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Characterization of Kaposi's Sarcoma-Associated Herpesvirus ORF11 as a Possible dUTPase

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CHARACTERIZATION OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS ORF11 AS A POSSIBLE dUTPASE

by

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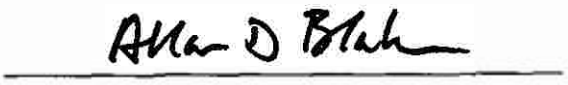

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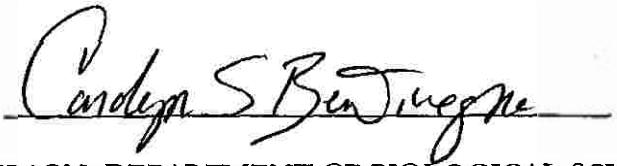

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ABSTRACT

A dUTPase is a crucial enzyme that hydrolyzes dUTP to dUMP. This reaction prevents the mutagenic or lethal misincorporation of uracil into DNA. For that reason, the enzyme is required for efficient DNA replication. Previous studies have shown that ORF11 has dUTPase-like motifs and thus may be a dUTPase. Generally, gammaherpesviruses contain six characteristic dUTPase motifs. In particular ORF11 contains motifs 1, 2, 4, and 6. While the characteristic motifs of gammaherpesviruses include motifs 1, 2, 3, 4, 5, and 6, the number of dUTPase-like motifs in ORF11's protein sequence is substantial. Thus, ORF11 may be a dUTPase. To further investigate this hypothesis, ORF11 was cloned, expressed in *E. coli*, and the protein was examined in a dUTPase assay. ORF11 was cloned into pGEX-5x-3 through a sequential process starting with a polymerase chain reaction (PCR) and ending with the isolation of plasmid DNA containing ORF11. The positive clones were sequenced and confirmed. Subsequently, ORF11 was expressed as a GST fusion protein in *E. coli*. Verification of expression was done by purifying the GST proteins using glutathione beads. The purified proteins were evaluated by SDS-PAGE. The SDS-PAGE demonstrated purification of proteins with their expected sizes. Finally, the lack of dUTPase activity was demonstrated by a dUTPase assay. Bacterial extracts expressing ORF11 were incubated with dUTP and the separation of nucleotides was evaluated using thin layer chromatography. In summary, our results demonstrate that Kaposi's sarcoma-associated herpesvirus ORF11 does not code for a functional dUTPase. Further studies are needed to determine the function of the protein.

INTRODUCTION

Kaposi's sarcoma, also known as KS, was identified in 1872 by Moritz Kaposi, a Hungarian dermatologist. He described the disease as an angiomatous neoplasm that affected men of Italian, Jewish or Mediterranean descent (Dourmishev *et al.*, 2003). Though the disease's characteristics were identified during this time not much was known about the causitive agent. It was not until 1994, when scientists from the Department of Pathology in Columbia University isolated fragments of a genome from the lesion of a Kaposi's sarcoma patient with AIDS. The scientists found that the sequences were homologous to several genes of the *Gammaherpesvirinae* sub-family (Chang *et al.*, 1994). The isolated genome was then completely sequenced in 1996 (Russo *et al.*, 1996). Kaposi's sarcoma-associated herpesvirus (KSHV), also known as humanherpesvirus 8 (HHV-8), was found to be the causative agent of Kaposi's sarcoma (Ambroziak *et al.*, 1995). Although scientists have advanced significantly since 1872 in identification of the virus and several of its mechanisms, there are still several unknown factors of the virus. These unknown factors include understanding latency, transmission, specific interaction with HIV, sex preference, and unknown functions of a number of viral genes.

KSHV belongs to the *Gammaherpesvirinae* sub-family and is the first known human Rhadinovirus (Knipe & Howley, 2007). Also, in the same sub-family is Epstein-Barr virus (EBV), which was found to be homologous to KSHV. KSHV is the causative agent of primary effusion lymphoma (Cesarman *et al.*, 1995), some instances of multicentric Castleman's disease (Soulier *et al.*, 1995), and Kaposi's sarcoma(Chang *et al.*, 1994). In the United States, the virus infects less than 5% of the population. In the Mediterranean and Europe, it infects 5%-20% and in Africa it infects greater than 50% of the population (Knipe & Howley, 2007). In the US, KSHV is mostly seen in patients whose immune system has been compromised, such as AIDS patients. However, not much is known about the correlation between HIV and KSHV.

KSHV's gene expression and replication is similar to that of EBV (Knipe & Howley, 2007). However, they differ greatly in their latency programs. The gene expression of the herpesviruses involves latent, immediate-early, early, and late gene transcription. Immediate-early genes code for transcription factors and play an important role in initiating lytic gene expression leading to viral replication. The early genes are involved with DNA replication and the late genes are involved with packaging the virus (Lacoste *et al.*, 2004). KSHV has a genome about 165 kbp long. Its genome codes for several cellular homologous proteins. These include IL-6, bcl-2, DNA polymerase, G-protein coupled receptor, uracil-DNA glycosylase (UNG), and dUTPase (Russo *et al.*, 1996).

One essential protein of the virus is a dUTPase. A dUTPase is an enzyme that prevents the misincorporation of uracil into DNA. It does this by maintaining a low ratio

of dUTP/dTTP (Chen *et al.*, 2002). Uracil can be incorporated into DNA by misincorporation of dUTP or by the deamination of cytosine. dUTPases exist in several herpesviruses. However, it is not clear why the virus needs this enzyme if the cell already contains it. The importance of dUTPases may be due to the ability of herpesviruses to replicate in non-dividing cells. The lack of the dUTPase gene in lentiviruses has been shown to show a defect in the replication of non-dividing cells (Turelli *et al.*, 1997). In another gammaherpesvirus, EBV, the dUTPase of the virus was shown to be essential in non-dividing cells as well (Fleischmann *et al.*, 2002). Therefore, dUTPases are required for efficient DNA replication in several herpesviruses.

Due to the importance of dUTPases in herpesviruses, it has been suggested that dUTPases may be a potential target for chemotherapy (Studebaker *et al.*, 2001). Currently, there are a few drugs which are used to target herpesviruses. These include acyclovir, famciclovir, ganciclovir, idoxuridine, valacyclovir, trifluorothymidine, and foscarnet. Specifically, ganciclovir and foscarnet are used to treat patients with KSHV (Porter, 2006). These drugs act on the DNA polymerase of the virus and inhibit DNA replication (Studebaker *et al.*, 2001). Consequently, drug resistance is becoming a problem with these drugs and new targets must be developed (Studebaker *et al.*, 2001).

Gammaherpesviruses have six characteristic dUTPase motifs. These motifs are termed 1, 2, 3, 4, 5 and 6. On the other hand, human dUTPases contain the characteristic motifs 1-5 and exist as trimers as opposed to herpesvirus dUTPases which exist as monomers (Tarbouriech *et al.*, 2005). The protein structure differences between human and herpesvirus dUTPases coincide with the gene length of both. Generally, the gene that

codes for dUTPases in herpesviruses is approximately double the size of the human gene (McGeehan *et al.*, 2001). Specifically, human, *E. coli*, and *S. cerevisiae* dUTPases are 141, 150, and 147 residues long, respectively, and exist as monomers (McIntosh *et al.*, 1992). On the contrary, herpesvirus dUTPases range from 318 residues to 388 residues in length (Kremmer *et al.*, 1999; McGeehan *et al.*, 2001). Although both enzymes differ greatly, they both have the same function.

There is one known functional dUTPase that is coded by KSHV, GRF54 (Kremmer *et al.*, 1999). However, KSHV GRF11 has been suggested to be a putative dUTPase (Davidson *et al.*, 2005) because it contains gammaherepesvirus dUTPase-like motifs 1, 2, 4 and 6. In order to further investigate this hypothesis, ORF11 was cloned, expressed as a GST fusion protein, and assayed for dUTPase activity.

GRF11 was cloned into pGEX-5x-3 and expressed in *E.coli* BL21 cells. The expressed GST fusion proteins were purified for verification. Finally, the protein extracts were assayed for dUTPase activity and visualized by thin layer chromatography. Our findings show that GRF11 does not code for a functional dUTPase and further studies are needed to determine the function of GRF11.

MATERIALS AND METHODS

Sequence Alignment

KSHV ORF11 and ORF54 were aligned using ClustalW2 (http://www.ebi.ac.uk/clustalw2/). The accession numbers used for the alignments were AAC57088 for ORF11 and AAB62631 for ORF54. They were manually aligned and characteristic dUTPase motifs 1, 2, 6 and 4 were designated according to Davidson *et al.* (2005).

Cloning KSHV ORF11

KSHV ORF11 was cloned into pGEX-5x-3 (GE Healthcare), a GST vector, for protein expression and characterization studies. ORF11 was amplified via the polymerase chain reaction using BCBL-1 genomic DNA as the template. Twenty five cycles of the following conditions were used to amplify the gene: 94°C, 2 m; 94°C, 30 s; 54°C, 30 s; 68°C, 2 m. The primer set used is: Forward-AAGGATCCAAGCGCAGGAGTCAGAGCAG and reverse-AAGAATTCCTAACTGCGTCCGGTGGC. The final reaction volume was 50 uL in a 1X reaction buffer which consisted of 1.75 mM $MgCl_2$, 50 ng BCBL-1 template DNA, 25 pmol of each primer, and Fideli Taq polymerase (USB).

Following PCR, the product was gel purified using a QIAquick Gel Extraction Kit (Qiagen). The purified product and the pGEX vector were singly digested with Bam HI (Promega) and Eco RI (Promega) respectively. Additionally, the vector was treated with Thermosensitive Alkaline Phosphatase (Promega). All of the digests were heat inactivated and purified using Charge Switch Kit (Invitrogen) before ligation. The ligation reaction volume was 20 uL in a 1X Ligase Buffer Mix containing T4 DNA Ligase (Promega), ORF1 1, and pGEX purified digest products. Another ligation reaction without ORF1 1 was done as the control. The reaction was incubated overnight at 4°C. The ligation was then transformed into *Escherichia coli* (*E. coli*) DH5α cells. The transformation is described in further detail in the next section. Ten colonies from the transformation were grown in Luria Broth and ampicillin (100 ug/uL) overnight at 37°C. Plasmid DNA was isolated using Quantum Prep Plasmid Mini-prep Kit (Bio Rad). The isolated DNA was digested using the previously stated restriction enzymes to verify the presence of an insert. The presumed positive clones were sent to be sequenced at Mclab (San Francisco, CA).

Transformation

E. coli DH5α and *E. coli* BL21 (DE3) pLysS competent cells were made according to Sambrook and Russell (2000). The competent cells were gently mixed and aliquoted at 50 uL in each tube. Two aliquots of competent cells were used for the transformation. Frozen *E. coli* DH5α competent cells were thawed on ice. Then, 5 uL of the ligation reactions were added to the aliquots. The ligations were transformed into *E*

coli DH5α cells. As a control, 1 uL of the parental plasmid was added to an aliquot as well. The cells were mixed and incubated on ice for thirty minutes. Next, the cells were incubated at 42°C for ninety seconds and rested on ice for two minutes. Afterward, 950 uL of room temperature Luria Broth media was added to each tube and cells were incubated at 37°C for one hour in a water bath. The cells were then plated on Luria Broth, agar and ampicillin (100 ug/uL) plates and incubated overnight at 37°C.

Expression and Purification of KSHV ORF11

The isolated pGEX-ORF11 DNA and pGEX-NdUTPase (human dUTPase) were transformed into *E. coli* BL21 (DE3) pLysS expression cells using the protocol described in the transformation section. pGEX-NdUTPase was graciously provided by Dr. Patrizia Caposio and colleagues from the Department of Public Health and Microbiology at the University of Torino, Italy (*Caposio et al.*, 2004). This plasmid contains the human nuclear form dUTPase and was used as a positive control.

Following transformation, the colonies were picked and grown overnight at 28°C in 10 mL Luria Broth, ampicillin (100 ug/uL), and 0.5% glucose. Then, 9 mL of overnight cultures were inoculated into 95 mL of Luria Broth, 5 mL of glucose (0.46%), and ampicillin (100ug/uL) and grown at 28°C. The cultures were grown to an optical density of 0.5 and induced with 0.5 mM IPTG for one hour. The 100 mL was separated into two aliquots of 50 mL; one was used in protein purification and the other was used in the dUTPase assay. The cells were harvested at 4°C, 10,000 r.p.m. for ten minutes. The cells were lysed in 3 mL of buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.01%

Igepal). The cells were sonicated three times for fifteen seconds with a ten second rest on ice in between. Samples were spun down at 4°C, 10,000 r.p.m. for fifteen minutes. The supernatant was collected in 3 mL aliquots. Next, 100 uL of glutathione beads (Sigma) at a 30% (w/v) slurry were added to the aliquots and rotated overnight at 4°C. The beads were washed three times at 4°C for five minutes with 600 mM NaCl HEPES buffer (20 mM HEPES, 600 mM NaCl, 5 mM $MgCl_2$, 0.01% Igepal). Afterward, the beads were rinsed once with 100 mM HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Igepal). Finally, the beads were resuspended in 150 uL of 100 mM NaCl HEPES buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Igepal). The proteins bound to the glutathione beads were run on a 10% SDS-PAGE gel. The gel was stained with Brilliant blue (Sigma) and visualized under white light.

dUTPase Assay

dUTPase activity was determined using the protocol described by Kremmer *et al.* (1999). First, 50 mL aliquots of the previous bacterial cultures were harvested 1 hour after induction of protein expression by centrifugation at 10,000 r.p.m. for ten minutes. The pellets were resuspended in 1 mL buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Igepal) and cells were lysed by a single fifteen second sonication.

dUTPase activity was determined by incubating 15 uL of *E. coli* cell extract, containing 1.5 ug/uL of protein, added to 10 uL of a 10 mM dUTP (Sigma) solution (dUTP in 20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Igepal) at 37°C for thirty minutes and one hour. The reaction was terminated by adding 7 uL of 0.2 M EDTA.

Thin Layer Chromatography

First, 16 uL of each sample was loaded onto a polyethyleneimine cellulose plate with fluorescent indicator (Sigma-Aldrich). The following were loaded on the PEI plate: 10 mM dUMP (Sigma), 10 mM dUTP (Sigma), a mixture of 10 mM dUMP and 10 mM dUTP, the parental vector, and the vector containing the human dUTPase form. The nucleotide phosphates generated by the dUTPase assay were separated by ascending chromatography in a buffer (0.25 M KH_2PO_4, pH 3.5). The PEI plate was visualized with 254nm of UV light and photographed in Gel-Doc-It system.

RESULTS

Detection of motifs in KSHV ORF11 and ORF54

There are two classes of dUTPases: Class 1, which includes bacteria, fungi, plants, metazoans, poxviruses, retroviruses, as well as invertebrate, fish, and amphibian herpesviruses and Class 2, which includes mammalian herpesviruses (McGeehan *et al.*, 2001). The two classes both contain motifs 1, 2, 3, 4, and 5. However, Class 1 motifs are ordered 1, 2, 3, 4, and 5 and Class 2 motifs are arranged as 3, 1, 2, 4, and 5. Also, Class 1 dUTPase proteins are approximately 150 amino acids long as opposed to Class 2 dUTPase proteins which are about twice as long (McGeehan *et al.*, 2001).

In particular, gammaherpesvirus dUTPases contain the conserved motifs 1, 2, 4, and 5 in the C-terminal half of the protein and motif 3 in the N-terminal half (McGeehan *et al.*, 2001). A proposed model has shown that motif 3 in the N-terminal half of the protein is replaced by motif 6; although, motif 3 is still present in the functional protein. (McGeehan *et al.*, 2001). Thus, if ORF11 is a dUTPase it should contain these dUTPase-like motifs.

In order to determine whether ORF11 contained dUTPase-like motifs, ORF11 and ORF54 (known KSHV dUTPase; Kremmer *et al.*, 1999) were aligned using ClustalW2.

The proteins were then manually aligned in accordance with Davidson and Stow (2005). ORF11 and ORF54 differ in length and the sequences needed to be manually aligned to show motif similarities. The sequence alignments of KSHV ORF11 and ORF54 showed similarity in motifs 1, 2, 4, and 6. Both ORF11 and ORF54 contain motifs 1, 2, 4 and 6. However, ORF54 also contains motifs 3 and 5 (Davidson & Stow, 2005). The alignment demonstrates that ORF11 contains a significant amount of dUTPase-like motifs. Thus, ORF11 may be a dUTPase. Figure 1A illustrates the sequence alignment between ORF11 and ORF54. In contrast, the length of each protein sequence differs substantially. ORF11 contains407 amino acids and ORF54 318 amino acids. This suggests ORF11 may have a different function which utilizes the additional amino acids. As stated previously, the herpesvirus dUTPases (Class 2) are about twice as long as human dUTPases (McGeehan *et al.*, 2001).

The highlighted regions show similar chemical structure between amino acids. Figure 1B takes a closer look at the carboxy terminus of each protein. The manual alignment illustrated the similarity of dUTPase motifs 1, 2, 4 and 6 between ORF11 and ORF54. These results suggest ORF11 may be a functional dUTPase.

Cloning of KSHV ORF11 into pGEX-5x-3 gives rise to five positive clones

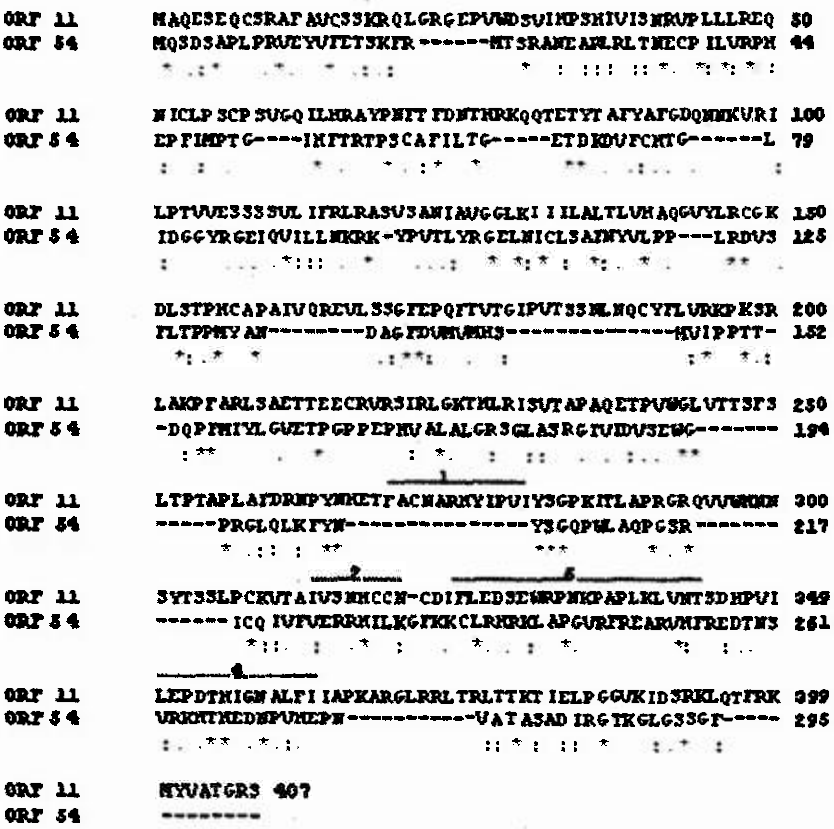
To determine the functionality of ORF11, the gene was first cloned and sequenced. The gene was amplified using PCR and cloned into pGEX-5x-3. PCR was performed using a pair of specific oligonucleotide primers and BCBL-1 genomic DNA which contains the genome KSHV. Figure 2A demonstrates the ability of the specific primer pair to amplify a gene with the expected size of 1.2 kbp. The gene amplified is

consistent with the length of ORF11's nucleotide sequence (*Russo et al.*, 1996). After PCR amplification, the gene was gel purified to prevent non-specific bands from interfering with future reactions. Figure 2B demonstrates DNA purification resulting in specific and intense bands for ORF11 (1.2 kbp). Gel purified ORF11 and pGEX were digested sequentially with Bam HI and Eco RI. In between each digest the DNA was purified.

Figure 2C demonstrates DNA purification resulting in specific and intense bands for ORF11 (1.2 kbp) and pGEX (5.0 kbp) doubly digested with Bam HI and Eco RI. Following digestion and purification, ORF11 and pGEX were ligated. The ligated DNA was transformed into *E. coli*. The DNA from the resulting colonies was isolated and tested with a double digestion using Bam HI and Eco RI, respectively. Figure 2D demonstrates five positive clones, pGEX-ORF11 clones 2, 3, 4, 6 and 7. In lanes 3,4,5,7 and 8 expected band sizes of 5.0 kbp (pGEX) and 1.2 kbp (ORF11) were observed.

The clones were sent for sequencing to Mclab (San Francisco, CA). The results showed clones 2 and 7 had 99% similarity and clones 3, 4, and 6 had 98% similarity to KSHV ORF11's known nucleotide sequence (data not shown). The remaining 1% and 2% regions respectively, are attributed to GC rich regions that were unable to be detected. Due to these results, clone 2 was used for further analysis.

A.



B.

Figure 1. *Sequence analysis of KSHV ORF11 and ORF 54.* Sequence analysis of KSHV ORF 11 (putative dUTPase) and ORF 54 (dUTPase) was performed using ClustalW2. A manual sequence alignment was done to designate dUTPase-like motifs in ORF11. The motifs are designated within the boxes. Key: * -nucleotides are identical in all sequences in the alignment, : -conserved substitutions have been observed, and . -semi-conserved substitutions are observed. The amino acids in red (AVFPMILW) are small and hydrophobic, in blue (DE) are acidic, in magenta (RK) are basic, in green (STYHCNGQ) are hydroxyl + amine + basic - Q, and in gray indicate other.

Expression and purification of KSHV ORF11

Positive protein expression and purification was imperative to demonstrate before continuing to the dUTPase assay. ORF11 was transformed into *E. coli* strain BL21 and purified using glutathione beads. The resulting OST fusion proteins were separated on a 10% SDS-PAOE gel. Figure 3 illustrates the expression and isolation of GST and GST fusion proteins: GST, GST-NdUTPase, and OST-ORF11, respectively. The GST protein, expressed from the pGEX-5x-3 plasmid, was shown to be approximately 26kDa (lane 2). The GST-NdUTPase isolated protein was found to be approximately 48 kDa (lane 3) (Caposio *et al.*, 2004). Finally, the GST-ORF11 protein was found to be approximately 72 kDa (lane 4). The combination of OST (26 kDa) and ORF11 (46 kDa) gave the expected size of 72 kDa. Noted in the 10% SDS-PAGE gel are a few non-specific bands. These bands are likely the result of co-purification of *E. coli* proteins. However, expression of all three proteins was confirmed.

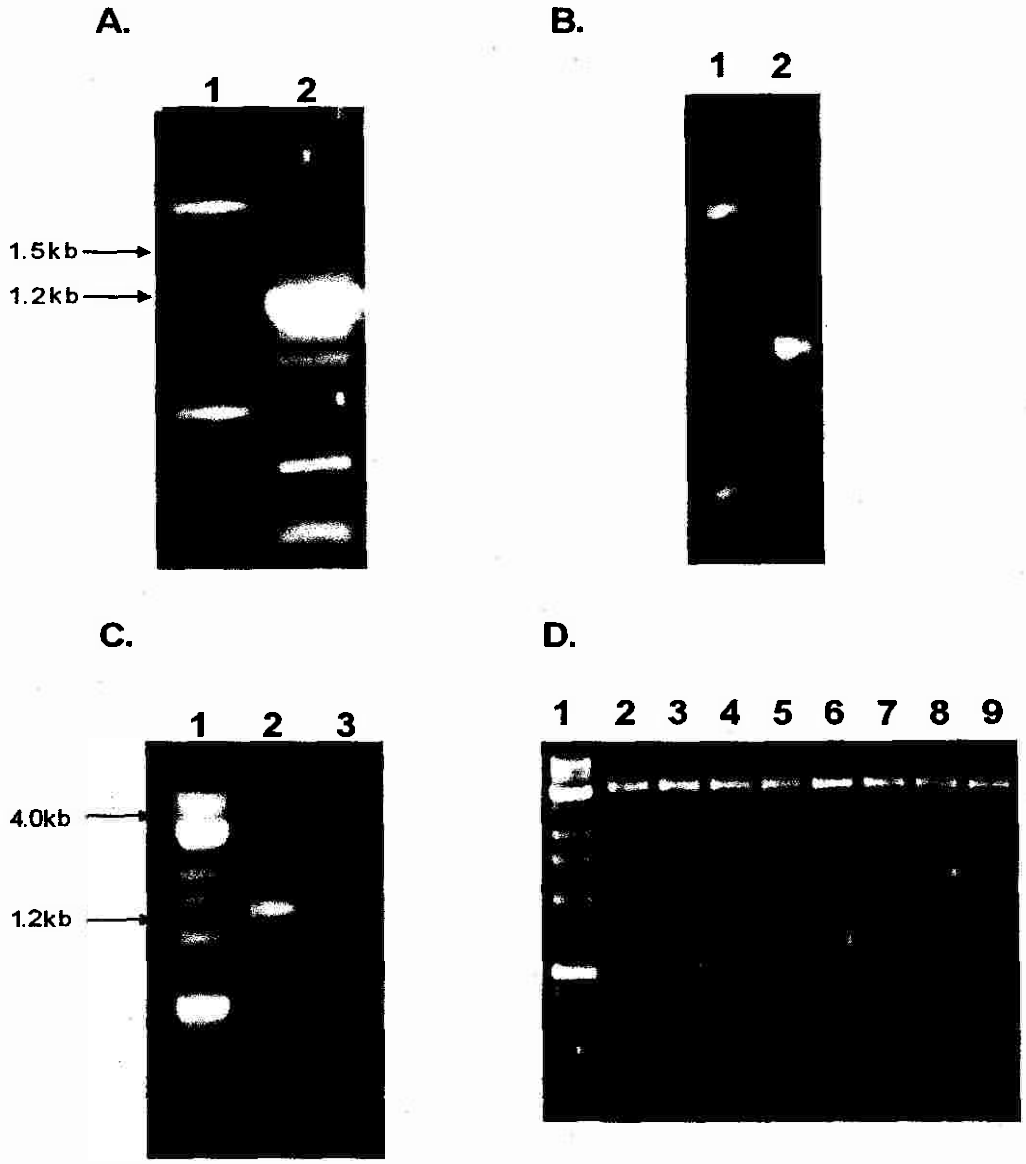


Figure 2. *Cloning of KSHV ORF11.* **A.** PCR amplification using BCBL-1 genomic DNA and designated primers revealed several copies of a 1.2 kbp PCR product (ORF11) (lane 2). To the left is a 1 kb plus ladder (lane 1). ORF11 was cloned into a pGEX-5x-3. **B.** ORF11 was gel purified revealing a single band of 1.2 kbp (lane 2). **C.** ORF11 and pGEX were sequentially digested with the enzymes Bam HI and Eco RI. Following digestions both were purified. Intense bands are seen for ORF 11 (1.2 kbp) (lane 2) and pGEX (5.0 kbp) (lane 3). **D.** The purified digests were used in a ligation and transformed into *E. coli.* The colonies obtained were grown in Luria broth including ampicillin (100ug/uL). DNA was isolated and double digested with Bam HI and Eco RI (lanes 2-9).

KSHV ORF11 is not a functional dUTPase

After demonstrating the positive expression of KSHV ORF11 and human NdUTPase, dUTPase activity was assayed by conducting a dUTPase assay and thin layer chromatography. GST, ORF11, and human NdUTPase were expressed in *E.coli* BL21 cells. The *E. coli* cell extracts were combined with dUTP and reaction buffer and incubated at 37°C for 30 minutes. The reaction was stopped and spotted onto a polyethyleneimine cellulose plate. Thin layer chromatography was used to separate the nucleotides in a phosphate buffer. The plate was visualized under UV light and photographed. Figure 4 demonstrates the dUTP hydrolysis by OST, GST-NdUTPase, and GST-ORF11. *E. coli* extracts containing pGEX-5x-3 and pGST-NdUTPase served as negative and positive controls, respectively. The bacterial extracts expressing pGST demonstrated a low background level of dUTP hydrolysis due to endogenous *E. coli* dUTPase activity (lane 4) (Kremmer *et al.*, 1999). While bacterial extracts expressing pGST-NdUTPase demonstrated the complete conversion of dUTP to dUMP in 30 minutes (lane 5). In contrast, bacterial extracts expressing pGST-ORF 11 did not show the complete conversion of dUTP to dUMP and showed dUTP hydrolysis similar to the bacterial extracts expressing pGST (lane 6). Therefore, ORF11 is not a functional dUTPase.

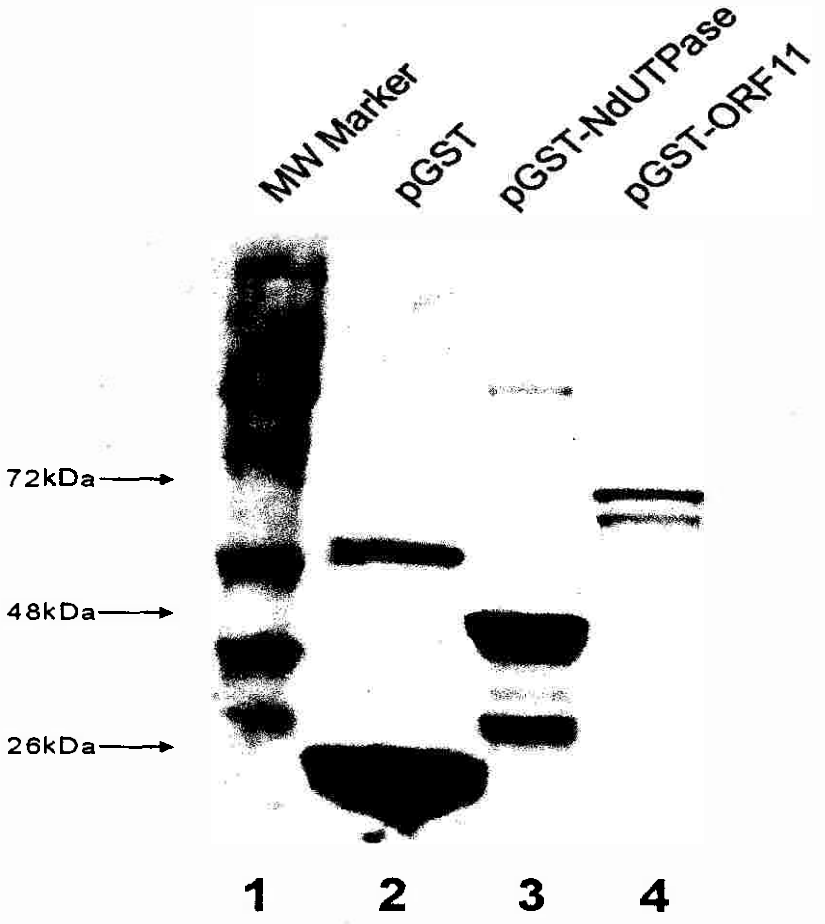


Figure 3. *Expression and Purification of KSHV ORF 11.* pGST (lane 2), pGST-NdUTPase (lane 3), and pGST-ORF 11 (lane 4) were expressed in *E. coli* BL21 (DE3) pLysS cells and purified using glutathione beads. The proteins and a rainbow molecular weight marker(lane 1, GE Healthcare) ladder were run on a 10 % SDS-PAGE gel and stained with Brilliant blue.

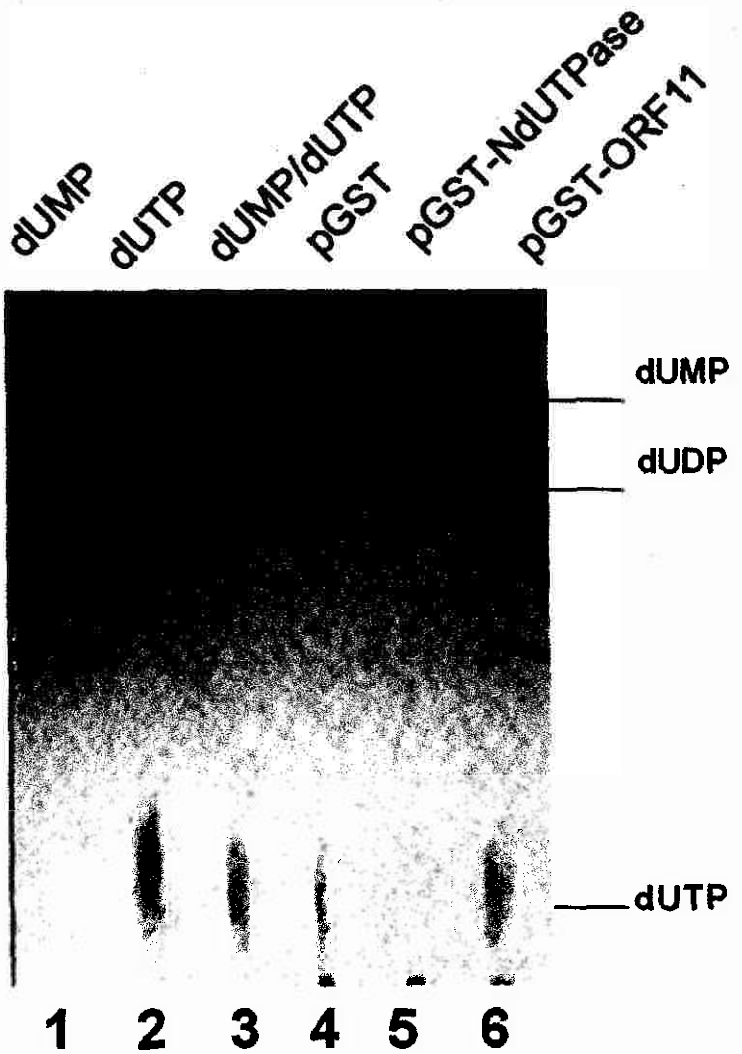


Figure 4. *KSHV ORF11 is a non-functional dUTPase. E. coli* protein extracts expressing pGST (lane 4), pGST-NdUTPase (lane 5), and pGST-ORF11 (lane 6) were incubated with 10 mM dUTP (Sigma) and the nucleotide phosphates generated were spotted on a PEI plate and separated by thin layer chromatography. Also spotted on the PEI plate were dUMP (lane1), dUTP (lane 2) and a mixture of both (lane 3) to be used in comparison of nucleotide separation. The PEI plate was visualized under 254 nm UV light.

DISCUSSION

Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease are three devastating diseases. They are caused by the gammaherpesvirus Kaposi's sarcoma-associated herpesvirus. Although the virus was recently discovered in 1994 and completely sequenced in 1996, there are several functions that are still unknown about many of the genes of the virus. As a result, there are not many therapeutic agents available for these lymphomas. Those that are available are nucleotide and non-nucleotide analogs. As previously mentioned, these include acyclovir, famciclovir, valacyclovir, ganciclovir, idoxuridine, trifluorothymidine, and foscarnet (Studebaker *et al.*, 2001). However, herpesviruses are becoming resistant to these particular drugs (Studebaker *et al.*, 2001). Hence, the importance of discovering new targets that can be used as chemotherapeutic agents. Potential targets to be used as chemotherapeutic agents in herpesviruses are dUTPases (Studebaker *et al.*, 2001).

A dUTPase is an enzyme which catalyzes the hydrolysis of dUTP to dUMP. This conversion is important in maintaining the ratio between dUTP and dTTP inside the cell. dUTP can be easily mistaken for dTTP by DNA polymerase and be misincorporated into the DNA of replicating genomes (Larsson *et al.*, 1996). Cells without the enzyme have been shown to have elevated recombination frequencies and abnormal mutation rates.

This ultimately leads to DNA fragments as intermediates in DNA metabolism and finally apoptosis (Larsson *et al.*, 1996). These effects demonstrate the importance of the enzyme in DNA replication. It also suggests that the enzyme may prove to be a significant chemotherapeutic target (Larsson *et al.*, 1996).

There are two different classes of dUTPases. The first, Class 1, is found in bacteria, plants, metazoans, and fungi. Also included in this class are poxviruses, retroviruses, adenoviruses, and invertebrate, fish and amphibian herpesviruses (McGeehan *et al.*, 2001). The second, Class 2, is found in mammalian herpesviruses (McGeehan *et al.*, 2001). Although both classes exhibit the same function, they differ greatly in protein sequence and structure.

Class 1 dUTPases are about 150 amino acids in length and contain five conserved motifs. These motifs are termed and ordered 1, 2, 3, 4, and 5 from the N-terminus to the C-terminus. Class 1 dUTPases are also active as trimers. In contrast, Class 2 dUTPases are approximately twice the size in length and contain six characteristic motifs. In particular, *Alpha-* and *Gammaherpesvirinae* contain the motifs termed and ordered 3, 1, 2, 4, and 5 from the N-terminus to the C-terminus. In addition, they also contain motif 6. The difference in length and motif order has been attributed to the genetic duplication of a standard dUTPase coding sequence and the later loss of one copy of each motif from the double-length chain (McGeehan *et al.*, 2001). Overall, a functional dUTPase should at least contain motifs 1-5 (McGeehan *et al.*, 2001).

In order to determine whether ORF11 contained dUTPase like motifs, ORF11 and ORF54 amino acid sequences were aligned using ClustalW2 (Figure 1A). The results of

the alignment (Figure 1B) showed that ORF11 contained dUTPase-like motifs 1, 2, 6, and 4. Still, it lacked conservation of motifs in the N-terminus. Also, the amino acid lengths between both genes differed by 89 amino acids. The ORF11 protein sequence contains 407 amino acids while the ORF54 contains 318 amino acids. However, the alignment showed a substantial number of dUTPase-like motifs; therefore ORF11 may function as a dUTPase.

Following the identification of dUTPase-like motifs in ORF11, the gene was positively cloned (Figure 2) and expressed in bacterial cells (Figure 3). Also positively expressed in bacterial cells, were GST and the human NdUTPase (Figure 3). Subsequently, the bacterial extracts of GST, GST-ORF11, and GST-NdUTPase were incubated with dUTP for 30 minutes and then spotted on a PEI plate in the dUTPase assay. The results of the dUTPase assay demonstrated that ORF11 is not a functional dUTPase (Figure 4), suggesting the protein has another function.

The dUTPase assay evaluated the enzyme activity of bacterial extracts which expressed GST, GST-NdUTPase, and GST-ORF11 respectively (Figure 4). The bacterial extracts containing the GST plasmid did not show a complete conversion from dUTP to dUMP. However, it showed endogenous *E. coli* dUTPase activity similarly observed by Kremmer *et al.* (1999). The GST-NdUTPase (positive control) showed a complete conversion from dUTP to dUMP. These results were similar to the dUTPase activity of the human dUTPase control and ORF54 demonstrated by Kremmer *et al.* (1999). Finally, the bacterial extracts containing GST-ORF11 did not show a complete conversion from dUTP to dUMP; though, it showed endogenous *E. coli* dUTPase activity due to the use of

bacterial extracts as oppose to purified protein.

In this experiment, we have clearly determined that ORF11 is not a functional dUTPase. As indicated previously, ORF11 lacked the conservation of motifs at the N-terminus of the protein (Davidson & Stow, 2005). This may explain the reason ORF11 did not demonstrate dUTPase activity. For that reason, its dUTPase-like motifs must serve an alternative function. Previous studies have shown ORF11 to be part of the tegument (Lu *et al.*, 2004) and most recently in (Rozen *et al.*, 2008).

Tegument proteins are encased in the region between the viral capsid and the viral envelope. The tegument contains a complex network of protein-protein interactions which includes capsid proteins, glycoproteins, and other viral and cellular proteins (Mettenleiter, 2002). These proteins are involved in assembly of the virion. According to Rozen *et al.* (2008), ORF11 was found to be a tegument protein. It was shown to interact with both ORF45 and ORF64 of the virus. ORF45 encodes an immediate-early protein and is expressed in the tegument (Zhu *et al.*, 2006). It interacts with interferon regulatory factor 7 and possibly plays a role in viral entrance and exit (Zhu *et al.*, 2006). ORF64 encodes a large tegument protein and may function as a hub protein which recruits other proteins such as ORF11 and ORF45 during virion assembly (Rozen *et al.*, 2008). ORF11's interaction with ORF45 and ORF64 may suggest it is expressed as an immediate-early gene in the lytic cycle and may play a role in virion assembly. In addition, ORF11 and ORF45 were both found to be expressed as primary lytic genes (Jenner *et al.*, 2001). This further suggests ORF11 may be expressed as an immediate-early gene. Still, it's interaction with these proteins does not offer insight into why

ORF11 contains dUTPase-like motifs.

ORF11 was just one of the proteins to be found to have dUTPase-like motifs. Another protein described to have dUTPase-like motifs was HCMV's UL84 (Davidson & Stow, 2005). Similar to ORF11, UL84 also lacks conservation of dUTPase motifs at the N-terminus of the protein. A study by Colletti *et al.* (2005) has shown that UL84 has homology to a family of helicases and demonstrates UTPase activity. However, a UTPase converts UTP to UDP yielding a phosphate. Thus, it is not enzymatically similar to a dUTPase (Davidson & Stow, 2005).

While we have clearly demonstrated that ORF11 is not a functional dUTPase future experiments are needed to determine a specific function. Possible future experiments include the determination of the expression stage of the protein during the lytic cycle, determining whether ORF11 has UTPase activity, and verification of specific protein interactions with ORF45 and ORF64. Determining the expression stage of ORF11 during the lytic cycle can offer insight as to what type of role in the virus life cycle the protein possesses. Immediate early proteins play a role in transcription regulation, early proteins play a role in DNA replication, and late proteins play a role in virion assembly (Knipe & Howley, 2007). Determination of UTPase activity would offer insight on the protein's putative role in DNA metabolism. Finally, verification of specific protein interactions with ORF45 and ORF64 will confirm ORF11's possible role in virion assembly.

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