

## Identification and Characterization of Pseudorabies Virus dUTPase

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**Sequence analysis within the long segment of the pseudorabies virus (PrV) genome identified an open reading frame of 804 bp whose deduced protein product of 268 amino acids exhibited homology to dUTPases of other herpesviruses. The gene was designated UL50 because of its colinearity with the homologous gene of herpes simplex virus type 1. An antiserum raised against a bacterially expressed fragment of PrV UL50 specifically detected a 33-kDa protein in lysates of infected cells, which is in agreement with the predicted molecular mass of the PrV UL50 protein. A UL50-negative PrV mutant (PrV UL50<sup>-</sup>) was constructed by the insertion of a  $\beta$ -galactosidase expression cassette into the UL50 coding sequence. A corresponding rescuant (PrV UL50<sup>resc</sup>) was also isolated. The interruption of the UL50 gene led to the disappearance of the 33-kDa protein, whereas restoration of UL50 gene expression restored detection of the 33-kDa protein. Enzyme activity assays confirmed that UL50 of PrV codes for a dUTPase which copurifies with nuclei of infected cells. PrV UL50<sup>-</sup> replicated with an only slightly reduced efficiency in epithelial cells in culture compared with that of its parental wild-type virus strain. Our results thus demonstrate that UL50 of PrV encodes a protein of 33 kDa with dUTPase activity which copurifies with nuclei of infected cells and is dispensable for replication in cultured epithelial cells.**

Herpesvirus genomes encode a set of enzymes involved in nucleotide metabolism. The alphaherpesvirus herpes simplex virus (HSV), for example, specifies a thymidine kinase, ribonucleotide reductase, and dUTPase, which increase deoxynucleoside triphosphate pool sizes, and a uracil DNA glycosylase important for DNA repair (for a review, see reference 27). These enzymes were shown to be nonessential for replication in cell culture but proved to be important virulence factors in the mouse model (3, 4, 8, 10, 11, 25, 26).

Pseudorabies virus (PrV), an alphaherpesvirus of swine, is the causative agent of Aujeszky's disease. Following infection of the nasal mucosa, PrV enters sensory neurons and spreads to the central nervous system, where it induces the severe neurological symptoms associated with Aujeszky's disease. Infection of the respiratory epithelia causes respiratory disorders. In most other susceptible species, which include nearly all mammals except higher primates and equines, Aujeszky's disease is characterized by a very high mortality (23, 31).

PrV also encodes a thymidine kinase and a ribonucleotide reductase which have both been shown to be nonessential for viral replication in cultured cells but are involved in determining viral virulence in vivo (7, 14, 16, 21, 31). The deduced protein product of the PrV UL2 gene exhibits homology with uracil DNA glycosylases; however, a functional characterization has not yet been performed (6, 15). So far, a protein with dUTPase activity has not been identified in PrV.

Analysis of the complete sequence of the *Bam*HI fragment 5' of the PrV strain Ka genome (13) led to the identification of 221 bp of an open reading frame whose deduced protein product exhibited homology to C-terminal regions of herpesvirus dUTPases (2). On the basis of the colinearity of this genomic region with the genome of HSV type 1 (HSV-1), the gene was designated UL50. Sequence determination within the adjacent

*Bam*HI fragment 1 (Fig. 1) identified the missing 5'-terminal part of the PrV UL50 gene. The complete UL50 open reading frame consists of 804 bp encoding a polypeptide of 268 amino acids (aa) with a calculated molecular mass of 29 kDa (GenBank accession number U38547). A putative TATA box was found 126 bp upstream of the translation initiation codon, and a perfect polyadenylation signal, 5'-AATAAA-3', is located 80 bp downstream of the stop codon (2). These properties suggest that PrV UL50 encodes a dUTPase.

A comparison with deduced amino acid sequences from other alphaherpesviruses revealed that the PrV UL50 gene product is most closely related to the homologous proteins of bovine herpesvirus 1 (17) and then to equine herpesvirus 1 (30), HSV-1 (20), and varicella-zoster virus (5) (Fig. 2). The deduced PrV UL50 protein contains the five sequence motifs conserved in all dUTPases sequenced so far in the rearranged order typical of herpesvirus dUTPases (19). In herpesviruses, motif 3 is located upstream from motif 1 so that the order of motifs is 3, 1, 2, 4, and 5 as counted from the N terminus. Herpesvirus dUTPases also differ in length from dUTPases of other species in that they are significantly longer. Epstein-Barr virus dUTPase contains 278 aa (1), bovine herpesvirus 1 dUTPase contains 325 aa (17), equine herpesvirus 1 dUTPase contains 326 aa (30), HSV-1 dUTPase contains 371 aa (20), and varicella-zoster virus dUTPase contains 396 aa (5). In contrast, dUTPase from *Escherichia coli* consists of 151 aa (18) and the human enzyme comprises 141 aa (22). The deduced PrV UL50 protein of 268 aa would thus represent the smallest herpesviral dUTPase.

The relatively high degree of homology between dUTPases of herpesviruses and those of other species is found mainly in the C-terminal part of the longer herpesvirus dUTPases. Therefore, we chose an N-terminal fragment of the predicted PrV UL50 polypeptide for prokaryotic expression as a fusion protein to obtain a PrV UL50 protein-specific rabbit antiserum (Fig. 1). In Western blot (immunoblot) analyses of PrV-infected bovine kidney (MDBK) cells (Fig. 3, lane 2) the antiserum generated against the PrV UL50 protein ( $\alpha$ -UL50 serum) specifically detected a protein of 33 kDa, which corresponds

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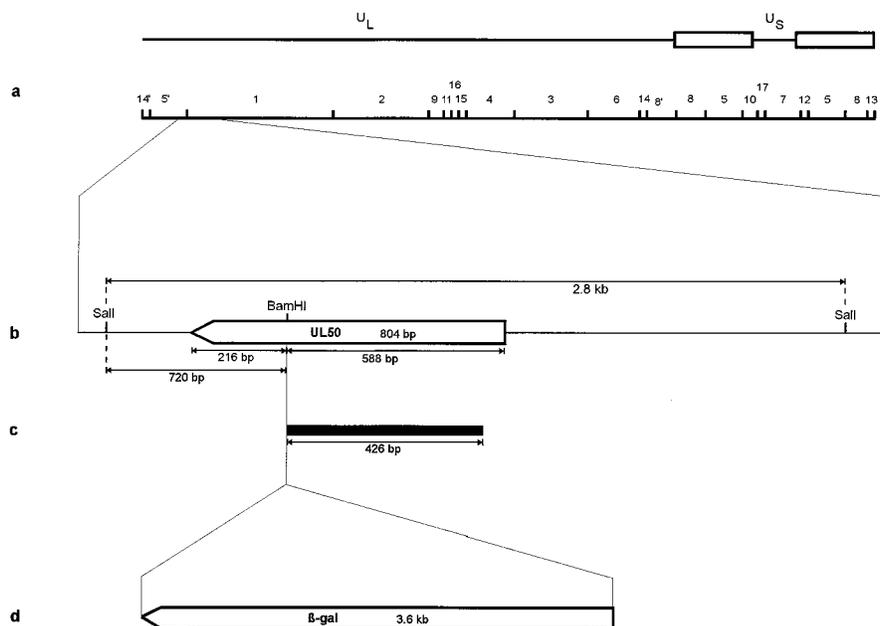


FIG. 1. Location of the PrV UL50 gene and construction of PrV UL50<sup>-</sup>. (a) Genome organization and *Bam*HI restriction map of the PrV genome. Open rectangles represent the inverted repeat regions that separate the genomic DNA into a unique long (U<sub>L</sub>) and a unique short (U<sub>S</sub>) portion. (b) Enlargement of the part of the long segment that includes UL50. Relevant restriction sites are shown, and sizes of fragments are indicated. (c) Fragment of UL50 that was expressed as fusion protein. (d) Insertion of the β-galactosidase (β-gal) expression cassette into the UL50 gene to isolate a PrV UL50<sup>-</sup> mutant.

well to the calculated molecular mass of the UL50 gene product. The antiserum apparently did not cross-react with dUTPase from mock-infected MDBK cells (Fig. 3, lane 1).

To inactivate the UL50 gene, the coding sequence was interrupted by the insertion of a β-galactosidase expression cassette (24) into the unique *Bam*HI site within UL50 (Fig. 1). A complete deletion of UL50 would also have deleted the N terminus of a small overlapping reading frame with an opposite orientation (12). This was avoided by the insertional mutagenesis which was to result in inactivation of the UL50 gene but leave adjacent genes intact. Following cotransfection with genomic DNA of wild-type PrV strain Ka (9, 13), progeny plaques which stained blue under a Blu-Gal (Gibco-BRL, Eggenstein, Germany) agarose overlay were picked and purified to homogeneity (PrV UL50<sup>-</sup>). A respective rescuant in which the expression of UL50 had been restored was isolated after cotransfection of DNA from PrV UL50<sup>-</sup> with a 2.8-kb *Sall* fragment (Fig. 1) containing the authentic UL50 gene (PrV UL50resc) by selecting for a white plaque phenotype with Blu-Gal. Correct insertion of the β-galactosidase cassette in PrV UL50<sup>-</sup> and restoration of wild-type UL50 in PrV UL50resc were verified by Southern blot hybridization (data not shown).

Figure 3, lane 3, shows that interruption of the UL50 gene in PrV UL50<sup>-</sup> resulted in the absence of the 33-kDa protein as detected by the α-UL50 serum in Western blot analyses. In contrast, the restoration of UL50 gene expression led to renewed detection of the 33-kDa protein. Thus, we provide convincing evidence that the 33-kDa protein recognized by the antiserum represents the PrV UL50 gene product. Since the α-UL50 serum was directed against the amino-terminal part of the PrV UL50 protein, which can theoretically still be expressed in the PrV UL50<sup>-</sup> mutant, the data also indicate that this expression, if it occurs at all, does not lead to a stable translation product detectable by the α-UL50 serum.

To assay whether PrV UL50 indeed encodes a protein with

dUTPase activity (32), confluent monolayers of  $2 \times 10^7$  MDBK cells were infected with either wild-type PrV Ka, PrV UL50<sup>-</sup>, or PrV UL50resc at a multiplicity of infection of 1 or mock infected and incubated for 20 h at 37°C. Thereafter, dUTPase activity was assayed in nuclear and cytoplasmic fractions. Figure 4 shows the averages of three independent experiments. Levels of dUTPase activity in nuclear fractions were three- to fourfold higher in PrV Ka- or PrV UL50resc-infected cells than in mock-infected cells and cells infected with PrV UL50<sup>-</sup> (Fig. 4A). In contrast, levels of cytoplasmic dUTPase activity did not differ significantly between infected and mock-infected cells (Fig. 4B). Although our assay did not differentiate between viral and cellular dUTPase, a comparison between mock-infected and infected cells, as well as between wild-type, rescuant, and UL50<sup>-</sup> mutant virus, clearly shows that an increase in the level of nuclear dUTPase activity was dependent on the presence of an intact UL50 gene in the infecting virus. From these results we conclude that PrV UL50 does code for a dUTPase and that the enzyme copurifies with and is most likely contained in the nuclei of infected cells. In contrast, bovine herpesvirus 1 dUTPase activity has been shown to be localized mainly in the cytoplasm but was also detectable in the nuclear fraction (17). HSV-1 dUTPase was found in the nucleus, whereas HSV-2 dUTPase appears to be confined to the cytoplasm of infected cells (32). Since all herpesviruses replicate in the nucleus, the significance of these different findings is unknown at present.

Isolation of the PrV UL50<sup>-</sup> mutant on normal epithelial cells already indicated that PrV dUTPase is not essential for replication of PrV in vitro. To analyze replication of the virus mutant in more detail, one-step growth kinetics were assayed after infection of MDBK cells at a multiplicity of infection of 5. Compared with wild-type PrV and UL50resc, the appearance of infectious progeny of PrV UL50<sup>-</sup> was slightly delayed (by approximately 2 h), resulting in ca. 10-fold lower titers at

	1		50
PrV	.....ME	ESAGATSA..	.....QSAATSVS
BHV-1	.....MA	NSAAATTATM	SGDRGILVVE LNAEAPWRL
EHV-1	.....	MASV	TNLVDSIVVV ECGERWRARA
HSV-1	.....	MSQWSSGAI	LVQPDLSLGRG YDGDWHTAVA
VZV	MNEAVIDPIL	ETAVNTGDMF	CSQTI PNRCLE KDTILILEVQP ECADTLQCVL
	51		100
PrV	E...SPAEE	.....ETILV	CASEPVTVDG GRLLVCRSPG PEGFYKVPFLG
BHV-1	E...SCCEPD	SLALWGPIAP	AAKRDETAPS GSLLYSR.....LINLN
EHV-1	E...AAGRLL	VLINNHTVEL	SGEHGSAGEF YSVL.....TDVG
HSV-1	T...RGGGVV	QLNLVNRRAV	AFMPKVS GDS GWAVG.....RVSLD
VZV	DDKVSRRHQP	LLRNHKKLEL	PSEKSVTRGG FYM.....QQLELL
	101		150
PrV	LKVALPTGYA	MLVAQRGGGR	T.....T N.....GIVDA
BHV-1	MKAAAPGGYA	IIMSQMRSGD	THMPRP PAVA V.....GIVDS
EHV-1	VRVACSSGYA	IVLTQISGLL	PVEPEPGNFS NVTFPENSAK YTYAYGIVDS
HSV-1	LRMAMPADFC	AIHAPALAS	P.....G HHVILGLIDS
VZV	VKSAPPNEYA	LLLIQCKDTA	LADED.....N FFWANGVIDA
		<b>motif 3</b>	
	151		200
PrV	<b>GFRGEVQ</b> AIV	.....APGRP	RAQFYCTPLR L.....
BHV-1	<b>GYSGILRA</b> IV	WAPESAAAAP	PAGL...ALR LTLARLTTTL PRLIAVDD..
EHV-1	<b>GYRGVVK</b> AVQ	FAPGINTSVP	PQOMSLGLVL VKLARKSIHV TSIGSTRD..
HSV-1	<b>GYRGTVM</b> AVV	VAPKRTREFA	PGLTRLDVTF LDILATPPAL TEPISLRQFP
VZV	<b>GYRGVIS</b> ALL	YRPGVTVIL	PGHLTIYLPF VKLRQSRLLP KNVLKHLDD..
	201		250
PrV	.....APG	IATDVPFPE	.....
BHV-1	...DANAGTE	AGVEVPFFA	.....
EHV-1	...GRTSEAN	L.....FYD	.....
HSV-1	QLAPPPPTGA	GIREDPWLEG	ALGAPSVTTA LPARRRGRSL VYAGELTFVQ
VZV	.....PIFK	SIQVQPLSNS	PSNYEKVPVIF EFADISTVQQ GQFLHRDSAE
	251		300
PrV	.....	<b>VFAPKRDE</b> D	<b>AGYDI</b> PCPRE LVLPPGGAET VTLP.V.HRT
BHV-1	.....	<b>TFAPKRDE</b> D	<b>AGYDI</b> AMPYT AVLAPGENLH VRLP.V.AYA
EHV-1	.....	<b>YFAPKRVE</b> D	<b>AGYDI</b> SAPED ATIDPDSEHF VDLP.I.VFA
HSV-1	TEHGDGVREA	IAPL <b>KRRED</b>	<b>AGFDI</b> VVRRP VTVFANGTTV VQPS.LRMLH
VZV	YHIDVPLTYK	HIIN <b>KRQED</b>	<b>AGYDI</b> CVPNY LYLKRNEPIK IVPPIIRDWD
		<b>motif 1</b>	
	301		350
PrV	DGRHWA..YV	<b>FGRSS</b> LNLRG	IVVFPTWES G.PCRFRIQN RGAHPVTLES
BHV-1	ADAHAAAPYV	<b>FGRSS</b> CNLRG	LIVLPTAWPP GEPCRFVLRN VTQEPVAAA
EHV-1	NSNPVATPCI	<b>FGRSS</b> MNRRG	LIVLPTRWVA GRTCFFFLIN VNKYPVSIITK
HSV-1	ADAGPAACYV	<b>LGRSSL</b> NARG	LLVVPTRWLP GHVCAFVVYN LTGVPTLLEA
VZV	LQHPISNAYI	<b>FGRSS</b> KSRSG	IIVCPTAWPA GEHCKFYVYN LTGDDIRIKT
		<b>motif 2</b>	
	351		400
PrV	<b>GQRVAQL</b> VLT	REPLG.WIT	.....GRSPFP ATPRA..PMQ HRPALFLARD
BHV-1	<b>GQRVAQL</b> LLL	ARRLE.WLPS	.....GLNDREPPF TSPPRA..APP APGAPRLRWR
EHV-1	<b>GQRVAQL</b> LLT	EDIDDALIPP	.....TVNYDNPPF TYSPS..EST KAPQSPVLWK
HSV-1	<b>GAKVAQL</b> LVA	GADALPWIPP	DNPHGTKALR NYPRGVPDST AEFRNPLLIV
VZV	<b>GDRLAQV</b> LLI	DHNTQIHL.K	HNVLNSIAFP YAIRGCKGIP G.....VQWY
		<b>motif 4</b>	
	401		435
PrV	FVA.....P	<b>SSARGAR</b> GFG	STGL.....
BHV-1	RVADLAAAVP	<b>PSARGPR</b> GFG	STGL.....
EHV-1	FTTDFDREAP	<b>SSLRADG</b> GFG	STGL.....
HSV-1	FTNEFDABAP	<b>PSEKRG</b> TGFG	STGI.....
VZV	FTKTLDLIAT	<b>PSEKRG</b> TGFG	STDKETNDVD FLLKH
		<b>motif 5</b>	

FIG. 2. Comparison of the amino acid sequences of the PrV UL50 gene product and homologous proteins of other alphaherpesviruses. Each strand of the genomic region containing the UL50 gene was sequenced at least once by the dideoxy chain termination method (28) with the regular set of nucleotides and once replacing dATP by c7-deaza-ATP and dGTP by c7-deaza-GTP. Sequences were analyzed with Wisconsin Package, version 8 (Genetics Computer Group, Madison, Wis.). Amino acid sequences were compared with the program Pileup. Conserved sequence motifs found in all dUTPases are indicated with boldface and italic type. The dUTPase sequences of the other herpesviruses were published previously (5, 17, 20, 30). BHV-1, bovine herpesvirus 1; EHV-1, equine herpesvirus 1; VZV, varicella-zoster virus.

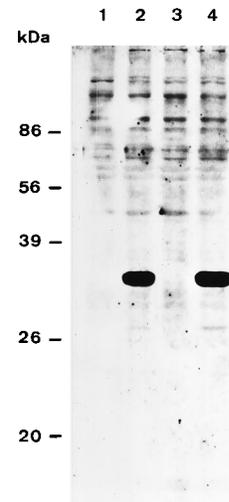


FIG. 3. Identification of the UL50 gene product. The procaryotic expression vector pET23 (Novagen, Madison, Wis.) was used for T7 promoter-controlled expression of a fusion protein consisting of a short leader tag and 426 bp from the 5' terminus of the UL50 gene (Fig. 1). Recombinant pET plasmid was transformed into *E. coli* BL21(DE3)pLysS, which contains a DE3 lysogen (T7 RNA polymerase under the control of the *lacUV5* promoter), and expression was induced by the addition of 2 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside). Bacterial lysates were used for immunization of a rabbit. To assay reactivity of the  $\alpha$ -UL50 serum, MDBK cells were infected at a multiplicity of infection of 2 with either wild-type PrV Ka (lane 2), PrV UL50<sup>-</sup> (lane 3), or PrV UL50resc (lane 4) or they were mock infected (lane 1). Twenty-four hours after infection, cells were lysed and proteins were separated by sodium dodecyl sulfate-13% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose for immunostaining with the rabbit  $\alpha$ -UL50 serum. Bound antibody was visualized by chemiluminescence (ECL detection system; Amersham, Braunschweig, Germany). Positions of marker proteins are indicated.

early time points (data not shown). However, final titers of PrV UL50<sup>-</sup> and PrV UL50resc ( $5 \times 10^7$  PFU/ml and  $6 \times 10^7$  PFU/ml, respectively) were similar to those of wild-type PrV Ka ( $1 \times 10^8$  PFU/ml).

dUTPases are a component of the de novo dTMP synthesis pathway, since their main function is to catalyze the dephosphorylation of dUTP to dUMP. They also reduce the intracellular level of dUTP, so that the misincorporation of uracil into newly synthesized DNA is minimized. In dividing cells these functions can be supplied in *trans* by the cellular dUTPase, so that a virus lacking its own enzyme is still able to replicate efficiently. In nondividing cells like neurons (29), however, the lack of dUTPase could be a significant disadvantage, possibly impairing replication of the mutant virus. This has implications for the neurovirulence of alphaherpesviruses, and it has been shown that dUTPase-deficient HSV-1 mutants display significantly reduced neurovirulence in the mouse model (25). Preliminary analyses indicate that this is also true for PrV neurovirulence in pigs (data not shown).

In summary, we identified the PrV UL50 gene and characterized its gene product as a 33-kDa protein with dUTPase activity which is detected in the nuclear fraction and is dispensable for PrV replication in cultured epithelial cells.

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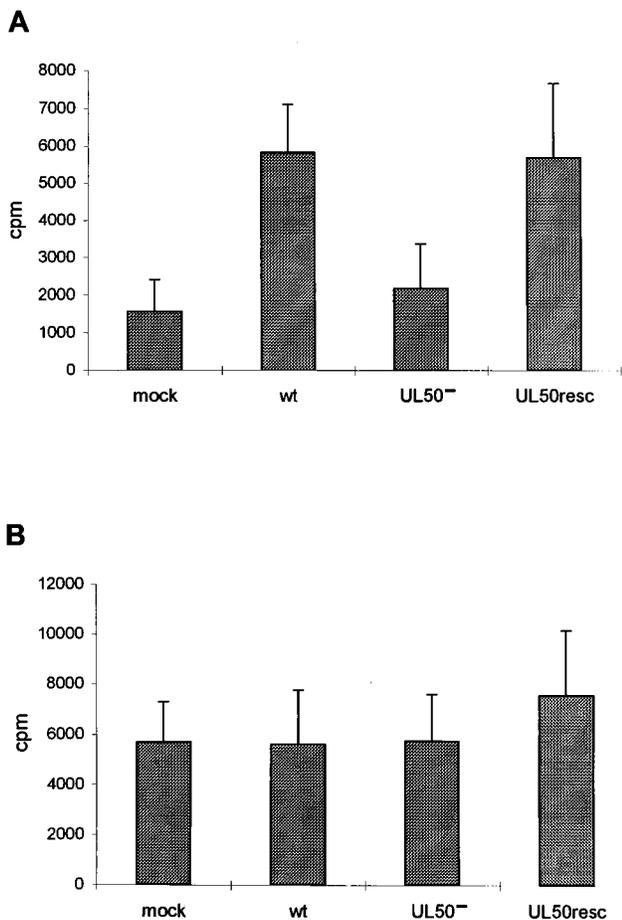


FIG. 4. Detection of dUTPase activity in infected cells. MDBK cells ( $2 \times 10^7$ ) were infected with either the wild-type (wt) PrV Ka, PrV UL50<sup>-</sup>, or PrV UL50<sup>resc</sup>, or they were mock infected (mock). After incubation for 20 h at 37°C, cells were rinsed with cold phosphate-buffered saline and scraped into 1 ml of hypotonic solution [20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer (pH 7.8), 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>]. Nonidet P-40 was added to a final concentration of 0.2%, and the suspension was incubated on ice for 20 min. Nuclear and cytoplasmic fractions were separated by centrifugation at  $500 \times g$  for 10 min. The nuclear fraction was washed once with hypotonic solution and resuspended in 300  $\mu$ l of the same solution. Potassium acetate was then added to both cytoplasmic and nuclear fractions to a final concentration of 80 mM. Five microliters of a stock solution containing 100 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 10 mM EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 20 mM ATP, and 0.5 mM [<sup>3</sup>H]dUTP (2 Ci/mmol; Amersham, Braunschweig, Germany) was added to 45  $\mu$ l of the extracts and incubated at 4°C for 1 h. The reaction was terminated by the addition of 20  $\mu$ l of 100 mM EDTA–105  $\mu$ l of methanol. [<sup>3</sup>H]dUMP and [<sup>3</sup>H]dUTP were separated by thin-layer chromatography on polyethyleneimine cellulose plates containing a fluorescence indicator. The dUMP spots were excised and [<sup>3</sup>H]dUMP was quantitated by scintillation counting. dUTPase activities of nuclear (Fig. 4A) and cytoplasmic (Fig. 4B) fractions were determined. Results represent the average of three independent experiments. Standard deviations are indicated with bars.

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