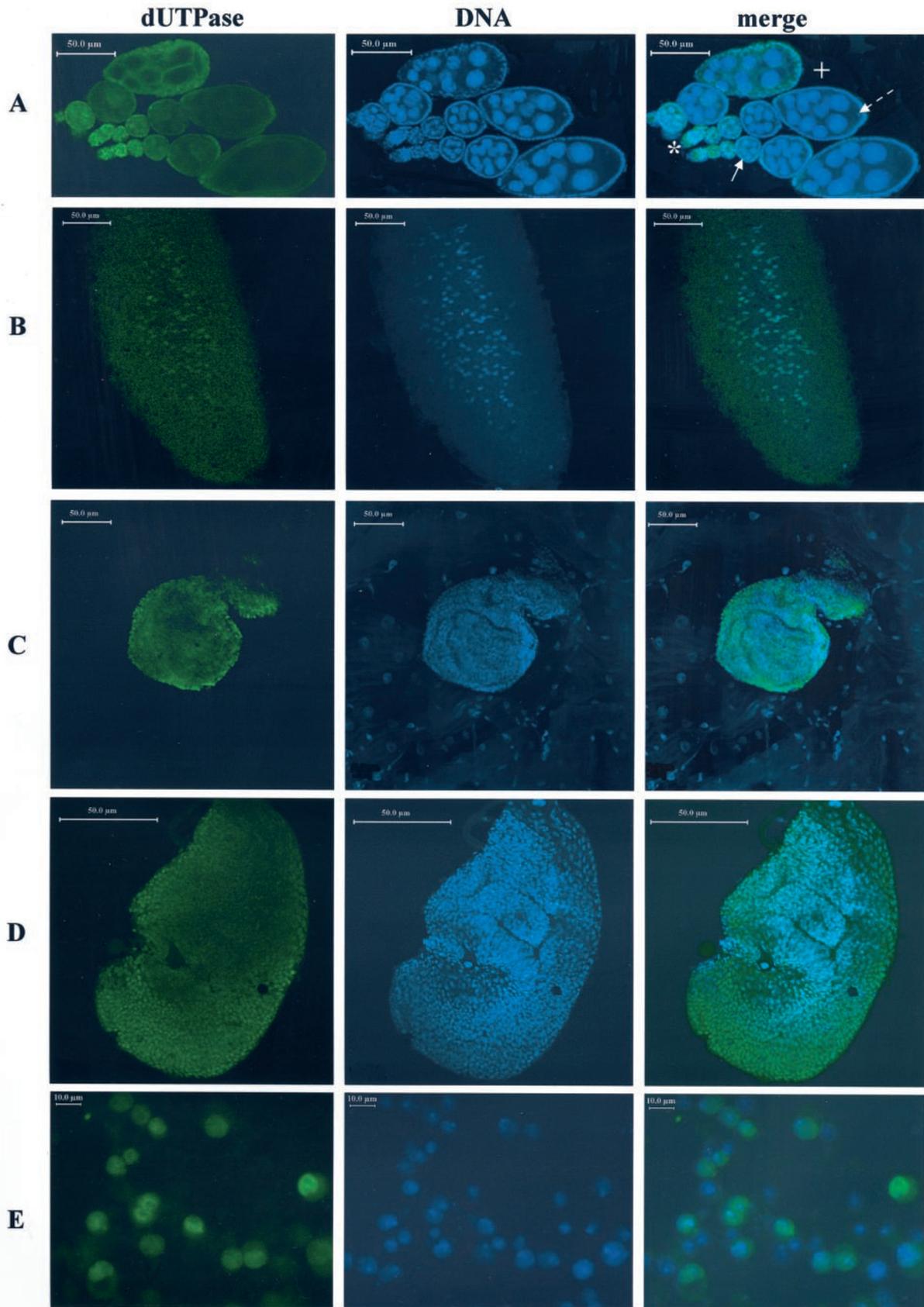
FIG. 4. **Immunofluorescence microscopy for localization of dUTPase in *Drosophila*.** *A*, ovaries. Early immature, medium sized, and final mature follicles are marked with *asterisk*, *arrow*, and *cross*, respectively. *Dashed arrow*, oocyte nucleus. *B*, early embryo. *C*, larva. Part of a dissected larva is shown, with an imaginal disk in the middle that is surrounded with other larval tissues. *D*, isolated imaginal disc. *E*, S2 cells. Double-stained specimens (dUTPase (*green*, *left*) and nuclear DNA (*blue*, *middle*), merge (*right*)) are presented.
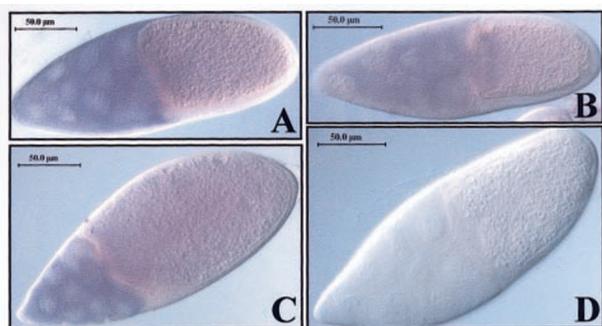
Developmental Regulation of Drosophila dUTPase

FIG. 5. *In situ* **mRNA hybridization of *Drosophila* embryos.** Three stained embryos are shown (*A–C*), together with a control (*D*), where the labeled dUTPase DNA was omitted. dUTPase mRNA is stained violet. Note the violet infiltration into the egg cell from the nurse cells.

mostly in the nucleus. Although the dUTPase protein pool of early embryos is derived from the cytoplasm of maternal nurse cells, the protein becomes quickly enriched in the embryonic nuclei. Beyond the putative NLS-driven process, this phenomenon argues for a different transport mechanism that possibly involves interacting protein partners of dUTPase.

In addition, interacting protein partner(s) were suggested to regulate enzyme activity (13). Experiments performed in our laboratory also indicated that several fractions of S2 cell extract have significant modulatory effect on the recombinant 23-kDa dUTPase isoform.[3]

These observations initiated investigations by two experimental approaches toward endogenous macromolecules capable of binding to dUTPase. Fig. 6, *A–D*, shows results of surface plasmon resonance measurements. Recombinant dUTPase lacking the extra C-terminal 28-residue segment was immobilized on a sensor chip, and heat-stable larval extract (13) was injected upon this surface. In this experimental set-up, significant binding of macromolecules was observed (Fig. 6*B*). This binding is clearly dependent on total protein concentration in the extract, according to thermodynamic expectations on association-dissociation processes. An increase in the baseline signal after injection, also dependent on total protein concentration, indicates that a portion of the bound macromolecules was retained on the dUTPase-conjugated chip. Specificity of the binding was checked by injection of serum albumin (not shown) and dUTPase (Fig. 6*A*) over naive dUTPase-conjugated chip, not yet probed with *Drosophila* larval extract. No binding was observed in either of these control experiments, arguing against nonspecific adsorption. Interestingly, when such a control experiment was repeated by injecting dUTPase upon a dUTPase-conjugated chip probed previously with *Drosophila* larval extract, some binding was clearly observed (Fig. 6*C*). In view of partial retention of *Drosophila* proteins on the dUTPase-conjugated chip, these results may indicate that the retained macromolecules (or their network) are multivalent dUTPase partners.

To check whether the *Drosophila*-specific C-terminal 28-residue segment has a major role in binding macromolecular partners from cellular extract of the larvae, immobilization of both full-length and truncated recombinant *Drosophila* dUTPase was performed on another sensor chip. Injection of heat-stable larval extract upon these two enzyme surfaces in parallel experiments showed practically the same characteristics (Fig. 6*D*), arguing against an exclusive role of the C terminus in the interaction processes.

In the above experiments, heat-stable larval extract was used to prevent the possibility of nonspecific precipitation. Having determined that in the heat-stable extract some specific binding may occur, experiments with injections of total extract were also performed. Binding phenomena were observed in these injections as well, although the markedly different protein compositions of the total and heat-stable extracts precluded direct comparisons. Detailed analysis of binding phenomena using such a heterogeneous population of possible binding partners is not straightforward in surface plasmon resonance techniques. Further quantitative experiments with partially purified dUTPase partner macromolecules are planned in our laboratory.

Despite the lack of quantitative analysis in the above surface plasmon resonance experiments, sensograms argued strongly in favor of the presence of macromolecules capable of physical interaction with dUTPase in *Drosophila* larval extract. These experiments, however, cannot decide whether the observed binding phenomenon is attributable to one single component or to a mixture of dUTPase-binding macromolecules. Far-Western blotting was therefore used additionally with the aim of separating the potential interacting components. This approach is limited to identification of those protein-protein interactions that persist under the relatively harsh experimental conditions. Comparative Western and far-Western blots (Fig. 6*E*) of extracts from *Drosophila* embryos and first-stage larvae show several distinct protein bands that appear only on the far-Western blots. A different pattern of far-Western blots of embryos and first-stage larvae is evident.

The two independent approaches consonantly indicate the presence of dUTPase partners in *Drosophila* that may depend on development. Co-immunoprecipitation and affinity experiments are in progress in our laboratory with the aim of identifying the interacting proteins and characterizing their potential regulatory functions.

DISCUSSION

Two Isoforms of dUTPase—Authentic localization signals for nuclear and mitochondrial isoforms of human dUTPase, as well as human uracil-DNA glycosylase, responsible for excising uracil from DNA, have been described (1, 30–32). Mammalian base excision repair enzymes are present in both nuclei and mitochondria (33–37) for independent repair of organellar DNA. *Drosophila* dUTPase partially conforms to this situation; it is shown here to possess two physiological isoforms with different N termini. The 23-kDa isoform contains a putative NLS (**PAAKK**M**KID**), which is a close homolog of both the experimentally confirmed human c-myc NLS (**PAAKR**V**KLD**) (38, 39) and human RanBP3 NLS (**P**PV**KRER**TS) (conserved residues shown in bold) (40). The homologous S**PSKRAR**PA signal is present in the human dUTPase N terminus, wherein mutation of basic residues completely abolishes nuclear localization (2). The similarities in these NLS sequences together with the experimental evidence available for three different proteins, including the human ortholog, strongly suggest that the PAAKKMKID segment is indeed the authentic NLS of *Drosophila* dUTPase.

The 21-kDa isoform is an N-terminally truncated version lacking this NLS segment; however, it does not contain any other known transport signal. Cellular fractionation experiments (Fig. 3*F*) indicated that isolated mitochondria from *Drosophila* embryos do not contain detectable amounts of dUTPase. In this respect, it is important to point out that mitochondrial base excision repair enzymes were mostly investigated in mammalian systems, and our knowledge on the generation and metabolism of uracil-DNA in *Drosophila* is not yet complete. The lack of the uracil-DNA glycosylase homolog, uracil-DNA glycosylase, in in-

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[3] A. Békési, I. Zagyva, M. Pukáncsik, É. Hunyadi-Gulyás, K. F. Medzihradszky, and B. G. Vértessy, manuscript in preparation.

Fig. 6. **Detection of macromolecular interactions between dUTPase and other cellular macromolecules.** *A–D*, surface plasmon resonance sensograms. *A*, injection of recombinant dUTPase over naive dUTPase (1–159) chip not yet probed with *Drosophila* extract. *B*, injection of heat-stable larval extracts (at 0.05, 0.10, and 0.15 mg/ml protein concentration shown in *light gray*, *dark gray*, and *black curve*, respectively) on the same chip. *C*, injection of dUTPase over dUTPase (1–159) chip already probed with *Drosophila* extract. *D*, Injection of 0.15 mg/ml heat-stable larval extract over full-length (*black trace*) and C-terminal truncated (*gray trace*, offset to aid visualization) dUTPase-conjugated chips. Flow rate in panels *A–C*, 5 μl/s; in panel *D*, 20 μl/s. *E*, Western (*I*) and far-Western (*II*) blots of *Drosophila* extracts from embryos (*E*), and first-stage larvae (*1L*). *Arrows* denote protein bands present exclusively on the far-Western blot. *Dashed arrow* denotes a position, already apparent as a faint *single band* on the Western blot, that becomes a stronger *double band* on the far-Western blot.
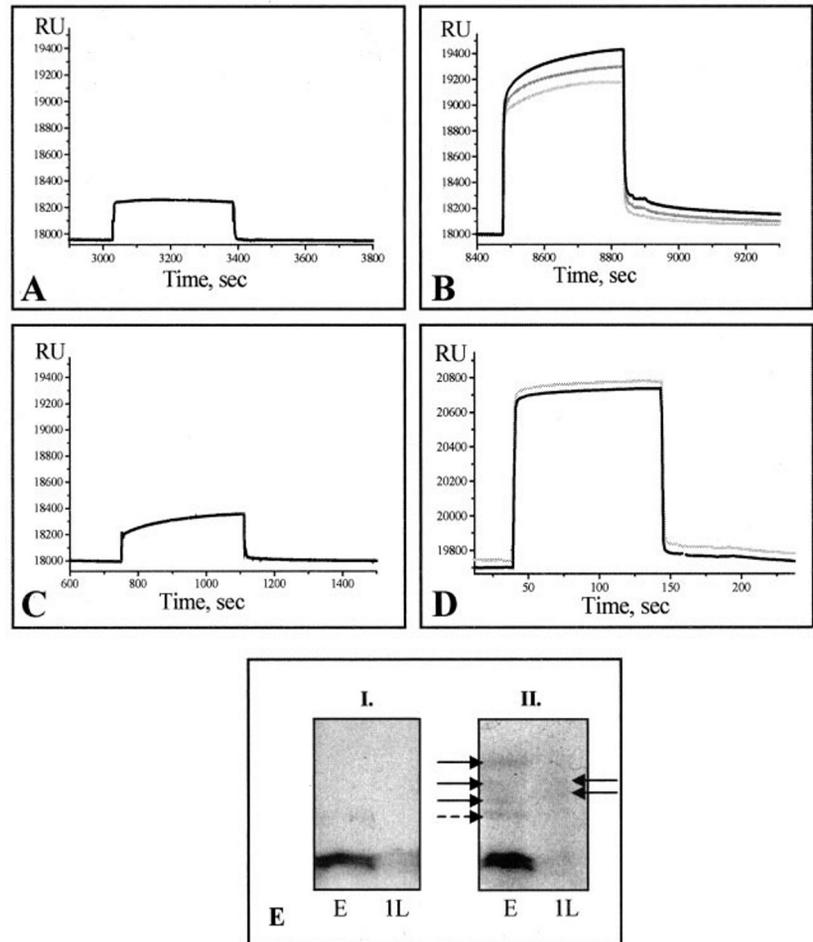
sects (41) does, in fact, indicate some possible differences as compared with mammalian organisms.

Nuclear Localization Does Not Show a Complete Dependence on the Putative NLS—Immunohistochemical staining of actively dividing S2 cells demonstrated that most dUTPase colocalizes with nuclear DNA. However, Western blot analysis of these cells showed a 1:1 ratio of the two isoforms; only one of them possessed the putative NLS, indicating possible additional transporter factors.

In agreement with the above results, the capability of dUTPase translocation both into and out of the nuclei is further supported by immunohistochemical and Western blot experiments on developing ovaries and embryos (compare Figs. 3 and 4, *A* and *B*). dUTPase translocation from the cytoplasm of nurse cells into the nuclei of the early embryo cannot be rationalized purely on the basis of the NLS signal because the very same dUTPase population is shown to change its intracellular localization. On both the protein and mRNA level, a surplus of the shorter isoform lacking the putative NLS is evident from Western blot of mature follicles and mRNA analysis of ovaries (Fig. 3, *C* and *E*). This would suggest mostly diffuse cytoplasmic localization for embryonic dUTPase, in contrast to the experimental results (compare Fig. 4*B*). Translocation of both dUTPase isoforms either into or out off the nucleus alludes to the existence of an additional transport mechanism, probably involving specific dUTPase-binding proteins.

Developmental Regulation of Enzyme Level—The drastic decrease in expression of both dUTPase isoforms in larvae is paralleled with confinement of residual dUTPase to the imaginal discs (compare Fig. 3*A* and 4*C*). This stage-specific control is in line with earlier results (42), based on activity measurements. Lack of dUTPase in larvae was suggested to result in uracil-substituted DNA and induced thymineless cell death, contributing to apoptosis required during metamorphosis (12). The absence of dUTPase in larvae is expected to induce stable replacement of thymine by uracil in DNA, because the fruit fly genome codes only for mismatch-specific uracil-DNA glycosylases (41) and lacks the uracil-DNA glycosylase homolog, uracil-DNA glycosylase. However, the third larval stage was suggested to be associated with the expression of an effective uracil-DNA endonuclease (43) that may induce stage-specific DNA degradation. In agreement, we observed that imaginal discs, not sentenced to death in metamorphosis, retained dUTPase, whereas other larval tissues, subjected to apoptosis in the prepupal stage, did not. A critical assessment of the role of thymineless cell death in developmental apoptosis in *Drosophila* requires further studies involving characterization of the uracil content in larval DNA and phenotype analysis of transgenic mutant strains with perturbed dUTPase content. Such experiments are in progress in our laboratory.

This strict regulation of the dUTPase protein isoforms is in striking contrast with the constitutive mRNA levels (see Fig. 3). This contradiction underlines the importance of such parallel detection techniques in developmental investigations (44). Several mechanisms might be invoked to account for this discrepancy. First, mRNA lifetime may be unusually extended by complexation to control elements. Second, the productive translational polysome complex may be subjected to multilevel modulation, and third, posttranslational modifications may significantly shorten protein lifetime. According to available databases, the 3′- or 5′-untranslated dUTPase mRNA regions do not contain any known translational control elements. However, within the metabolic pathway of thymidylate biosynthe-

sis to which dUTPase also belongs, thymidylate synthase regulates not only its own mRNA (by feedback) but also mRNA of dihydrofolate reductase (45–47). Lack of the methionine residue at the N terminus of the 23-kDa isoform may shorten protein lifetime (see N-end rule (48)).

Cell Cycle-dependent Regulation—In both *Homo sapiens* (30) and *Candida albicans* (49), dUTPase transcription is under cell cycle control. The present data suggest similar proliferation-related expression of fly dUTPase (Fig. 3*D*). In addition to a Zeste site (14, 50), the potential promoter region up to −250 bp of both transcripts contains two tandem *Drosophila*-specific DNA replication-related elements (TATCGATA) (51), present also in many cell cycle-controlled genes (*e.g.* proliferating cell nuclear antigen and DNA polymerase α) (52). This promoter region suggests same regulation for both isoforms. In contrast, only the nuclear isoform of human dUTPase is subjected to cell cycle-dependent regulation, whereas the mitochondrial isoform is constitutive, corresponding to the character of DNA synthesis in these two organelles.

A complete depletion of dUTPase in nonreplicating nuclei would seriously compromise constitutive (*e.g.* transcription-coupled) repair processes (53). Small constitutive amounts of dUTPase, escaping experimental detection and presumably coupled to DNA repair complexes (36, 54), might resolve this problem. This underlines the importance of further investigations on dUTPase-interacting proteins, both in *Drosophila* and human cell lines.

Presence and Putative Role of the Extra C-terminal Region— The 28-residue *Drosophila*-specific C-terminal segment, present in both isoforms (see Fig. 1, *C–E*) is highly flexible and has no significant effect on the activity of the purified enzyme (14). However, several fractions of S2 cell extract modulated the activity of recombinant *Drosophila* dUTPase strictly, depending on the presence of the unique C terminus.[3] This unique region may therefore mediate regulation of dUTPase activity by other protein factors. dUTPases from various organisms contain species-specific, flexible N- or C-terminal extensions that provide interaction surface for cellular macromolecules (17–19). According to surface plasmon resonance experiments, the conserved dUTPase domain also participates in protein-protein interactions (Fig. 6*D*). Far-Western data suggested a stage-specific pattern of dUTPase interacting proteins (Fig. 6*E*). Effector proteins of dUTPase are suggested to exist in *Drosophila* and in *Bacillus subtilis* (13, 55).

In conclusion, we identified the *Drosophila* dUTPase isoforms both at mRNA and protein levels and characterized their expression, localization, and developmental patterns. In agreement with the observed multiple regulation of the enzyme, independent experiments supported the existence of endogenous macromolecular dUTPase partners. Further investigation and identification of dUTPase interacting proteins in *Drosophila* as well as in human cell lines is expected to provide novel insights into the mechanism of thymineless death.

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