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Vaccinia Virus Encodes a Functional dUTPase

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The sequence of the vaccinia virus open reading frame F2L predicts a polypeptide with significant similarity to cellular dUTPases. To determine whether the F2L gene product has this activity, it was expressed in bacteria as a fusion with glutathione S-transferase. Affinity purified fusion protein was shown to hydrolyze dUTP yielding dUMP as the product. While the dUTPase was not completely dependent on the addition of divalent cations, its activity was stimulated markedly by Zn^{2+}, Mg^{2+}, and Mn^{2+}. The nucleotide substrate specificity of the enzyme was limited to dUTP. These results demonstrate that vaccinia virus encodes a functional dUTPase whose role in viral infection is suggested to be the augmentation of DNA nucleotide precursors and the minimization of cytoplasmic dUTP concentrations.

Vaccinia virus is a member of the poxvirus family that is characterized as large DNA viruses that replicate in the cytoplasm of the host cell. Since vaccinia virus does not enter the nucleus during the course of its life cycle, it must provide the entire spectrum of proteins functioning in DNA replication and messenger RNA synthesis (reviewed in Ref. *1*). These proteins include a DNA polymerase, a RNA polymerase, and the accessory factors that regulate the activity of the polymerases.

Vaccinia virus also encodes several enzymes functioning in the synthesis of nucleotide precursors for DNA synthesis. These include a thymidine kinase (*2, 3*), a thymidylate kinase (*4*), and a ribonucleotide reductase (*5*). In addition, the F2L open reading frame of vaccinia predicts a polypeptide with significant similarity to the dUTPases of bacteria (*6*), tomato (*7*), and humans (*8*). The F2L gene product has also been suggested to be similar to retroviral pseudoproteases (*9*) that are now known to be dUTPases (*10*). The F2L gene is transcribed as an early class gene (*9*), consistent with a role in viral DNA synthesis. To determine whether the vaccinia virus F2L gene product is a dUTPase, it was expressed bacteria in recombinant form.

The F2L gene was isolated from the vaccinia genome by polymerase chain reaction (PCR) using Taq polymerase and inserted into a vector designed to express glutathione S-transferase (GST) fusion proteins using methodologies described previously (*11*). The cloned gene was sequenced in its entirety to ensure that no mutation was introduced by the PCR. When bacterial cells were induced to synthesize the fusion protein, an new abundant 40 kDa polypeptide was produced. This is close to the size expected for the combination of the 15 kDa F2 polypeptide and the 27 kDa GST. Approximately 50% of the fusion protein remained soluble after lysis of the cells. The GST-F2L fusion protein was purified by affinity chromatography on glutathione agarose (Fig. 1). The protein preparation was estimated to be greater than 95% homogeneous with respect to the fusion protein. The yield of protein was determined to be about 6 mg from a 0.5 liter culture of bacteria.

The F2-GST fusion protein was tested for activity toward dUTP by incubation with [^{3}H]-dUTP and thin layer chromatography (TLC) of the products on polyethyleneimine plates. Plates were developed using 0.9 *M* LiCl, 0.8 *M* acetic acid. Nucleotides were eluted from the plates by washing excised sections with 2 *M* LiCl and radiolabel was quantitated by scintillation counting (*12*). Enzyme reactions were conducted for 30 min at 37° in solutions consisting of 50 m*M* Tris–HCl, pH 7.5, 1 m*M* [^{3}H]-dUTP (specific activity of 500–1000 cpm/nmol), and 0.5 μg fusion protein. Analysis of radiolabel on the thin layer plate showed two major labeled species (Fig. 2). The slower moving product had the mobility of the input dUTP. The more rapidly moving product had the mobility of dUMP. Very little radiolabeled material co-migrated with dUDP. Therefore the GST-F2L protein preparation was capable of hydrolyzing dUTP yielding dUMP as a product. Presumably the other product of the reaction was pyrophosphate, although this was not demonstrated.

The vaccinia dUTPase was characterized as to solution conditions for optimal enzyme activity. dUTPase activity was determined by a DE81 filter binding assay (*13*). In this assay, the substrate dUTP remains bound to the filter, whereas the product dUMP is released by washing the filter in 4 *M* formic acid, 1 m*M* ammonium formate. The enzyme was equally active over the range

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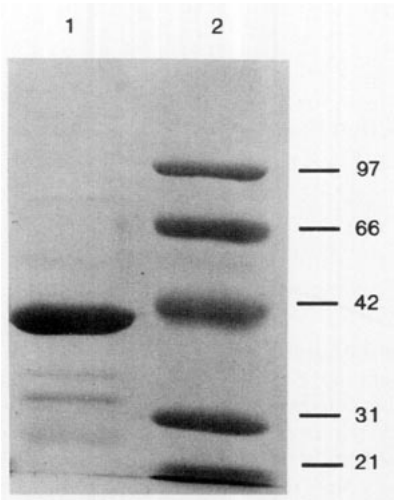

FIG. 1. Polypeptide composition of the GST-F2L fusion protein. Five micrograms of affinity purified fusion protein was analyzed by electrophoresis on a 10% SDS–polyacrylamide gel electrophoresis and staining with coomassie blue. Lane 1 is the fusion protein. Lane 2 is size standards as indicated on the right (in kDa).

of pH from 6.0 to 9.5 (data not shown). As shown above, the vaccinia dUTPase did not require the addition of divalent cations for activity. This is somewhat unusual since most nucleotidyl phosphohydrolases require divalent cations. Therefore, the effect of divalent salts on the activity of the vaccinia dUTPase was determined. Titration of the dUTPase reaction with $MgCl_2$ showed that the activity indeed increased with maximal activity observed above 8 m*M* $MgCl_2$ (data not shown). Various other divalent cations as chloride salts at a concentration of 10 m*M* were also tested for their effect on dUTPase activity. Zn^{2+} was tested as a sulfate salt because of its superior solubility. The choice of anion proved to be inconsequential since $MgCl_2$ and $MgSO_4$ had identical effects on enzyme activity (not shown). Of the cations tested, Zn^{2+} was most effective, stimulating dUTPase activity 15-fold (Table 1). Mg^{2+} and Mn^{2+} were less effective, and Ca^{2+} and Co^{2+} had little stimulatory effect. Chelators of divalent cations were also tested for their effect on the dUTPase. Ethylenediaminetetraacetic acid (EDTA), a nonspecific chelator, was found to inhibit the activity. Ethylene glycol-bis-[β-aminoethyl ether] (EGTA), a calcium-specific chelator, and *o*-phenanthroline, a zinc-specific chelator, had no effect on the activity of the dUTPase. These results indicate that the dUTPase activity of the vaccinia F2L gene product is dependent on divalent cations. The metal dependence of the vaccinia virus dUTPase is very similar to that of the human enzyme (*13*). All further experiments were performed in the presence of 10 m*M* $MgCl_2$.

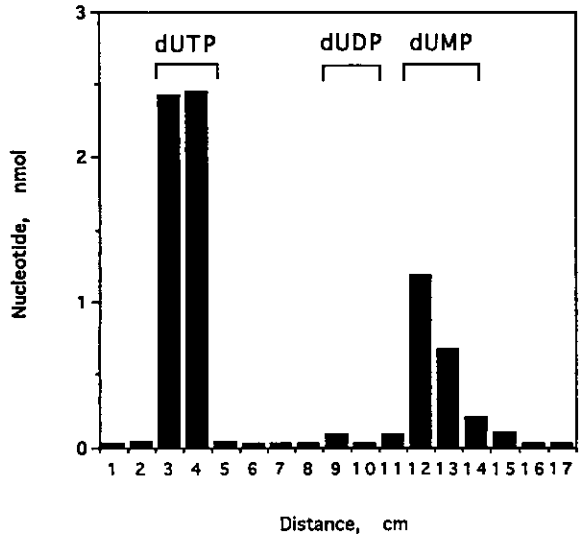

FIG. 2. Thin layer chromatography of products of a reaction of 0.5 μg of GST-F2L fusion protein with [^{3}H]-dUTP. The reaction solution did not contain divalent cations. Reaction products were chromatographed on polyethyleneimine thin layer plates. The thin layer plates were cut into 1-cm strips and radiolabel was quantitated by scintillation counting. Distance was measured from the origin of chromatography. The mobility of dUMP, dUDP, and dUTP standards are indicated above.

TABLE 1

EFFECT OF DIVALENT CATIONS AND CHELATORS ON dUTPase ACTIVITY

Compound	dUTPase Activity (nmol product)
None	0.47
$MgCl_2$	5.5
$MnCl_2$	4.2
$ZnSO_4$	7.2
$CaCl_2$	0.70
$CoCl_2$	0.31
EDTA	ND[a]
EGTA	0.71
o-Phenanthroline	0.55

Note. dUTP hydrolysis was determined using 100 ng enzyme and 10 m*M* of the indicated compound. Reactions were conducted at 37° for 10 min. Enzyme activity is expressed as reduction of dUTP bound to DE81 filters.

[a] ND, Not detectable.

The GST-F2L fusion protein hydrolyzed dUTP in a time dependent manner (Fig. 3). From these data, it was determined that the fusion protein had a specific activity of 1000 units/mg protein (one unit is defined as that which hydrolyzes 1 μmol dUTP per min). The turnover number for the enzyme was estimated to be about 43 molecules/min. As a control, GST was purified from bacteria harboring the expression vector with no DNA insert. GST devoid of any fusion did not exhibit detectable dUTPase activity. This indicates that the dUTPase activity of the purified GST-F2L fusion protein was due to the F2L gene product. The preparations also appeared not to contain any bacterial dUTPase activity.

The kinetic properties of the vaccinia dUTPase were determined from reaction rates at varying substrate

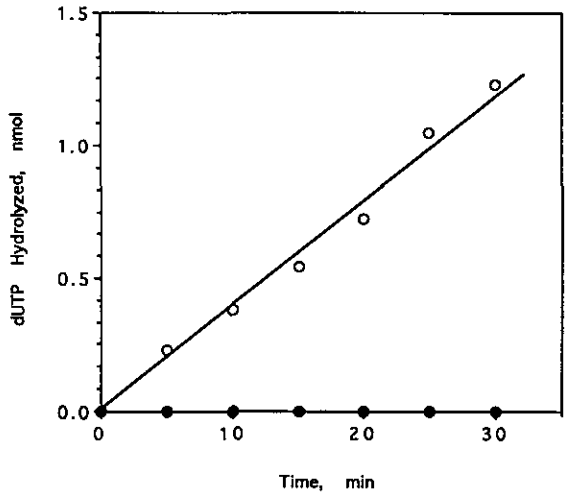

FIG. 3. dUTPase activity of the GST-F2L fusion protein. Forty ng of GST-F2L fusion protein (○) or GST alone (●) were incubated with 1 m*M* [^{3}H]-dUTP in 50 m*M* Tris–HCl, pH 7.5, 10 m*M* $MgCl_2$.

concentrations. Lineweaver-Burk analysis of the data indicated that the enzyme had a K_M of about 100 μM for dUTP (data not shown).

The substrate specificity of the vaccinia F2L gene product was examined by testing α[^{32}P]-labeled nucleoside triphosphates as substrates and analysis of the reaction products by TLC. Only dUTP was found to serve as an efficient substrate for hydrolysis. ATP, dATP, GTP, dGTP, CTP, dCTP, UTP, and TTP were not hydrolyzed to a detectable extent (data not shown).

The studies described here demonstrate that the vaccinia virus F2L gene product has dUTPase activity. This activity was predicted by the fact that the vaccinia virus F2L open reading frame encodes a protein with 30% identity to *Escherichia coli* dUTPase (*6*), 52% identical to a tomato dUTPase (*7*), and 63% identity to human dUTPase (*8*). dUTPases are apparently encoded by many viruses whose genome replicates via a DNA intermediate. In the herpes virus family, herpes simplex virus type I, Epstein–Barr virus, and varicella zoster virus encode a dUTPase (*14*). In addition, the nonprimate lentiviruses and type D retroviruses have been shown to encode a functional dUTPase (*10*). The results described here suggest that other members of the poxviruses also encode a dUTPase. Of all the dUTPases described to date, the vaccinia sequence by far most closely resembles the human dUTPase sequence. The high identity between the human and vaccinia dUTPases suggests that poxviruses acquired the dUTPase gene from a mammalian host's genome rather recently in their evolution.

dUTPases are believed to catalyze the hydrolysis of dUTP for two different purposes. First, they produce dUMP that is an intermediate in one pathway for the biosynthesis of TTP. dUTPases also are believed to hydrolyze dUTP as a protective mechanism to prevent the incorporation of dUTP into the DNA genome (*15*). Uracil residues in cellular DNA are targets for uracil N-glycosylase, and as a result, are mutagenic. Vaccinia virus has been shown to encode a functional uracil N-glycosylase that is essential for virus viability (*16*). By hydrolyzing dUTP to dUMP, the intracellular dUTP concentrations are kept to a minimum. Avoiding mutagenic events such as those caused by incorporation of uracil residues would seem to be particulary beneficial for poxviruses whose life-cycle is restricted to the cell cytoplasm, and hence cannot likely utilize the host's DNA repair capabilities. It is not yet known whether the vaccinia virus dUTPase indeed has a role in modulation of mutation. This, however, is eminently testable since the dUTPase gene is known to be dispensable for virus replication in culture (*17*).

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