Biochemical and Phylogenetic Characterization of the dUTPase from the Archaeal Virus SIRV*

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The derived amino acid sequence from a 474-base pair open reading frame in the genome of the Sulfolobus islandicus rod-shaped virus SIRV shows striking similarity to bacterial dCTP deaminases and to dUTPases from eukaryotes, bacteria, Poxviridae, and Retroviridae. The putative gene was expressed in Escherichia coli, and dUTPase activity of the recombinant enzyme was demonstrated by hydrolysis of dUTP to dUMP. Deamination of dCTP by the enzyme was not detected. Phylogenetic analysis based on amino acid sequences of the characterized enzyme and its homologues showed that the dUTPase-encoding dut genes and the dCTP deaminase-encoding dcd genes constitute a paralogous gene family. This report is the first identification and functional characterization of an archaeal dUTPase and the first phylogeny derived for the dcd-dut gene family.

Little is known about the biosynthesis of nucleic acid precursors in archaea. It has been demonstrated that the extremely thermophilic archaean Sulfolobus acidocaldarius is unable to utilize exogenous thymidine for biosynthesis of nucleic acids, presumably due to a lack of thymidine kinase (1). Thus, the de novo pathway must be the only supplier of thymidine nucleotides in Sulfolobus. Generally, the immediate thymidine nucleotide precursor in the endogenous de novo synthesis is dUMP. Three pathways for dUMP synthesis are known: (i) deamination of dCTP to dUTP by dCTP deaminase (2-deoxycytidine-5′-triphosphate amidohydrolase, EC 3.5.4.13) and successive hydrolysis of dUTP to dUMP by dUTPase (2-deoxycytidine-5′-triphosphatase, EC 3.6.1.23); (ii) deamination of dCMP to dUMP by dCMP deaminase (2-deoxycytidine-monophosphate amidohydrolase, EC 3.5.4.12); and (iii) reduction of UDP or UTP to dUDP or dUTP, respectively, by ribonucleotide reductases followed by hydrolysis of dUTP to dUMP by dUTPase. The conversion of dUDP to dUTP is most probably done via phosphorylation to dUTP. In bacteria, the latter pathway contributes much less to dUMP synthesis than the pathways through deamination of deoxycytidine nucleotides (2). In higher organisms and in certain bacteria as well as in T-even phage-infected Escherichia coli cells, the deamination occurs at the monophosphate level, whereas in most enterobacteria, it occurs at the triphosphate level (3). Only one enzyme involved in the de novo pathway of thymidylate biosynthesis has recently been detected in Sulfolobus cells, thymidylate synthetase (4), the ubiquitous enzyme that catalyzes the conversion of dUMP to dTMP.

In the course of sequencing the genome of the Sulfolobus islandicus rod-shaped virus SIRV (5), we found an open reading frame (ORF)† that encodes a putative protein with considerable similarity to both bacterial dCTP deaminases and dUTPases from eukaryotes, bacteria, Poxviridae, and Retroviridae. In the latter case, the homologous region is part of the gag-pol polyproteins (6–10). This dual similarity led us to functionally characterize the novel SIRV gene, which was accomplished by expression of the encoded protein in E. coli, with subsequent enzymatic tests. It also inspired a phylogenetic analysis of the dcd-dut gene family. Furthermore, the recent discovery of two members of this gene family in the whole genome sequence of Methanococcus jannaschii (11) made it obvious that a biochemical characterization of at least one archaeal member of this gene family is crucial for functional discrimination of these genes.

MATERIALS AND METHODS

Strains and Plasmids—SIRV was grown in cells of S. islandicus isolate REN2H1 (5) and purified by polyethyleneimine precipitation followed by cesium chloride gradient centrifugation. The pGEX-2T expression vector, containing the Schistosoma japonicum glutathione S-transferase gene under the control of the tac promoter (12), was purchased from Pharmacia Biotech Inc.

DNA Purification Procedures and Oligonucleotides—Virus DNA was prepared as described earlier (5). Plasmids were prepared with a plasmid kit from QIAGEN Inc. Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer according to the manufacturer's instructions.

DNA Sequencing—Double-stranded DNA was sequenced using the U. S. Biochemical Corp. Sequenase 2.0 kit following the manufacturer's instructions. Oligonucleotide primers were complementary either to adjacent vector sequences or to portions of previously sequenced parts of the cloned DNA. To ensure accuracy, both DNA strands were sequenced.

Plasmid Construction and Transformation—The putative dut gene was amplified from SIRV DNA by the polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs Inc.). Primer P1 (5′-TATGGAATTCATCTTCCTTTTCTCAATGTC-3′) corresponded to the 5′-end of the putative gene, and primer P1r (5′-ATCGAGGATCATGATTCTTTCAGATAG-3′) corresponded to the 3′-end of the coding region. To facilitate the subsequent cloning steps, a BamHI restriction site was introduced in the former primer, and an EcoRI site was introduced in the latter primer. PCR was performed in a GTC-2 genetic thermal cycler (Precision Scientific) under the following conditions: 3 min of initial denaturation at 94 °C followed by 33 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C each. The amplified DNA

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The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction.
Characterization of SIRV dUTPase

**Fig. 1.** Nucleotide sequence of the SIRV dut gene and deduced amino acid sequence of SIRV dUTPase. The stop codon is indicated by the *lightface asterisk*. Sequence motifs for the typical archaeal box A promoter element (17) and the transcription terminator (18) are printed in underlined upper-case letters. The putative transcription start site is indicated by the *boldface asterisk* above the corresponding nucleotide and is underlined.

**Table I**

| [Enzyme] in assay mixture | Radioactivity in spots corresponding to: | cpm |
|---------------------------|----------------------------------------|-----|
|                          | dUTP                                    | 22,100 |
|                          | dUDP                                    | 210  |
|                          | dUMP                                    | 110  |

with 4.5% polyacrylamide in the stacking gel and 16% in the separating gel. Gels were stained with Coomassie Blue R-250.

dUTPase Assay—The standard assay was carried out by 1 h of incubation at 56 °C in a 75-μl volume containing 20 mM potassium phosphate buffer, pH 6.8, 1.35 mM dithiothreitol, 7 mM MgCl₂, 0.15 mM [3H]dUTP (specific activity of 1.65 μCi/μmol), and the appropriate amount of the recombinant enzyme. Five-μl samples of the reaction mixtures were applied to polyethylenimine-cellulose plates (Merck). Prior to the application of the samples, 30 nmol each of dUTP, dUDP, and dUMP were applied to the start line of the chromatogram. The plates were developed in one dimension with 1 M formic acid and 0.5 M LiCl and, after drying, examined under UV light. The marker spots corresponding to dUTP, dUDP, and dUMP were cut out; nucleotides were eluted by washing excised sections with 2 M LiCl; and radiolabel was quantified by scintillation counting.

dCTP Deaminase Assay—dCTP deaminase activity was determined by a spectrophotometric method based on the difference between the molecular extinction coefficients of deoxyctidine and deoxyuridine (3). The assay was carried out at 56 °C in time intervals between 10 and 120 min. The reaction volume was 400 μl and contained 20 mM potassium phosphate buffer, pH 6.8, 1.35 mM dithiothreitol, a range of MgCl₂ concentrations (0–25 mM), 1 mM dCTP, and different amounts of the recombinant enzyme (2.5–50 μg).

**Phylogenetic Analysis**—Amino acid sequences were retrieved from public data bases. Multiple alignment of the sequences was performed with the help of the computer program CLUSTAL (14). Final adjustments considering obvious similarities not indicated by the alignment program were done manually after visual inspection. Phylogenies were inferred and analyzed for statistical confidence using distance, parsimony, and bootstrapping programs from the PHYLIP package (Version 3.572PC) (15) and PAUP (Version 3.1.1) (16).

**RESULTS**

The ORF analyzed in this report was found in a 2101-base pair-long EcoRI-ClaI restriction fragment of SIRV DNA. The nucleotide sequence of the ORF and its flanking regions is presented in Fig. 1. The GC content of the coding region is 27%, close to the average GC content of SIRV DNA (25%).

The putative translation start codon (ATG) is 1482 base pairs from the EcoRI cloning site, and the TTA stop codon is 145 base pairs from the ClaI cloning site. A typical archaeal “box A” promoter motif, TTAAA (17), is found 61–66 nucleotides upstream of the translation start codon. A typical Sulfolobus terminator motif, TTTAAAT.

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FIG. 3. Aligned amino acid sequences of dUTPases and dCTP deaminases. Positions that appear to be invariable in all sequences are **boldface**. Positions characteristic for dCTP deaminases are *underlined*. Positions characteristic for archaeal dUTPases are *italic*. Ecol C, *E. coli* dCTP deaminase (21); Hinf C, *H. influenzae* dCTP deaminase (22); Mjan C, *M. jannaschii* dCTP deaminase (MJ0430) (11); Ecol U, *E. coli* dUTPase (23); Cbur U, *C. burnetii* dUTPase (Thiele, D., Willems, H., Oswald, W., and Kraus, H. (1994) EMBL accession number S44300); Hinf U, *H. influenzae* dUTPase (22); Mjan U, *M. jannaschii* dUTPase (MJ1102) (11); Desu U, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap U, *H. sapiens* dUTPase (26); Lesc U, *S. lycopersicum* dUTPase (27); Scer U, *S. cerevisiae* dUTPase (28); OrfV U, ORF virus strain NZ2 dUTPase (29); VacV U, vaccinia virus strain WR dUTPase (30); VarV U, variola virus dUTPase (31); SfkU, *S. flexus* (32); Ecol M, *E. coli* dCTP deaminase (21); Mjan M, *M. jannaschii* dCTP deaminase (25); Cbur M, *C. burnetii* dCTP deaminase (24); Hinf M, *H. influenzae* dCTP deaminase (22); Damb U, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap M, *H. sapiens* dUTPase (26); Lesc M, *S. lycopersicum* dUTPase (27); Scer M, *S. cerevisiae* dUTPase (28); OrfV M, ORF virus strain NZ2 dUTPase (29); VacV M, vaccinia virus strain WR dUTPase (30); VarV M, variola virus dUTPase (31); SfkM, *S. flexus* (32); Ecol L, *E. coli* dCTP deaminase (21); Mjan L, *M. jannaschii* dCTP deaminase (25); Cbur L, *C. burnetii* dCTP deaminase (24); Hinf L, *H. influenzae* dCTP deaminase (22); Damb L, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap L, *H. sapiens* dUTPase (26); Lesc L, *S. lycopersicum* dUTPase (27); Scer L, *S. cerevisiae* dUTPase (28); OrfV L, ORF virus strain NZ2 dUTPase (29); VacV L, vaccinia virus strain WR dUTPase (30); VarV L, variola virus dUTPase (31); SfkL, *S. flexus* (32); Ecol H, *E. coli* dCTP deaminase (21); Mjan H, *M. jannaschii* dCTP deaminase (25); Cbur H, *C. burnetii* dCTP deaminase (24); Hinf H, *H. influenzae* dCTP deaminase (22); Damb H, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap H, *H. sapiens* dUTPase (26); Lesc H, *S. lycopersicum* dUTPase (27); Scer H, *S. cerevisiae* dUTPase (28); OrfV H, ORF virus strain NZ2 dUTPase (29); VacV H, vaccinia virus strain WR dUTPase (30); VarV H, variola virus dUTPase (31); SfkH, *S. flexus* (32); Ecol T, *E. coli* dCTP deaminase (21); Mjan T, *M. jannaschii* dCTP deaminase (25); Cbur T, *C. burnetii* dCTP deaminase (24); Hinf T, *H. influenzae* dCTP deaminase (22); Damb T, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap T, *H. sapiens* dUTPase (26); Lesc T, *S. lycopersicum* dUTPase (27); Scer T, *S. cerevisiae* dUTPase (28); OrfV T, ORF virus strain NZ2 dUTPase (29); VacV T, vaccinia virus strain WR dUTPase (30); VarV T, variola virus dUTPase (31); SfkT, *S. flexus* (32); Ecol D, *E. coli* dCTP deaminase (21); Mjan D, *M. jannaschii* dCTP deaminase (25); Cbur D, *C. burnetii* dCTP deaminase (24); Hinf D, *H. influenzae* dCTP deaminase (22); Damb D, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap D, *H. sapiens* dUTPase (26); Lesc D, *S. lycopersicum* dUTPase (27); Scer D, *S. cerevisiae* dUTPase (28); OrfV D, ORF virus strain NZ2 dUTPase (29); VacV D, vaccinia virus strain WR dUTPase (30); VarV D, variola virus dUTPase (31); SfkD, *S. flexus* (32); Ecol G, *E. coli* dCTP deaminase (21); Mjan G, *M. jannaschii* dCTP deaminase (25); Cbur G, *C. burnetii* dCTP deaminase (24); Hinf G, *H. influenzae* dCTP deaminase (22); Damb G, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap G, *H. sapiens* dUTPase (26); Lesc G, *S. lycopersicum* dUTPase (27); Scer G, *S. cerevisiae* dUTPase (28); OrfV G, ORF virus strain NZ2 dUTPase (29); VacV G, vaccinia virus strain WR dUTPase (30); VarV G, variola virus dUTPase (31); SfkG, *S. flexus* (32); Ecol I, *E. coli* dCTP deaminase (21); Mjan I, *M. jannaschii* dCTP deaminase (25); Cbur I, *C. burnetii* dCTP deaminase (24); Hinf I, *H. influenzae* dCTP deaminase (22); Damb I, *D. ambivalens* dUTPase (20, 25 (as dCTP deaminase)); Hsap I, *H. sapiens* dUTPase (26); Lesc I, *S. lycopersicum* dUTPase (27); Scer I, *S. cerevisiae* dUTPase (28); OrfV I, ORF virus strain NZ2 dUTPase (29); VacV I, vaccinia virus strain WR dUTPase (30); VarV I, variola virus dUTPase (31); SfkI, *S. flexus* (32).
Characterization of SIRV dUTPase

**dUTPases (dUTPase-dCTP deaminase enzyme family)**

**Figure 4. Phylogenetic diagram of the SIRV dUTPase.** See legend to Fig. 3 for definitions of abbreviations. The numbers indicate the statistical support for the existence of the corresponding branches determined by bootstrapping (200 rounds).

**TTTTTYT** (18), is located 11–17 nucleotides downstream of the translation termination codon (Fig. 1).

A polypeptide presumably encoded by this ORF has 158 amino acid residues and an inferred molecular mass of 16.2 kDa. When the PIR protein sequence data base (19) was searched with this amino acid sequence, the highest sequence similarities were found with hypothetical protein-3 from Desulfurolobus ambivalens (88%) (20); with dCTP deaminase from *E. coli* (at that time, the only known dCTP deaminase; 58%); and with dUTPases from retroviruses, lentiviruses, and poxviruses (49–59%). Statistical tests for the significance of the similarities to these sequences resulted in scores significantly higher than the mean scores between random sequences.

To find out the enzymatic properties of the protein presumably encoded by the ORF, this region of the viral genome was amplified by PCR and inserted into pGEX-2T, an expression vector containing the glutathione *S*. *pae*.

The recombinant protein expressed in *E. coli* was induced and had an apparent molecular mass of ~42 kDa (Fig. 2), close to that predicted by the fusion of the 26-kDa glutathione S-transferase gene and the ~16.2-kDa SIRV protein. The fusion protein was bound to glutathione, and after removal of the nonspecifically bound material, the expressed SIRV protein was released by thrombin cleavage. It had an apparent molecular mass of ~15 kDa, slightly smaller than the predicted mass. Analysis on a 16% SDS-polyacrylamide gel showed it to be 94% pure (Fig. 2).

The synthesized protein was tested for putative dUTPase activity by incubation with 3H-labeled dUTP and subsequent chromatography of the reaction products on polyethyleneimine thin-layer plates. In spots corresponding to dUTP, dUDP, and dUMP, radioactivity was quantified by scintillation counting; the results are shown in Table I. The label was found in two species, one having the mobility of the input dUTP and the other having the mobility of dUMP. No significant radioactivities were observed as a result of incubation of dCTP with the expressed protein, as described under "Materials and Methods" (data not shown). Therefore, dCTP deaminase activity could be ruled out for the recombinant enzyme.

Sequence alignments are the basis for molecular phylogenetic analyses. Fig. 3 shows the amino acid sequence of the novel SIRV dUTPase aligned with all presently known archaean, bacterial, and eukaryotic homologues and most viral homologues found in the public sequence data bases by BLAST search (34). Thirteen positions are characteristic for the three putative archaeal dUTPases, and four positions are characteristic for the three putative archaeal dUTPases: SIRV, *D. ambivalens* (hypothetical protein-3), and *M. jannaschii* (gene MJ1102 product). Four amino acids are universally conserved throughout all sequences (aspartic acid 57, serine 147, aspartic acid 165, and glycine 170) (Fig. 3). All four belong to the previously described conserved sequence motifs of dUTPases (40): aspartic acid 57 belongs to motif 1, serine 147 to motif 2, and aspartic acid 165 and glycine 170 to motif 3. The latter two amino acids correspond in human dUTPase structure to residues in the beta-hairpin, suggested to be responsible for binding the deoxypyrimidine portion of dUTP (41); aspartic acid 165 is believed to be important for discrimination between dUTP and dUMP (42). Motif 4 described by McGeoch (40) is less conserved in the three putative archaeal dUTPases and in dCTP deaminases. Even less conserved in archaeal dUTPases is motif 5 (40), suggested to be important for the catalytic activity of dUTPases (43, 44); moreover, in the enzyme from *M. jannaschii*, the region where motif 5 is found in other dUTPases is completely absent.

swinepox virus strain KASZA dUTPase (32); *HsV1*, *Ictalurid* herpesvirus 1 dUTPase (33); *VisV*, *visna* lentivirus strain KV1772 pol polypol (10); *OmoV*, ovine lentivirus strain SA-OMVV pol polypol (8); *MntV*, mouse mammary tumor virus strain BR6 pol polypol (7); *SpaV*, ovine pulmonary adenocarcinoma virus pol polypol (9); *SmpV*, Mason-Pfizer monkey virus pol polypol (6); *SirV*, SIRV dUTPase. Five conserved sequence motifs of dUTPases described by McGeoch (40) are indicated.

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Characterization of SIRV dUTPase

It seems reasonable that SIRV encodes its own dUTPase: pool sizes of dTTP in host cells might not be sufficiently high to support rapid growth of a virus with a genomic GC content of only 25%. (The GC content of the chromosomesal DNA of Sulfolobus solfataricus, a close relative of the SIRV host strain, is 38%.) It is also possible that dUTPase from SIRV is involved in keeping the intracellular dUTP concentration at a low level such that incorporation of dU into DNA is minimized, and thus, the DNA repair processes are not invoked (24).

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