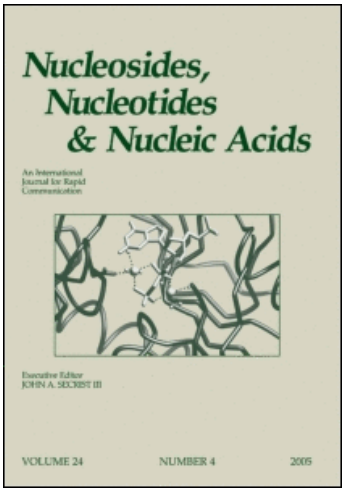

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Characterization of the Catalytic Subunit of the Human Herpesvirus 6 (HHV-6) DNA Polymerase Expressed in an In Vitro Transcription/Translation Assay

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INTRODUCTION

Human herpesvirus 6 (HHV-6) is closely related to human cytomegalovirus (HCMV). After primary infection at early age, the virus returns to a latent state (the overall seropositivity in adults is ~80%), from which it may be reactivated during episodes of immune suppression, thus causing disseminated infections of various organs.[1] To date, no antiviral therapy has been formally approved for the treatment of HHV-6 infections. Based on the experience with HCMV, the drugs most commonly used are ganciclovir (GCV), foscarnet and, to a lesser extent, cidofovir and acyclovir (ACV), all targeting the viral DNA polymerase. All (except for ACV) produce major adverse effects; moreover, long-term use may in some instances give rise to drug-resistant virus strains.

The HHV-6 DNA polymerase (DNA pol) exists as a heterodimer consisting of a catalytic subunit (encoded by the U38 gene of HHV-6) and a processivity factor (encoded by the U27 gene), which allows the synthesis of extended stretches of DNA without dissociation from the DNA template. Bapat et al.[2] have reported

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the characterization of the HHV-6 DNA polymerase, obtained from infected cell cultures by chromatographic purification. We here report the development of a eukaryotic in vitro transcription/translation assay for the production of the catalytic subunit of the enzyme.

METHODS

Plasmids

The plasmid containing the HCMV UL54 gene (encoding the catalytic subunit of the HCMV DNA pol) was kindly provided by Dr. T. Cihlar. The HHV-6B U38 gene, preceded by the truncated alfalfa mosaic virus 5′ UTR, was cloned into the pGem3Z vector (Promega) under control of the SP6 promoter according to published procedures.[3]

In Vitro Expression

In vitro expression was carried out using Promega's TNT SP6 Quick coupled transcription/translation rabbit reticulocyte kit, according to the Manufacturer's instructions. Apart from a master mix containing the SP6 RNA polymerase and a mix of NTPs, amino acids and cellular components, the reaction mixture contained 10 mM potassium acetate, 0.25 mM $MgCl_2$ and 10 ng/μL of the appropriate plasmid.

Enzyme Characterization

Approximately 0.4 units of enzyme (one unit was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol [^{3}H]dGTP into acid-insoluble material in 30 min at 37°C) were incubated for 20, 40 and 60 min at 37°C with a reaction mixture that was optimized previously and consisted of 25 mM Tris.HCl (pH 8.00), 100 mM $(NH_4)_2SO_4$, 0.5 mM DTT, 10 mM $MgCl_2$, 0.2 mg/mL BSA, 5% glycerol, 100 ng/μL activated calf thymus DNA and 100 μM of each unlabeled dNTP. Tritium-labaled dGTP, dCTP or dTTP were added at 0.25–2 μM (specific activity: $\leq$40 Ci/mmol) to determine K_m values. Incorporation of radiolabeled nucleotides was then determined by precipitation of nucleic acids on filters and scintillation counting.

Evaluation of Inhibitors

Enzyme (0.4 U) was incubated for 40 min at 37°C with the reaction mixture described above, containing the radiolabeled nucleotide (at a concentration approximately twice its K_m value) and various concentrations of the test compounds.

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RESULTS AND DISCUSSION

Compared to the traditional isolation of viral DNA polymerases from infected cell cultures, the assay described here represents a fast and straightforward approach for the evaluation of new (and existing) compounds for their HHV-6 DNA polymerase-inhibiting properties. Moreover, the assay can easily be extended to mutant forms of the enzyme, reported to confer resistance to nucleoside analogs such as GCV. The A961V mutant, the only GCV-resistant form of the HHV-6 DNA polymerase described to date,[4] was generated by site-directed mutagenesis in our laboratory and will be included in future enzyme studies.

K_m values were determined for the HHV-6 DNA pol and were 0.3 μM, 1.3 μM and 1.2 μM for dGTP, dCTP and dTTP, respectively. This is in good accordance with the values published earlier for the native (i.e., cell extract-derived and HPLC-purified) HHV-6 DNA.

Enzyme activity was significantly inhibited by foscarnet. The IC_{50} values for foscarnet were 0.98 ± 0.04 μM and 6.6 ± 0.77 μM for the HHV-6 and HCMV DNA polymerase, respectively. For the HCMV DNA polymerase, this value corresponds well the one obtained using the native enzyme (0.45 μM).[3]

In conclusion, the assay presented here provides a reproducible and timesaving cell-free enzyme system that can be used for the screening of novel antiviral compounds targeted at the HHV-6 DNA polymerase.

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