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Source: Avian Diseases, 52(2):324-331. 2008.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/8169-110607-ResNote.1
URL: http://www.bioone.org/doi/full/10.1637/8169-110607-ResNote.1

AVIAN DISEASES 52:324–331, 2008

Research Note—

Identification and Characterization of Duck Enteritis Virus dUTPase Gene

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Received 9 November 2007; Accepted and published ahead of print 11 January 2008

SUMMARY. Deoxyuridine triphosphatase (dUTPase) is a ubiquitous and important enzyme that hydrolyzes dUTP to dUMP. Many viruses encode virus-specific dUTPase, which plays an essential role in maintaining the integrity of the viral DNA both by reducing the dUTP levels and by providing the substrate for the thymidylate synthase. A 1344-bp gene of duck enteritis virus (DEV) homologous to herpesviral dUTPase was first reported in this paper. The gene encodes a protein of 477 amino acids, with a predicted molecular mass of 49.7 kDa. Multiple sequence alignment suggested that DEV dUTPase was quite similar to other identified herpesviral dUTPase and functioned as a homotrimer. The five conserved motifs of DEV dUTPase with 3-1-2-4-5 arrangement have been recognized, and the phylogenetic analysis showed that DEV dUTPase was genetically close to the avian herpesvirus. Furthermore, RNA dot blot, western blot, and immunofluorescence analysis indicated that the enzyme was expressed at early and late stages after infection. Immunofluorescence also confirmed that DEV dUTPase localized in the cytoplasm of DEV-infected duck embryo fibroblasts as early as 4 hr postinfection (hpi). Later, the enzyme transferred from cytoplasm to nucleus at 8 hpi, and then reached its expression peak at 12 hpi, both in the cytoplasm and nucleus. The results suggested that the DEV dUTPase gene might be an early viral gene in DEV vitro infection and contribute to ensuring the fidelity of genome replication.

RESUMEN. *Nota de Investigación*—Identificación y caracterización del gen dUTPasa del virus de la enteritis de los patos.

La dUTPasa es una enzima ampliamente distribuida y muy importante que hidroliza dUTP a dUMP. Muchos virus codifican dUTPasas virales específicas, que juegan un papel esencial en el mantenimiento de la integridad del ADN viral reduciendo los niveles de dUTP y proporcionando el sustrato para la enzima timidilato sintasa. En este trabajo se reportó por primera vez un gen de 1344 pares de bases del virus de la enteritis de los patos homólogo al gen de la dUTPasa de los herpesvirus. El gen codifica para una proteína de 477 aminoácidos con una masa molecular predicha de 49.7 kilodaltons. Múltiples alineamientos de secuencias sugirieren que la dUTPasa del virus de la enteritis de los patos es parecida a otra dUTPasa de herpesvirus y que funciona como un homotrímero. Se reconocieron los cinco motivos conservados de la dUTPasa del virus de la enteritis de los patos con un arreglo de 3-1-2-4-5 y el análisis filogenético demostró que la dUTPasa del virus de la enteritis de los patos es genéticamente cercana a la de los herpesvirus de las aves. Adicionalmente, el análisis mediante inmunotransferencia puntual para ARN, inmunotransferencia puntual para proteína e inmunofluorescencia indicaron que la enzima se expresó en las etapas temprana y tardía después de la infección. La inmunofluorescencia también confirmó que la dUTPasa del virus de la enteritis de los patos puede ser localizada en el citoplasma de fibroblastos de embrión de pato tan temprano como cuatro horas posteriores a la infección. Mas tarde, a las ocho horas posteriores a la infección, la enzima se transfiere al núcleo y alcanza su máximo de expresión tanto en el citoplasma como en el núcleo a las 12 horas posteriores a la infección. Los resultados sugieren que el gen de la dUTPasa del virus de la enteritis de los patos puede ser un gen viral temprano en la infección *in vitro* del virus de la enteritis de los patos y que contribuye a garantizar la fidelidad de la replicación del genoma.

Key words: duck enteritis virus, bioinformatics analysis, clone, expression, transcriptional analysis, subcellular localization

Abbreviations: BoHV = bovine herpesvirus; CeHV = cercopithecine herpesvirus 1; DAPI = 4′,6-diamidino-2-phenylindole; DEF = duck embryo fibroblast; DEV = duck enteritis virus; dUTPase = deoxyuridine triphosphatase; EHV = equid herpesvirus; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; GaHV = gallid herpesvirus; HHV = human herpesvirus; hpi = hours postinfection; HSV = herpes simplex virus; IPTG = isopropyl β-D-thiogalactoside; MEM = minimal essential medium; NCBI = National Center for Biotechnology Information; NTA = nitrilotriacetic acid; ORF = open reading frame; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate

Deoxyuridine triphosphatase (dUTPase; EC3.6.1.23), is a key nucleotide metabolic enzyme in a variety of organisms, and in several virus families. It catalyzes the hydrolysis of dUTP to dUMP and PPi, and thereby prevents the incorporation of uracil into DNA during replication. (9,12,19). Herpesviruses, including herpes simplex virus (HSV)-1 and -2; equine and bovine herpesviruses; poxviruses, including vaccinia virus and Orf virus; and certain retroviruses also encode a functional dUTPase (5,11,13,39). Studies on mutants of the equine infectious anemia virus and feline immunodeficiency virus dUTPase gene showed that the enzyme is not required for replication in fetal equine kidney cells and Crandell feline kidney cells, respectively (35,37). Interestingly, it has been found that some viral dUTPases act as an anti-mutation factor, to be free from the regulatory constraints of normal cellular processes and to prevent the synthesis of mutagenic uracil-substituted DNA. And, the herpesviral

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Table 1. Abbreviations and accession no. of 30 dUTPase from different species

Species	Virus name (Abbreviation)	Natural host	GenBank accession no.
Unclassified *Herpesviridae*	Duck enteritis virus (DEV)	Duck	DQ 486149
Alphaherpesvirinae	Meleagrid herpesvirus 1(MeHV-1)	Meleagrid	NP_073345
	Gallid herpesvirus 2(GaHV-2)	Avian	ABF72293
	Gallid herpesvirus 3(GaHV-3)	Avian	NP_066883
	Equid herpesvirus 1(EHV-1)	Equid	YP_053054
	Equid herpesvirus 4(EHV-4)	Equid	Q00030
	Bovine herpesvirus 5(BoHV-5)	Bovine	NP_954897
	Bovine herpesvirus 1(BoHV-1)	Bovine	NP_045308
	Suid herpesvirus 1(SuHV-1)	Swine	YP_068324
	Pseudorabies virus(PRV)	Swine	AAO38918
	Cercopithecine herpesvirus 1(CeHV-1)	Cercopithecine	BAC58091
	Cercopithecine herpesvirus 2(CeHV-2)	Cercopithecine	YP_164494
	Psittacid herpesvirus 1(PsHV)	Psittacid	NP_944384
	Gallid herpesvirus 1(GaHV-1)	Avian	YP_182340
Betaherpesvirinae	Human herpesvirus 6(HHV-6)	Human	CAA63171
	Human herpesvirus 7(HHV-7)	Human	AAC40759
Gammaherpesvirinae	Human herpesvirus 8 (HHV-8)	Human	YP_001129357
	Epstein-Barr virus (EBV)	Human	AAA45898
	Cercopithecineherpesvirus 15 (CeHV-15)	Cercopithecine	YP_067960
	Bovine herpesvirus 4 (BoHV-4)	Bovine	NP_076546
	Equid herpesvirus 2 (EHV-2)	Equid	NP_042651
	Saimiriine herpesvirus 2 (SaHV-2)	Squirrel	CAC84350
	Ateline herpesvirus 3 (AtHV-3)	Ateline	NP_048026
	Alcelaphine herpesvirus 1 (AtHV-1)	Alcelaphine	NP_065553
	Ovine herpesvirus 2 (OvHV-2)	Ovine	YP_438178
Orthoretrovirinae	Mouse mammary tumor virus (MmTV)	Mouse	BAA03766
	Equine infectious anemia virus (EIAV)	Equid	AAA83028
Asfarviridae	African swine fever virus (ASFV)	Swine	NP_042823
Nimaviridae	Shrimp white spot syndrome virus (WSSV)	Shrimp	AAK77740
Enterobacteriaceae	*Escherichia coli* (*E.coli*)	Bacteria	BAE77652

dUTPases were proved to be important determinants of neurovirulence, neuroinvasiveness, and establishment of or reactivation from latency (29).

Duck enteritis virus (DEV) is a member of the *Herpesviridae*, which can induce duck viral enteritis (DVE) in ducks, and this virus has produced significant economic losses in domestic and wild waterfowl as a result of mortality, elimination, and decreased egg production (31). Most previous research has focused on the epidemiology and prevention of duck enteritis, whereas DEV genome and molecular biology research has been rare. Currently, some DEV gene fragments have been found, including complete UL6, UL23, and UL24 genes, but DEV genomic organization remains unclear. The majority of DEV sequences reported were limited to a single open reading frame (ORF) in the UL region. So far, a DEV genomic library was constructed successfully in our laboratory (10). Sequence analysis showed that DEV encoded several enzymes involved in nucleotide metabolism, including a dUTPase (data not shown).

In our study, the dUTPase gene was analyzed by bioinformatics methods, and then it was cloned into the prokaryotic expression vector pET-32a and expressed in *Escherichia coli*. Moreover, RNA dot blot and western blot, as well as immunofluorescence analysis, were used to determine the gene temporal transcription/translation course and subcellular localization in DEV-infected cells. This work may provide a foundation for further studies on the function of DEV dUTPase.

MATERIALS AND METHODS

Virus and cells. DEV CH strain is a high-virulence field strain of DEV, obtained from Key Laboratory of Animal Disease and Human Health of Sichuan Province. Duck embryo fibroblasts (DEFs) were cultured in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 C. For virus infection, MEM supplemented with 2–3% FBS was used.

Bioinformatics analysis of DEV dUTPase. First, the National Center for Biotechnology Information (NCBI) BLAST and ORF Finder servers were used to sequence similarity search. Second, amino acid sequence comparisons and homology searches were performed using the computer-assisted NCBI BLASTP (1). Multiple sequence alignment and phylogenetic analysis were obtained on the bases of 30 dUTPase sequences (Table 1) by using NCBI conserved domains finder, CLUSTAL-X (16), and DNAstar6.0 (6) programs, respectively.

Polymerase chain reaction (PCR) amplification and plasmid construction. The primer sequences for PCR amplification of DEV dUTPase gene were as follows: forward primer 5′-GAATTCATGG-CAACTGGAAATTGTGGAGCT-3′ and the reverse primer 5′-GCGGCCGCTTATATCCCAGTTGATCCGAAACCTCCT-3′. The amplified PCR products were digested with *Eco*RI and *Not*I before being cloned into prokaryotic vector PET32a(+) (Novagen, Madison, WI), which was digested previously with the same restriction enzymes. Then, the recombinant plasmid PET32a-DUT was confirmed by PCR, restriction enzyme digestion, and DNA sequencing (Takara, Kyoto, Japan). This plasmid allows expression of a recombinant protein containing a hexahistidine tag to facilitate purification by Ni^{2+} affinity resins.

Prokaryotic expression, purification, and antibody preparation. The *E. coli*. BL21(DE3) cells transformed with the recombinant plasmid of PET32a-DUT were induced with isopropyl β-D-thiogalactoside (IPTG), which was performed essentially as described by Sambrook (32). The recombinant His-tagged proteins were purified by nickel affinity chromatography according to the manufacturer's protocol (Bio-Rad, Hercules, CA), and they were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie Blue (27), Antibodies against the purified His-tagged

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GaHV-2   -MDVHESHPRRDRDLENITLEALTVPWRLQFRVDEALYAVNPNG----WTCYIEERDQRCLRVTNNCVISLLKSDLKRKY   75
GaHV-3   MMDVGQTHPCGN-ALEKMSSHASGAPWRMQFSVDEAIYARNPEG----WTCYIEERDPRCLRVINDCAISLPKRDVKRKY   76
MeHV-1   MMDTNGAHLRIETEPEPAPSHTTRVPWRLKYRVDETLYTLDHER----WTCYIEDRDRRCLRVINNQVISLCKSDKREKY   76
DEV      -MATGNCGAPKRVIQDLRSNESTSPTGPVCFCVEEAIVSHDRTGEGGYWFCGVDRTDPRCLRLDNTKAIDTPDSCRPGEI   79
EHV-1    -------------------------MASVTNLVDSIVVVECGER----WRARAEAAGRLLVLINNHTVELSGEHGSAGEF   51

                                                                  Motif 3
                                                                 *********
GaHV-2   QTCTLNIGIRVAVPQNYVVILAKLTDP---DPTSRGIPIIQVAN---------GLIDSGYRGSIRAVLFFEKSCI-IPKN   142
GaHV-3   EICALDLGVRVAVPRNYVVVLAKLTDP---DPTSRGVPVIRVAN---------GLIDSGYRGNVRVVLLYEAACT-IPKN   143
MeHV-1   QICVLDLGLRVAVPQEYAVVLAKLADP---DPTSRGIPVIGVAN---------GVIDSGYRGTIKAILFYEKSCV-IPKN   143
DEV      LVALIETGVRVAFPNNYVVLVTRLLLSS--DSIGGGSPFLRIAN---------GVVDAGYRGTIRLVVYYDGSITKIPPN   147
EHV-1    YSVLTDVGVRVACSSGYAIVLTQISGLLPVEPEPGNFSNVTFPENSAKYYTAYGIVDSGYRGVVKAVQFAPGINTSVPPG   131

GaHV-2   GLAIRLSLVKLASP-NLNTRVLFNLSDITPHLECGPDFSTSIETAVRLGSGETKP---LLPPSGGGIWAGTGCRALACLYN   219
GaHV-3   GLVIRLALVQLAYP-DFNSRVLFDLADITPHLDCGPNFSMSIATAAKSHSAQARP---LLPPGGEKLWPGTGCRALVCLYS   220
MeHV-1   GLAIRLSLVKLVSP-NMDSRILFDLSAITTHLECGPSFSSSIATAARSGASELVP---IIPASANGIWNGTGCRTLVCLYD   220
DEV      ALSVRLALVKLSDDQEIQRRILFDLYDASNYYECGGKFIESVRDATVNCSPETQLSLLRLPPDNAKIWPGTQCESMVVITS   228
EHV-1    QMSLGLVLVKLARK------------------S--IHVTSIGSTRDGRTSEAN----L----------------------   165

                                                                            Motif 1
                                                                          ***********
GaHV-2   DRVCKASHYTSSDKNIAFVVRYNDSTSVLGLKDFPT-AEDETFVRFYTSGQFATLIPPFFETFTPKRTEDAAYDIAAPGDIR   300
GaHV-3   DRVSRATHYNTLDSNVIFAVRYNDSTTVIGLKDVPK-YVHKTFVRFYTSGQFATFVPFYETFNTKRHEDAAYDIFAPSDIV   301
MeHV-1   NRASNASHFTSSDKNVAFAVRYNDESVVMGLKDIPK-PDTQTFVRFYTSGTFGTVIPFDETFTPKRSEDAGYDIPAPRDIL   301
DEV      DAAAATTQYIADPKQVSFAVKHS-RYVVLGLYKVPDDKEAKPSIMFSSCGKMSTLRPFFDTFNRKRLEDAGYDIPLPRDLE   309
EHV-1    ----------------------------------------------------------FYDYFAPKRVEDAGYDISAPEDAT   155

                             Motif 2                                   Motif 4
                              ****                                     ******
GaHV-2   LGALSSTTIMIQQRYVCMDDSVIPCIFGRSSMNLRGLIIIPSRWLPNSWLTITICNLTEMTVMIRCGDRIAQLLLVDHESA   381
GaHV-3   LESMSSVTIAIQQRYACADKSMVPWIFGRSSMNLRGLIISPSRWMPDSWLTLTLCNLTEAKATIKRGDRIAQLLLVNQEAA   382
MeHV-1   LEPLSSTAIVIQQRYTCLDASTIPCIFGRSSLNMRGVIVYPSRWSANSWLILTLCNTTTYEIKIKSGERIAQLLLVDQDCA   382
DEV      LQPRTFTEVKIRQIYNCKTSDVLPCIFGRSSMNAKGLTVLPTRWLEGEWLTFLIYNFTRKTVFLNAGDRVAQLVLISRDAN   390
EHV-1    IDPDESHFVDLPIVFANSNPAVTPCIFGRSSMNRRGLIVLPTRWVAGRTCCFFILNVNKYPVSITKGQRVAQLLLTEDIDD   236

                                                      Motif 5
                                                     *********
GaHV-2   TLIPPTNDTTR-MFPTV GKCRRPGASVGEPKWRETLEFDTEAYSSERQFSGFGSTGI   437
GaHV-3   ALLPTEGGTVR-CSLQ------------------------------------------   397
MeHV-1   TLLPSENNTTN-AFPTV GKCKRPISLSGEPNWRETTMFDKEAPPSQRQHAGFGSTGY   438
DEV      LWIPPHHNYSDPCPPAM LSTSAPYVTYPSPVWRFTLHYDTEAMTSERREGGFGSTGI   447
EHV-1    ALIPPTVNYDN-PFPTY SPSESTKAPQSPVLWKFTTDFDREAPSSLRADGGFGSTGL   292
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Fig. 1. Multiple amino acid sequences alignment of DEV dUTPase and selected members of the dUTPase family. Numbers on top of the alignment indicated the amino acid position relative to the N terminus of each dUTPase. Five highly conserved motifs are named as motifs 1 to 5 above the alignment and boxed.

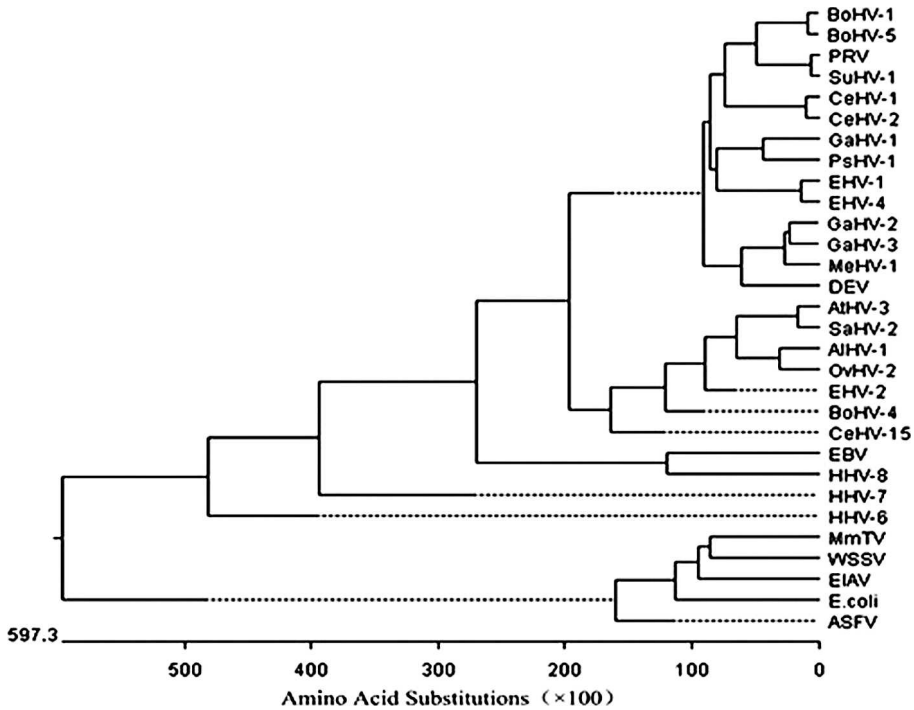

Fig. 2. Phylogenetic tree of the dUTPase based on 30 dUTPase amino acid sequences from different species (see Table 1) by using the MEGALIGN program in LaserGene (DNAStar, Madison, WI) with Clustal V method multiple alignment and sequence distance indicated by the scale was calculated using weight matrix PAM250.

dUTPase (recombinant dUTPase) were raised in rabbits. The immune serum obtained recognized the recombinant protein on western blots. Then, the purified IgG polyclonal antibodies were obtained by purification using caprylic acid and ammonium sulfate precipitation (21) and High-Q anion exchange chromatography (23).

Preparation and temporal analysis of RNA. To examine the dUTPase transcriptional courses in infected cells in vitro, total RNA was isolated from mock- or DEV-infected cells at different times (30 min, 60 min, 90 min, 2 hr, 4 hr, 12 hr, 24 hr, 36 hr, 48 hr, and 72 hr postinfection [hpi]) by using the Total RNA Isolation System (Takara). Cell volume equivalent amount of total RNA (15 μl) was digested by the RNase-free DNase I (Takara) to eliminate contamination of chromosomal DNA before dot blotting. Biotin-labeled oligonucleotides (150 ng/ml) were used as the probe, and hybridization was performed overnight at 42 C. Later steps were carried out as reported previously (8,41).

Western blotting. DEFs were either mock infected or infected with DEV as described above, and they were harvested at indicated times (2 hpi, 4 hpi, 8 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi, and 72 hpi). The cells were lysed on ice for 30 min with an equal volume of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mmol/L phenylmethylsulfonyl fluoride) (15). Equivalent amounts of the cell lysates (15 μl) were electrophoresed on 12% SDS-PAGE and subsequently transferred to a polyvinylidene fluoride membrane. The following steps were performed according to previous reports (27,32) and by using the ECL high-sensitivity electrochemiluminescence system (Pierce Chemical, Rockford, IL).

Subcellular localization. DEFs, grown in a coverslip in six-well plates, were either mock infected or infected with DEV CHv strain. The cells were harvested at different times postinfection (0 hpi, 2 hpi, 4 hpi, 8 hpi, 12 hpi, 24 hpi, 36 hpi, 48 hpi, and 72 hpi), and then they were fixed with 4% paraformaldehyde for 15 min at room temperature. Next, the cells were washed once with phosphate-buffered saline (PBS) and blocked for 1 hr in PBS containing 10% bovine serum albumin at 37 C. They were then incubated with purified rabbit polyclonal antibodies IgG (1:200 dilution) specific for recombinant DEV dUTPase at 4 C overnight, washed for 10 min three times in PBS, and then treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sino-American Biotechnology Co., Shanghai, China) for 45 min at 37 C. As described by Zhe Zhao and William Miller (26,43), the cell nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) counterstaining (5 μg/ml; Beyotime Institute of Biotechnology, Shanghai, China).

RESULTS

Bioinformatics analysis of DEV dUTPase. The 1344-bp nucleotide sequence encodes a polypeptide of 477 amino acids, with a putative molecular mass of 49.3 kDa. By subsequent searches for amino acid sequence similarities, it has been reported that the complete DEV ORF was a possible new member of the dUTPase family. The prediction of a virus-encoded dUTPase was tested by the multiple alignment of these sequences with some of the members of the dUTPase family (Fig. 1). Previously published comparisons of the primary structure of dUTPase from different α-herpervirus (7,17,27) indicated the presence of a rearranged order 3-1-2-4-5 of the five conserved motifs along the alignment, which were counted from the N terminus and are boxed in Fig. 1. A phylogenetic tree of 30 species of dUTPase is shown in Fig. 2; the general branching pattern was coincident with other previously published phylogenetic analysis (27,28), where the DEV protein was not included. The dUTPase of DEV and some fowl herpesviruses, such as gallid herpesvirus 2, gallid herpesvirus 3, and meleagrid herpesvirus 1, which were clustered within a monophyletic clade. Generally, the DEV dUTPase was grouped with some α-herpesviruses, and they were clustered close to the γ-herpesviruses and β-herpesviruses.

Prokaryotic expression. The *E. coli* BL21(DE3) cells transformed with the recombinant plasmid PET32a-DUT were highly expressed with 0.8 mM IPTG at 30 C. A polypeptide with a size corresponding approximately to that expected for the recombinant protein (about 66.8 kDa) was found in large amounts in crude cell extracts (Fig. 3). Based on the His-tag present at its N-terminal end,

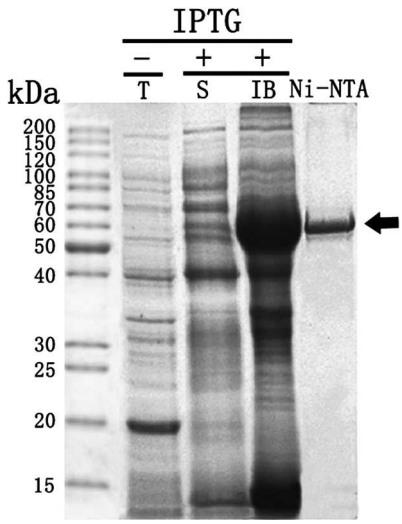

Fig. 3. Expression and purification of DEV recombinant dUTPase. Coomassie Blue staining after SDS-PAGE separation of total extracts from control noninduced (−T), IPTG-induced supernatant fraction (+S), and IPTG-induced inclusion body fraction (+IB) *E. coli* BL21(DE3) cells transformed with PET32a-DUT and purified fraction (Ni-NTA). The marker is on the left.

the recombinant dUTPase was purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography (Fig. 3).

Transcriptional analysis of DEV dUTPase gene. To study the expression of the DEV dUTPase gene during viral infection, RNA dot blot was carried out with the biotin-labeled oligonucleotide probe specific for this ORF. As shown in Fig. 4A, the DEV dUTPase gene transcripts showed up as early as 30 min postinfection, and then the hybridization signal intensity increased steadily, reaching a peak at 4 hpi, and thereafter declining slowly.

DEV dUTPase induction during infection. A polypeptide band migrating at the expected position for protein DEV dUTPase (about 49 kDa) was first observed at 8 hpi, with maximal amounts between 12 hpi and 24 hpi (Fig. 4B), and then declining gradually.

Immunolocalization of DEV dUTPase in infected cells. Immunofluorescence experiments were carried out to determine the subcellular localization of the protein encoded by DEV dUTPase gene in DEFs at different stages. As illustrated in Fig. 5, infected cells (D–R) showed a specific green fluorescent nuclear and/or cytoplasmic staining pattern, whereas essentially no signal was detected in mock-infected cells (Fig. 5A–C). The specific fluorescence could be detected only in the cytoplasm of infected cells as early as 4 hpi (Fig. 5D–F). At the later stage, it transferred from cytoplasm to nucleus, and it was dispersed both in cytoplasm and nucleus (Fig. 5G–I). At 12 hpi, a large amount of fluorescence was concentrated on the nucleus, showing the patch-like staining pattern. Some blue staining first occurred in the cytoplasm, corresponding with the irregular shape of nucleus (Fig. 5J–L). After 24 hr, fluorescence was gently dispersed both in cytoplasm and nucleus, and then it gradually diminished (Fig. 5 M–O). Interestingly, infected cells with an abnormal profile that were swollen or condensed, as well as nuclear fragments, that were found associated with the specific fluorescence diminished significantly in the late stage of DEV infection (Fig. 5Q).

DISCUSSION

In the last decade, the genes of many dUTPase have been characterized. According to the previous report, different species

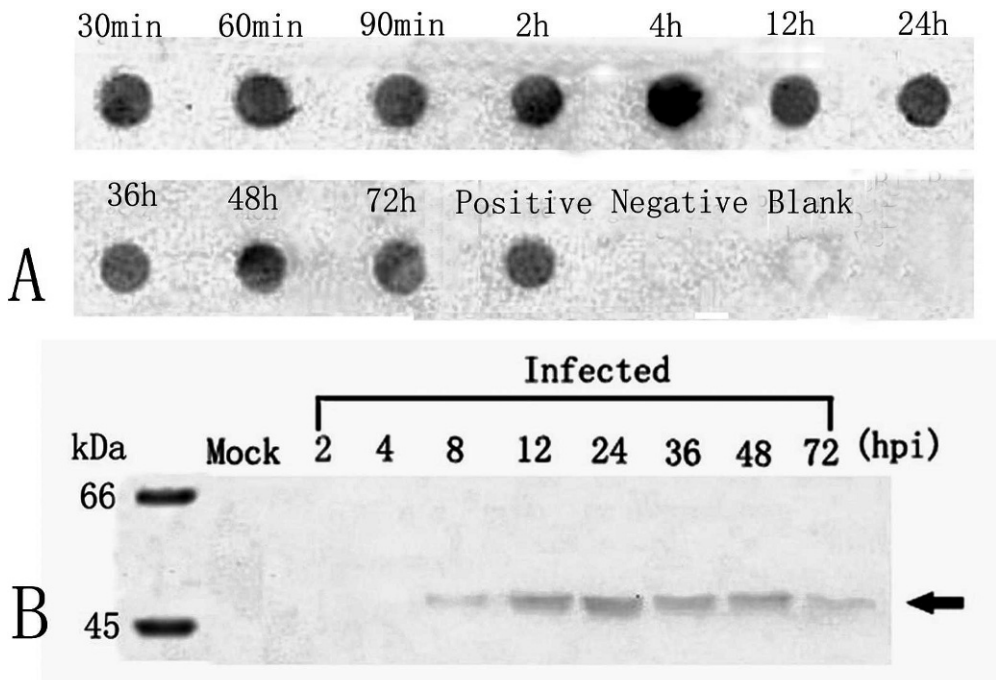

Fig. 4. (A) Transcriptional analysis of DEV dUTPase gene. Total RNA at different times after infection from DEV-infected cells, total RNA from mock cells (negative), DNA from cells infected with DEV (positive), and 2× standard saline citrate as the substitution (blank) were established. The biotin-labeled oligonucleotide was used as a probe. (B) DEV dUTPase expression in infected cells analyzed by western blot. Mock- or DEV-infected DEFs were lysed at different times after infection and subjected to western blot analysis by using an anti-recombinant dUTPase serum. The hpi corresponding to each infected cells extraction are indicated above the lanes. The arrow shows the expected position for DEV dUTPase (about 49 kDa). The electrophoresis migration of molecular mass markers is shown on the left.

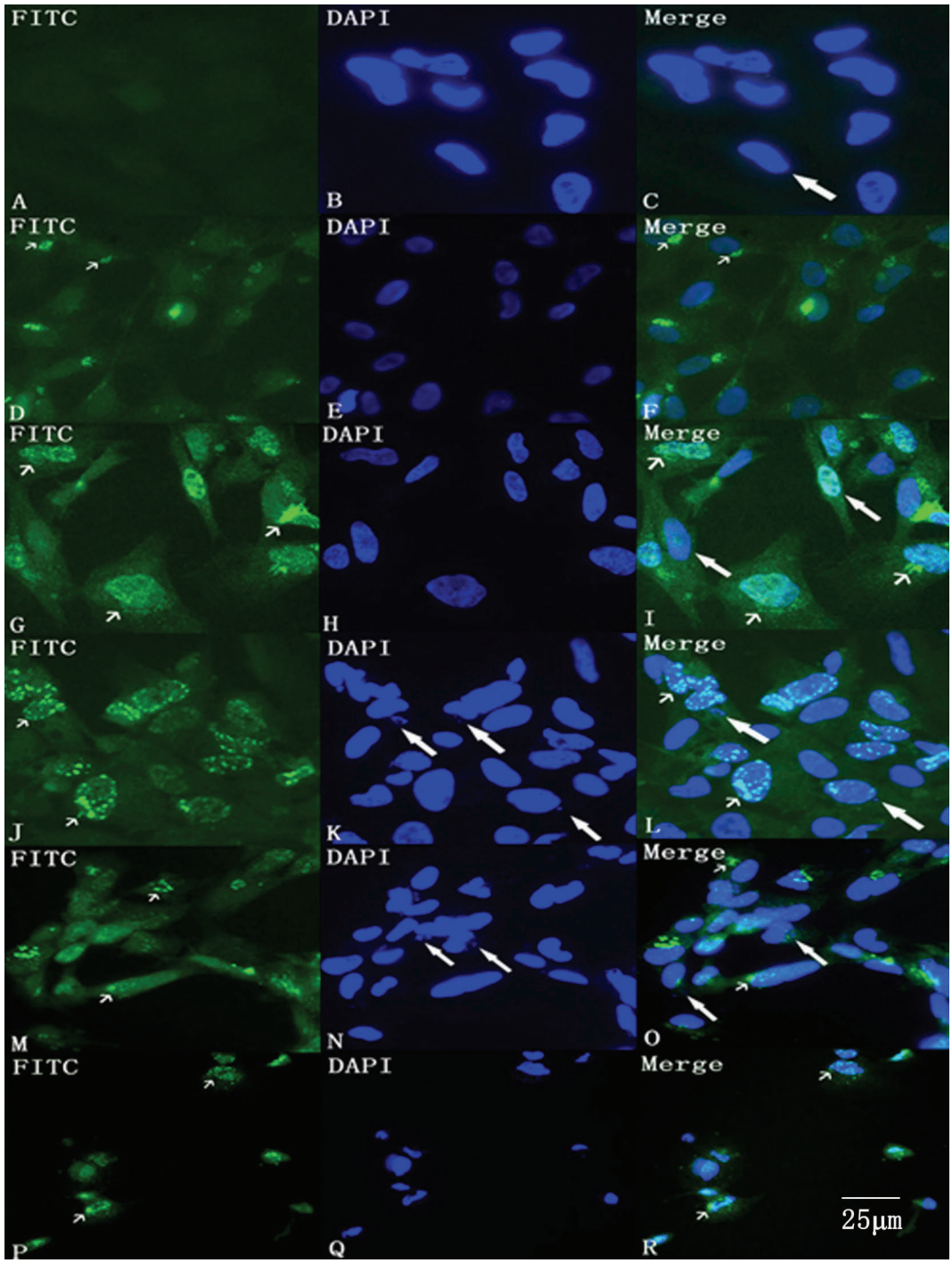

Fig. 5. Immunofluorescence detection of DEV dUTPase in infected DEF cells. Mock-infected or DEV-infected DEF cells were fixed at different stages (0 hpi, 4 hpi, 8 hpi, 12 hpi, 24 hpi, and 48 hpi), incubated with anti-recombinant dUTPase serum, and then stained with FITC-goat antibody and a fluorescent DNA dye (DAPI) as described in Materials and Methods. A, D, G, J, M, and P show the FITC staining; and B, E, H, J, M, and Q show the DAPI staining. C, F, I, L, O, and R are the merged images of FITC and DAPI staining. The short arrows indicate the DEV dUTPase FITC fluorescence staining, and the long arrows indicate the nucleic acid DAPI staining. Bars = 25 μm (A–R).

dUTPase are classified into two types: class 1 and class 2. Class 1 dUTPases have a chain length of around 150 amino acid residues, and the arrangement of the five conserved motifs is 1-2-3-4-5, which is known from bacteria, fungi, plants, metazoans, and a range of viruses. The mammalian herpesvirus dUTPase, which we now term class 2 dUTPases, have amino acid residues around twice as long those in class 1. They also have the same motif set but in a different arrangement, namely, 3-1-2-4-5. Moreover, the class 2 dUTPases with the 3-1-2-4-5 motif array are found only in the α- and γ-Herpesvirinae (22). The multiple alignment and phylogenetic analysis have allowed us to examine the evolution of the DEV dUTPase gene in detail. In addition to the origin listed in Table 1, dUTPase has also been detected in rhesus monkeys, dogs, cows, rabbits, and chickens (2,24). The evolutionary origin of viral dUTPase has been well studied, and the evidence for horizontal transfers between host and DNA viruses has also been revealed

(2,34,43). Phylogenetic analysis showed that DEV CHv strain dUTPase clearly clusters with the fowl herpesvirus (gallid herpesvirus 2, gallid herpesvirus 3, and meleagrid herpesvirus 1) and mammalian dUTPase (Fig. 2). Moreover, sequence comparisons of DEV and 29 reference strains (Table 1) of the dUTPase gene showed that the DEV CHv strain was more similar to α-herpervirus than to β-herpervirus or γ-herpervirus. Although it has been proven that the DEV clone-03 strain was grouped in the subfamily α-herpesvirinae recently (20), the highly conserved DEV CHv strain dUTPase gene phylogenetic analysis result was consistent with the previous study. So, it is also very important to confirm that DEV is a member of α-herpervirus. The five conserved motifs of DEV dUTPase with arrangement order 3-1-2-4-5 belong to the class 2 dUTPase characterization (3,17,18,28). Our preliminary analysis of this sequence shows that the DEV dUTPase gene probably also has a long evolutionary history and close similarity with eukaryotic dUTPase in general, indicating that it is another possible example of horizontal transfer, possibly from its host.

It has been reported that the dUTPase-encoding gene is a β (early) gene, and it is regulated by the presence of immediate early (α) gene product, specifically α4 (9,30,34,43), but little is known about the transcription and translation of the viral dUTPase. Zhe Zhao (43) reported that the RGV dUTPase earliest transcripts can be detected by reverse transcription-PCR at 4 hpi, whereas the Chlorella virus dUTPase gene transcribed as early as 15 min after infection, and its protein first occurred at 45 min after infection (42). Here, a preliminary result of temporal analysis showed the DEV dUTPase gene was an early gene during the in vitro infection, which was consistent with the results of the immunofluorescence assay. Similarly, expression of DEV dUTPase at the early stage during in vitro infection may play a surveillance role in fidelity of DEV DNA synthesis. However, further research is required to verify this hypothesis.

It has been confirmed that the viral enzyme subcellular localization involves a temporal change in cytoplasm, nucleus, or both of DEV-infected cells and that the enzyme is expressed at both early and late stages of infection, which is consistent with a role in maintaining a high TTP/dUTP ratio to minimize the introduction of uracil into the viral DNA. Although DEV dUTPase was not been found in the nucleus at the early stage, it is likely that the surveillance role performed by the cytoplasmic enzyme could be sufficient to ensure the fidelity of the viral DNA synthesis, which occurs in the nucleus, or maybe the viral dUTPase in the cytoplasm was synthesized earlier than the virus DNA replication. Currently, little is known about the subcellular localization of the herpesviruses dUTPase, although it has been reported previously that dUTPase was nearly exclusively a cytoplasmic enzyme (36). Presumably, the herpesviruses dUTPase are localized in the nucleus, because herpesviruses replicate their viral DNA and assemble virus capsids in the nucleus, e.g., HSV-1 (34,38,39,40) and Epstein–Barr virus (33)-encoded dUTPase. But, several studies have revealed that the HSV-2 encoded dUTPase was located primarily in the cytoplasm of infected cells (38,40). It is likely that the discrepancy may reflect different viruses cultivated in different cells (34).

According to our observations, the specific blue straining that first occurred in the cytoplasm at 12 hpi might be the progeny virus nucleic acid straining or the nuclear fragments. We hypothesize that the progeny nucleocapsids assembled in the nucleus and reached the cytoplasm by an envelopment-de-envelopment mechanism on the nuclear membrane (25). Previous studies reported that the nucleocapsids were first noted at 12 hr in nucleus and cytoplasm of attenuated DEV-infected cells (4,14). So, we can conclude that 12 hpi is an important time, because at that moment the induction of viral dUTPase may have a great effect on preventing dUTP incorporation into viral DNA when the activity of the host dUTPase is low or absent. Otherwise, it is possible that the appearance of infected cells with an abnormal profile that were swollen or condensed, and nuclear fragments, are related to the cytopathic effect induced by DEV infection.

As described here, this study demonstrates that the DEV-induced dUTPase differs from the host dUTPase via several primarily physical and biochemical properties. Further studies involving the construction of a dUTPase gene DEV mutant are required, which will reveal whether uracil is incorporated into the viral DNA during virus replication and if so, whether this incorporation results in higher mutation levels. Moreover, in vivo experiments with ducks infected with dUTPase-defective virulent isolates of DEV should also be of importance to assess the possible role of dUTPase in viral pathogenesis.

REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402. 1997.

2. Baldo, A. M., and M. A. McClure. Evolution and horizontal transfer of dUTPase-encoding genes in viruses and their hosts. J. Virol. 73:7710–7721. 1999.

3. Bjornberg, O., A. C. Bergman, A. M. Rosengren, R. Persson, I. R. Lehman, and P. O. Nyman. dUTPase from herpes simplex virus type 1; purification from infected green monkey kidney (Vero) cells and from an overproducing *Escherichia coli* strain. Protein Expr. Purif. 4:149–159. 1993.

4. Breese, S. S. J., and A. H. Dardiri. Electron microscopic characterization of duck plague virus. Virology 34:160–169. 1968.

5. Broyles, S. S. Vaccinia virus encodes a functional dUTPase. Virology 195:863–865. 1993.

6. Burland, T. G. DNASTAR's Lasergene sequence analysis software. Methods Mol. Biol. 132:71–91. 2000.

7. Camacho, A., R. Arrebola, J. Pena-Diaz, L. M. Ruiz-Perez, and D. Gonzalez-Pacanowska. Description of a novel eukaryotic deoxyuridine 5′-triphosphate nucleotidohydrolase in *Leishmania major*. Biochem. J. 325:441–447. 1997.

8. Chen, D. F., W. Endres, S. A. Meyer, and W. Stangel. A polymerase chain reaction-sequence-specific oligonucleotide procedure for HLA class II typing using biotin- and digoxigenin-labeled probes simultaneously in hybridization. Hum. Immunol. 39:25–30. 1994.

9. Chen, R., H. Wang, and L. M. Mansky. Roles of uracil-DNA glycosylase and dUTPase in virus replication. J. Gen. Virol. 83:2339–2345. 2002.

10. Cheng, A. C., M. S. Wang, M. Wen, W. G. Zhou, Y. F. Guo, R. Y. Jia, C. Xu, G. P. Yuan, and Y. C. Liu. Construction of duck enteritis virus gene libraries and discovery, cloning and identification of viral nucleocapsid protein gene. High Technol. Lett. 16:948–953. 2006.

11. Cottone, R., M. Buttner, C. J. McInnes, A. R. Wood, and H. J. Rziha. Orf virus encodes a functional dUTPase gene. J. Gen. Virol. 83:1043–1048. 2002.

12. Dauter, Z., R. Persson, A. M. Rosengren, P. O. Nyman, K. S. Wilson, and E. S. Cedergren-Zeppezauer. Crystal structure of dUTPase from equine infectious anaemia virus; active site metal binding in a substrate analogue complex. J. Mol. Biol. 285:655–673. 1999.

13. Elder, J. H., D. L. Lerner, C. S. Hasselkus-Light, D. J. Fontenot, E. Hunter, P. A. Luciw, R. C. Montelaro, and T. R. Phillips. Distinct subsets of retroviruses encode dUTPase. J. Virol. 66:1791–1794. 1992.

14. Guo, Y. F., A. C. Cheng, M. S. Wang, Y. Zhou, and G. F. Yuan. Study on morphogenesis of duck enteritis virus CH virulent isolate in infected duck embryo fibroblasts. Acta Vet. Zootechniac Sin. 37:274–280. 2006.

15. Haimovich, B., B. J. Aneskievich, and D. Boettiger. Cellular partitioning of beta-1 integrins and their phosphorylated forms is altered after transformation by Rous sarcoma virus or treatment with cytochalasin D. Cell Regul. 2:271–283. 1991.

16. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23:403–405. 1998.

17. Jons, A., and T. C. Mettenleiter. Identification and characterization of pseudorabies virus dUTPase. J. Virol. 70:1242–1245. 1996.

18. Liang, X., M. Tang, B. Manns, L. A. Babiuk, and T. J. Zamb. Identification and deletion mutagenesis of the bovine herpesvirus 1 dUTPase gene and a gene homologous to herpes simplex virus UL49.5. Virology 195:42–50. 1993.

19. Lichtenstein, D. L., K. E. Rushlow, R. F. Cook, M. L. Raabe, C. J. Swardson, G. J. Kociba, C. J. Issel, and R. C. Montelaro. Replication in vitro and in vivo of an equine infectious anemia virus mutant deficient in dUTPase activity. J. Virol. 69:2881–2888. 1995.

20. Liu, S., S. Chen, H. Li, and X. Kong. Molecular characterization of the herpes simplex virus 1 (HSV-1) homologues, UL25 to UL30, in duck enteritis virus (DEV). Gene 401:88–96. 2007.

21. Liu, X., M. Cai, X. Wang, and X. Li. One simple and efficient method for purification of IgG McAb from mice ascites: caprylic acid/ammonium sulfate precipitation. Hua Xi Yi Ke Da Xue Xue Bao 30:455–464. 1999.

22. McGeehan, J. E., N. W. Depledge, and D. J. McGeoch. Evolution of the dUTPase gene of mammalian and avian herpesviruses. Curr. Protein Pept. Sci. 2:325–333. 2001.

23. McGuire, J. M., M. Douglas, and K. D. Smith. The resolution of the neutral N-linked oligosaccharides of IgG by high pH anion-exchange chromatography. Carbohydr. Res. 292:1–9. 1996.

24. McIntosh, E. M., D. D. Ager, M. H. Gadsden, and R. H. Haynes. Human dUTP pyrophosphatase: cDNA sequence and potential biological importance of the enzyme. Proc. Natl. Acad. Sci. U S A 89:8020–8024. 1992.

25. Mettenleiter, T. C. Herpesvirus assembly and egress. J. Virol. 76: 1537–1547. 2002.

26. Miller, W. J., J. A. Skinner, G. S. Foss, and K. E. Davies. Localization of the fragile X mental retardation 2 (FMR2) protein in mammalian brain. Eur. J. Neurosci. 12:381–384. 2000.

27. Oliveros, M., R. Garcia-Escudero, A. Alejo, E. Vinuela, M. L. Salas, and J. Salas. African swine fever virus dUTPase is a highly specific enzyme required for efficient replication in swine macrophages. J. Virol. 73:8934–8943. 1999.

28. Prangishvili, D., H. P. Klenk, G. Jakobs, A. Schmiechen, C. Hanselmann, I. Holz, and W. Zillig. Biochemical and phylogenetic characterization of the dUTPase from the archaeal virus SIRV. J. Biol. Chem. 273:6024–6029. 1998.

29. Pyles, R. B., N. M. Sawtell, and R. L. Thompson. Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. J. Virol. 66:6706–6713. 1992.

30. Roizman, B., and A. E. Sears. Herpes simplex viruses and their replication. In: Fundamental virology, 3rd ed. B. N. Fields, and D. M. Knipe, eds. Lippincott-Raven Publishers, Philadelphia, PA. pp. 1043–1107. 1996.

31. Sandhu, T. S., and L. Leibovitz. Duck plague. In: Diseases of poultry, 11th ed. Y. M. Saif, ed. Iowa State University Press, Ames, IA. pp. 354–363. 2003.

32. Sambrook, J., and D. W. Russell. Molecular cloning: a laboratory manual, 3rd ed, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, New York. pp. 1228–1232. 2001.

33. Sommer, P., E. Kremmer, S. Bier, S. Konig, P. Zalud, M. Zeppezauer, J. F. Jones, N. Mueller-Lantzsch, and F. A. Grasser. Cloning and expression of the Epstein-Barr virus-encoded dUTPase: patients with acute, reactivated or chronic virus infection develop antibodies against the enzyme. J. Gen. Virol. 77:2795–2805. 1996.

34. Studebaker, A. W., G. K. Balendiran, and M. V. Williams. The herpesvirus encoded dUTPase as a potential chemotherapeutic target. Curr. Protein Pept. Sci. 2:371–379. 2001.

35. Threadgill, D. S., W. K. Steagall, M. T. Flaherty, F. J. Fuller, S. T. Perry, K. E. Rushlow, S. F. Le Grice, and S. L. Payne. Characterization of equine infectious anemia virus dUTPase: growth properties of a dUTPase-deficient mutant. J. Virol. 67:2592–2600. 1993.

36. Vilpo, J. A., and H. Autio-Harmainen. Uracil-DNA glycosylase and deoxyuridine triphosphatase: studies of activity and subcellular location in human normal and malignant lymphocytes. Scand. J. Clin. Lab. Invest. 43:583–590. 1983.

37. Wagaman, P. C., C. S. Hasselkus-Light, M. Henson, D. L. Lerner, T. R. Phillips, and J. H. Elder. Molecular cloning and characterization of deoxyuridine triphosphatase from feline immunodeficiency virus (FIV). Virology 196:451–457. 1993.

38. Williams, M. V., and D. S. Parris. Characterization of a herpes simplex virus type 2 deoxyuridine triphosphate nucleotidohydrolase and mapping of a gene conferring type specificity for the enzyme. Virology 156:282–292. 1987.

39. Wohlrab, F., and B. Francke. Deoxyribopyrimidine triphosphatase activity specific for cells infected with herpes simplex virus type 1. Proc. Natl. Acad. Sci. U S A 77:1872–1876. 1980.

40. Wohlrab, F., B. K. Garrett, and B. Francke. Control of expression of the herpes simplex virus-induced deoxypyrimidine triphosphatase in cells infected with mutants of herpes simplex virus types 1 and 2 and intertypic recombinants. J. Virol. 43:935–942. 1982.

41. Yadetie, F., A. K. Sandvik, H. Bergum, K. Norsett, and A. Laegreid. Miniaturized fluorescent RNA dot blot method for rapid quantitation of gene expression. BMC Biotechnol. 4:12. 2004.

42. Zhang, Y., H. Moriyama, K. Homma, and J. L. Van Etten. Chlorella virus-encoded deoxyuridine triphosphatases exhibit different temperature optima. J. Virol. 79:9945–9953. 2005.

43. Zhao, Z., F. Ke, J. Gui, and Q. Zhang. Characterization of an early gene encoding for dUTPase in Rana grylio virus. Virus Res. 123:128–137. 2007.

ACKNOWLEDGMENTS

The reported nucleotide sequence has been deposited in GenBank and the assigned accession no. DQ486149. The research were supported by the National Natural Science Foundation of China (3047129/30771598), New Century Excellent Talents program in University (NCET-04-0906/NCET-06-0818), Scientific and Technological Innovation Major Project Funds in University (706050), Sichuan Province Outstanding Youths Fund (03ZQ026-029), Sichuan Province Basic ResearchProgram (04JY021-100/04JY029-006-1/05JY029-109/05JY029-003/07JY029-016/07JY029-016), and Program for Key Disciplines Construction of Sichuan Province (SZD0418).