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Human Herpesvirus 8 Gene Encodes a Functional Thymidylate Synthase

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We demonstrate that human herpesvirus 8, obtained from the lymphoma cell line BC-3 as well as from Kaposi's sarcoma lesions, carries a gene that encodes a functional thymidylate synthase (TS). The particular characteristics of this enzyme are studied and compared to the characteristics of TSs encoded by other organisms.

Human herpesvirus 8 (HHV-8) is a gammaherpesvirus that is related to herpesvirus saimiri (HVS) and rhesus rhadinovirus (RRV) (17). HHV-8 replication can be selectively inhibited by several antiherpesvirus agents, among which cidofovir proved to be the most effective (19, 6). The HHV-8 genome encodes several enzymes that are involved in nucleoside and nucleotide biosynthesis. These include a thymidine kinase, a ribonucleotide reductase, a dihydrofolate reductase, and a thymidylate synthase (TS) (21). The HHV-8-encoded thymidine kinase (3, 7, 16) and dihydrofolate reductase (5) have been shown to be functional.

TS (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) is an enzyme that catalyzes the reaction of 5,10-methylenetetrahydrofolate and dUMP to dTMP. TS is the only enzyme leading to the de novo synthesis of dTMP, dTDP, and dTTP. The enzyme is a key target for the action of antitumoral drugs (8). HVS and varicella-zoster virus (VZV) have been previously reported to encode a functional TS (9, 22). Human cytomegalovirus, a virus that belongs to the betaherpesvirus family, does not encode a TS but markedly stimulates cellular TS activity in infected human embryonic lung cells (18).

HHV-8 encodes a TS gene (21), and the protein was previously shown to be expressed in the cytoplasm of cells transfected with an expression vector carrying the HHV-8 TS gene, although no proof of functional enzymatic activity was given (5). The gene encoding the TS of HHV-8 is 1,014 bp long, that of HVS is 885 bp long, that of RRV is 1,002 bp long, and that of VZV is 906 bp long. These TSs are markedly shorter than the human counterpart, which is encoded by a gene of 1,533 bp. In Table 1 we present the alignment of the TS of the different herpesviruses with the human TS. This alignment reveals a high degree of amino acid sequence similarity, in particular for the region extending from amino acids 174 to 224 (Table 1). In the region containing the catalytic site, the HHV-8 TS and its human counterpart are 88% identical.

We cloned and expressed the TS gene of HHV-8 by using DNA isolated from the HHV-8-containing BC-3 cell line (ATCC CRL-2277), as well as from biopsies of Kaposi's sarcoma lesions obtained from a Belgian case and from a Hungarian case of classic Kaposi's sarcoma. In addition, the respective TS genes of HVS (C488 strain) (virus kindly provided by H. Fickenscher, University of Erlangen, Erlangen, Germany), VZV (OKA strain) (isolated from fibroblasts infected with the virus), and RRV DNA (DNA kindly provided by Scott Wong, Oregon Health Sciences University, Portland) were cloned and expressed. The forward primers used for PCR amplification of the TS genes contained an *Eco*RI site, and the reverse primers contained a *Sal*I site. The resulting amplicons were cloned in pCR4-TOPO TA vector (Invitrogen), and the cloned fragments were sequenced on both strands by using the Big Dye terminator cycle DNA sequencing kit (ABI PRISM, Applied Biosystems). The HVS, VZV, RRV, and BC-3 HHV-8 TS sequences proved to be 100% identical to the sequences available from the National Center for Biotechnology Information Blast GenBank. HHV-8 DNA from the Belgian patient carried an A-G substitution at nucleotide position 21047 and at nucleotide position 20824. HHV-8 DNA isolated from the Hungarian patient carried a C-T substitution at position 21012, an A-G substitution at position 20776, and a T-A substitution at position 20177 (positions refer to the numbering used in GenBank U75698). The TS genes were subcloned in the bacterial expression vector pGEX4. This vector allows the production of glutathione *S*-transferase fusion proteins (Amersham Biosciences). The resulting plasmids were then used to transform competent *Escherichia coli* BL21 cells. Cultures were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma) for 6 h at 25°C. Cells were pelleted at 10,000 × *g* for 10 min at 4°C and resuspended in BugBuster protein extraction reagent (Novagen). Benzonase nuclease (Novagen) was added for 20 min at room temperature. Clarified lysates were pelleted for 20 min at 15,000 × *g* at 4°C and incubated with glutathione Sepharose 4B (Amersham Biosciences). The recombinant enzymes were eluted with glutathione according to the manufacturer's instructions. The protein concentrations were determined, and the size of the protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TS activity was assessed by means of the tritium release assay according to a modified version of a method used earlier (1).

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TABLE 1. Alignment of part of the TS gene products from various herpesviruses, as well as from *E. coli* and humans, in the neighborhood of the catalytic site[a]

TS Source	Start position	Amino acid sequence	End position	% Identity with human TS
Human	174	DRRIIMCAWNPRDLPLMALP**PCH**ALCQFYVVNSELSCQLYQRSGDMGLGVP	224	
HHV-8	198	DRRIIMCAWNPADLSLMALP**PCH**LLCQFYVADGELSCQLYQRSGDMGLGVP	248	88
RRV	194	DRRIVMCAWNPADLARMALP**PCH**VLCQFYVARGELSCQLYQRSADMGLGVP	244	82
VZV	163	RRMIISSWNPKDIPLMVLP**PCH**TLCQFYVANGELSCQVYQRSGDMGLGVP	212	78
HVS	155	DRRMLMCAWNVSDIPKMVLP**PCH**VLSQFYVCDGKLSCQLYQRSADMGLGVP	205	72
E. coli	126	RRIIVSAWNVGELDKMALA**PCH**AFFQFYVADGKLSCQLYQRSCDVFLGLP	175	64

[a] The catalytic site, PCH, is marked in boldface type.

The appropriate amount of enzyme was incubated with 14.6 Ci of [5-^{3}H]dUMP (Amersham Pharmacia Biotech)/mmol in 50 mM Tris-HCl buffer (pH 7.5) containing 5.0 mM formaldehyde, 15 mM β-mercaptoethanol, 0.1 mM NaF, and (6*R,S*)-5,10-methylene-5,6,7,8-tetrahydrofolate (Schircks Laboratory). According to the particular experimental conditions, various concentrations of dUMP and 5,10-methylene-5,6,7,8-tetrahydrofolate were used. The enzymatic reactions were initiated, following a 2-min preincubation of the reaction mixture at 37°C, by addition of the enzyme and were then incubated in triplicate at 37°C for the appropriate time. The enzymatic reaction was stopped on ice, and 1 ml of active carbon (100 mg/ml in 2% trichloroacetic acid) was added to the tubes. Following a 2-min incubation period on ice, the carbon was pelleted by centrifugation at 3,000 rpm in a Hereaus Minifuge T for 10 min (4°C). Tritium release was measured by determining radioactivity in 200-μl fractions of the supernatant. Enzyme kinetics were calculated by using Graphpad Prism version 3.02 for Windows (GraphPad Software, San Diego, Calif.).

HHV-8 TSs, obtained from three sources as described above, i.e., HHV-8 isolated from the BC-3 lymphoma cell line as well as from two different patients with Kaposi's sarcoma, were expressed. Recombinant HHV-8 TSs obtained from these three sources all proved functionally active. Recombinant TSs that were derived from HVS as well as from VZV also exhibited, as expected, functional TS activity. We failed, however, to detect functional TS activity associated with the RRV TS even though alternative cloning strategies were used and a protein of the correct size was expressed. The question remains whether the RRV TS is indeed functionally active or whether the particular RRV strain that was used here encodes a defective TS.

We next studied and compared the particular kinetics of the TS of HHV-8, HVS, and VZV. K_m values, with dUMP and methylenetetrahydrofolate (CH_2H_4-folate) as a substrate, for the TSs encoded by HHV-8 (DNA obtained from the Kaposi's sarcoma lesion of a Belgian patient), HVS, and VZV are summarized in Table 2. K_m values of *E. coli* TS (enzyme kindly provided by Paola Costi, University of Modena, Modena, Italy) and of human TS (data obtained from the literature) are also listed. K_m values for both substrates (dUMP and CH_2H_4-folate) were very comparable to those of the three herpesvirus enzymes and were in the same range as the K_m values reported for human TS (14).

We then investigated the inhibitory effects of three well-known TS inhibitors, i.e., 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) (Sigma), 5-iodo-2′-deoxyuridine-5′-mono-phosphate (IdUMP) (Sigma), and 5-(2-bromovinyl)-2′-deoxyuridine-monophosphate (BVdUMP) (kindly provided by P. Herdewijn, Rega Institute, Leuven, Belgium), and of dTMP (Sigma) (which is a product inhibitor of TS) on the three viral enzymes. For all three dUMP analogues studied, the type of inhibition was competitive with respect to dUMP. The TS encoded by HHV-8, HVS, and VZV proved as susceptible to the compounds as the human, murine, and lactobacillus thymidylate synthases (Table 3). This makes it unlikely that HHV-8 is a specific target for antiviral or antitumoral therapy against HHV-8 or tumors associated with HHV-8.

Evidence that cellular TSs from diverse species influence the regulation of p53 expression by decreasing the translational efficiency of p53 mRNA has been provided (11). It is also assumed that inhibition of p53 by the HHV-8 latency-associated nuclear antigen may be involved in HHV-8-induced oncogenesis by suppression of cell death (12). It would be tempting to speculate that HHV-8 TS plays a role in HHV-8-induced oncogenesis. In such a case, the viral TS could be one of the many factors, including the viral G protein-coupled receptor, the viral interferon regulatory factor, viral D-type cyclin, viral interleukin-6, viral bcl-2, viral FLICE-inhibitory protein, laten-

TABLE 2. K_m values for dUMP and CH_2H_4-folate of TSs from different sources[a]

TS source	K_m dUMP (μM)	K_m CH_2H_4-folate (μM)	k_{cat} (s^{-1})	k_{cat}/K_m dUMP (s^{-1} μM^{-1})	k_{cat}/K_m CH_2H_4-folate (s^{-1} μM^{-1})
HHV-8 (Belgian patient)	1.72 ± 0.92	19.6 ± 7.9	4.6 ± 0.6	2.67	0.23
HVS (C488 strain)	2.38 ± 0.57	23 ± 8.5	3.8 ± 0.4	1.59	0.16
VZV (OKA strain)	1.92 ± 0.81	15.2 ± 3.8	3.5 ± 0.2	1.82	0.23
E. coli	3.54 ± 0.41				
Human[b]	1.8 ± 0.7	14 ± 5	3.9	2.2	0.28

[a] Rate constants for the viral TS are based on three independent determinations (K_m dUMP) or two independent experiments (K_m CH_2H_4-folate).
[b] From reference 14.

TABLE 3. Comparison of rates of inhibition of herpesvirus, human, murine, and *Lactobacillus casei* TSs by 5-substituted dUMP analogues and dTMP

Source of TS	K_i (K_1/K_m) with indicated compound			
	FdUMP (nM)	IdUMP (nM)	BVdUMP (nM)	dTMP (μM)
HHV-8 (Belgian patient)	24.5 ± 2.2 (0.014)	1,095 ± 79 (0.64)	3,953 ± 307 (2.3)	12.8 ± 1.1 (7.44)
HVS	25.6 ± 3.4 (0.010)	876 ± 74 (0.37)	3,641 ± 283 (1.53)	33.4 ± 2.3 (14)
VZV	26 ± 1.9 (0.013)	1,301 ± 48 (0.68)	2,099 ± 172 (1.09)	9.1 ± 0.2 (4.739)
Human	6.4[a] (0.0025)		4,500[b] (0.58)	9.6[c] (7.3)
Murine	18.2[d] (0.001)	6,016[d] (3.27)	1,214[e] (0.66)	7.3[c] (3.72)
L. casei	14[e] (0.0027)	1,600[e] (0.30)	2,000[f] (0.66)	22[g] (7.58)

[a] From reference 15.
[b] From reference 13.
[c] From reference 20.
[d] From reference 23.
[e] From reference 1.
[f] From reference 2.
[g] From reference 4.

cy-associated nuclear antigen, K1, and latency-associated membrane protein, that are associated with virus-induced oncogenesis (10).

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