ENZYMES OF HUMAN HERPESVIRUSES

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The properties of human herpesvirus-encoded enzymes are reviewed and the importance of sequence analysis of viral genomes as well as the experiments on characteristics of enzymes isolated from infected cell cultures are emphasized. The following enzymes are described in detail: DNA replication complex consisting of DNA polymerase, DNA helicase-primase, single-stranded DNA binding protein and origin binding protein, further thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase as well as uracil-DNA-glycosylase, deoxyribonuclease and protein kinase. The importance of these enzymes from the point of view of antiviral chemotherapy is discussed.

1. INTRODUCTION

Much effort has been devoted to develop effective drugs against human herpesvirus infection. However, of different compounds exhibiting an antiviral activity in tissue culture or in experimental animals only few drugs have been introduced into clinical use[1]. The use of acyclovir (ACV) [9-(2-hydroxyethoxymethyl)guanine] (ref.[2]), probably the most often applied antiherpeticum, was undoubtedly a considerable progress in the treatment of several viral diseases[3,4]. At present it is clear that none of the drugs currently used, including the acyclic analogues of nucleotides, recently designed antiherpetics[5-8], are ideal chemotherapeutics which could interfere with viral replication at specific sites without adverse side-effects.

The identification of specific targets of antiviral drugs is possible on the one hand by studying their particular effects at the molecular level, and on the other by investigating the viral multiplication in general. A considerable progress has been recently achieved by a complete sequence analysis of different human herpesvirus genomes and the identification of genes functions. This access has resulted in a new insight into the set-up of herpesvirus-encoded proteins which may be helpful for a rational design of antiviral drugs taking in account the sterical, structural and electrostatic requirements of their active sites, their interaction and regulatory mechanisms.

Figure 1 illustrates a simplified view of herpes simplex virus replication and the numbers indicate virus-specific processes whose modulation by a suitable chemotherapeutic agent could interrupt the development of viral infection[19].

The most efficient antiviral drugs, analogues of nucleosides and nucleotides, probably act upon the synthesis of DNA precursors which is catalyzed both by viral and cellular enzymes, and also during the process of viral genome replication. Therefore, with regard to specificity of an antiviral agent, it is fundamental to know which enzymes are virus-encoded, whether they share any properties within the group of human herpesviruses and how they differ from their cellular counterparts.

The present contribution is intended to review the properties of enzymes encoded by human herpesviruses. It comprises both gene sequence analysis data as well as the information on isolation and characterization of enzyme activities from infected cells. Much attention has been devoted to enzymes encoded by herpes simplex viruses. The

last part of the present review summarizes the characteristics of less well defined enzymes of other human herpesviruses.

2. SHORT CHARACTERIZATION OF HUMAN HERPESVIRUSES

At present we distinguish 6 types of herpesviruses which infect humans and which are classified according to their biological and pathological properties in three subfamilies (Table I) (ref.[9]).

Five members of human herpesvirus group (HSV-1, HSV-2, VZV, HCMV, EBV) are described more in detail[9 - 12]; less information is available on human herpesvirus-6, discovered only in 1986 (ref.[13]), which was originally called human B-lymphotropic virus (HBLV) (ref.[14]) and was classified among *gammaherpesvirinae*[15]. Recent data, however, cast certain doubt on this classification since the genomic organization of HBLV is much closer to cytomegalovirus than to EBV or other human herpesviruses[16].

Human herpesviruses can generally be described as particles with linear double-stranded DNA genome of more than 120 000 nucleotide base pairs (bp) which is replicated in nuclei of infected cells. The genome is localized in an icosahedral

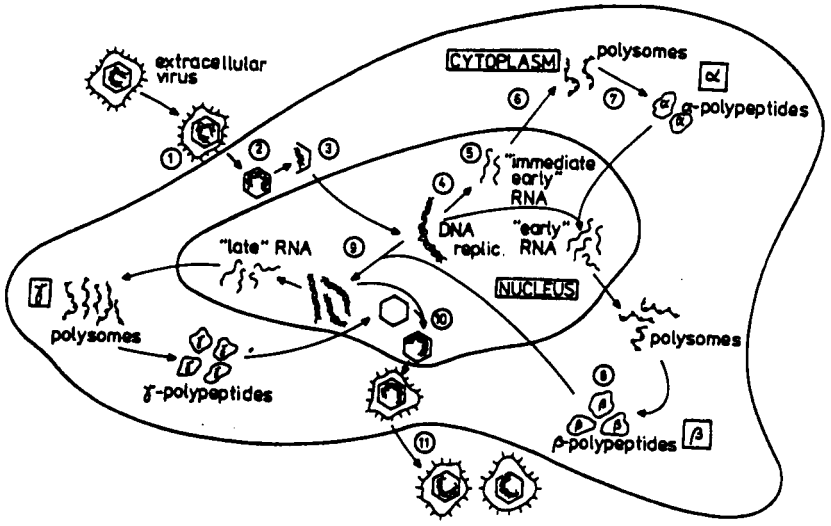

FIG. 1

Replication of herpes simplex virus (modified according to Darby and Field[18]): 1 adsorption of the virus to the cell surface; 2 transport of the virus across the cell membrane and uncoating; 3 release of the viral genome into the cytoplasm and its transport into the nucleus; 4 transcription; 5 post-transcriptional modification of RNA; 6 translocation of nuclear RNA into cytoplasm; 7 protein synthesis and processing; 8 virus-encoded enzymes; 9 replication of the viral genome; 10 assembly of macromolecules into the viral envelope; 11 release of virion from the cell

nucleocapsid with a diameter of more than 100 nm and surrounded by amorphous protein tegument and lipidic envelope originating in cell membrane and possessing virus-specific glycoproteins. This formation represents the final, extracellular infectious virus species[17].

3. SEQUENCE ANALYSIS OF HUMAN HERPESVIRUS GENOMES AS AN INITIAL STEP FOR THE IDENTIFICATION OF VIRUS-ENCODED PROTEINS

The main structural features of human herpesvirus genomes[20] are given in Fig. 2. Each of them is composed of unique (U) and repetitive (R) sequences.

Complete nucleotide sequence was hitherto published for HSV-1 genome, separately for the region U_L (ref.[21]), U_S (ref.[22]), R_L (ref.[23]), R_S (ref.[24]), furthermore, complete nucleotide sequences were also reported for VZV (ref.[25]) and EBV (ref.[26]). It seems that the organization of the HSV-2 genome is indistinguishable from that of HSV-1; the coding sequences are 70 – 80% identical[20]. Partial sequence is also known for HCMV genome; the sequences TR_S, U_S, IR_S, incomplete IR_L (ref.[27]) and the region including about 20 000 bp from U_L, which contains seven genes including DNA polymerase[28], are also known. Complete nucleotide sequence of HCMV has been published quite recently[29]. Recently part of HHV-6 genome has been sequenced[16]. The summary of data characterizing individual genomes is shown in Table II.

Theoretically, the nucleotide sequence can supply the information on the number of virus-encoded proteins. It should follow from the computer-assisted identification of open reading frames by means of localization of theoretically possible transcription promotors, transcription polyadenylated signals, signals for translation initiation and

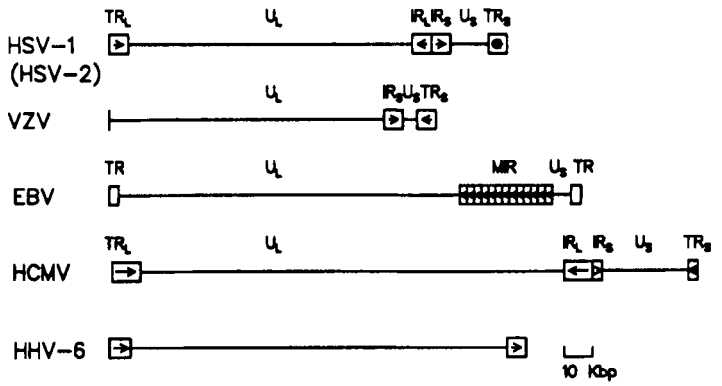

FIG. 2

Structure of the genomes of the human herpesviruses (according to McGeoch[20]): full lines indicate unique sequences, rectangles repetitive sequences and arrows show their mutual orientation. U_L long unique sequence; U_S short unique sequence; TR_L, IR_L terminal and internal repetitive region neighbouring with U_L; TR_S, IR_S terminal and internal repetitive sequence neighbouring with U_S; MIR major internal repetitive region

stop codons in convenient triplet periodicity. Nevertheless, such an approach can hardly distinguish very small or overlapping genes or genes with complex exon–intron structures. The real number of genes in the genome may thus to a certain extent differ from numbers indicated in Table II.

The identification of the individual gene function presents a more formidable problem. It can be investigated by the combination of the results from previous extensive genetic and biochemical work with HSV and analysis of structural motifs of amino acid sequences which may be found in proteins with a known function originating from other sources. In this respect, the data are rather scarce; in highly characterized HSV-1 genome which codes for 70 polypeptides only 27 of them are functionally specified. The properties of proteins from other herpesviruses are determined by comparing the amino acid homology of HSV-1 polypeptides with the corresponding counterparts situated in the analogous site of genome. Table III summarizes the data on proteins participating in the nucleotide metabolism and in the DNA replication process of HSV-1

TABLE I
Classification of human herpesviruses

Family *Herpesviridae*	
Subfamily	Type
Alphaherpesvirinae	Herpes simplex virus 1 (HSV-1) Herpes simplex virus 2 (HSV-2) Varicella zoster virus (VZV)
Betaherpesvirinae	Human cytomegalovirus (HCMV)
Gammaherpesvirinae	Epstein–Baar virus (EBV) Human herpesvirus 6 (HHV-6)

TABLE II
The size and composition of human herpesvirus genome

Virus	Genome size (bp)	% (G–C)	No. of genes
HSV-1	152 260	68.3	72
VZV	124 884	46.0	70
EBV	172 281	59.9	84
HCMV	229 354	57.2	200
HHV-6	170 000	41	?

TABLE III

Enzymes in HSV-1 and their homologues in VZV, EBV, HCMV. Relative molecular weight of proteins (M_r) is calculated on the basis of their amino acid sequences

Function	HSV-1			VZV		EBV		HCMV	
	gene	M_r	ref.	gene	M_r	gene	M_r	gene	M_r
Uracil-DNA-glycosylase	UL2	36 326	30, 31	59	34 375	BKRF3	31 606		
DNA helicase-primase	UL5	98 710	32 – 34	55	98 844	BBLF4	89 853		
DNA helicase-primase	UL8	79 921	32 – 34	52	86 343	BBLF3	22 605		
Origin-binding protein	UL9	94 246	32, 33	51	94 370	BBLF2	60 364		
Deoxyribonuclease	UL12	67 503	35	48	61 268	BGLF5	52 666		
Protein kinase	UL13	57 193	36, 37	47	54 347	BGLF4	51 291	HSRF3	78 233
Thymidin kinase	UL23	40 918	38, 39	36	37 815	BXLF1	67 193		
ssDNA-binding protein	UL29	128 342	40	29	132 133	BALF2	123 122	DB129	129 005
DNA polymerase	UL30	136 413	40	28	134 041	BALF5	113 419	HFLF2	137 103
Ribonucl. reductase large s.[a]	UL39	124 043	41	19	86 823	BORF2	93 030		
Ribonucl. reductase small s.[a]	UL40	38 017	42	18	35 395	BaRF1	34 358		
dsDNA-binding protein	UL42	51 156	32, 33	16	46 087	BMRF1	43 373		
dUTPase	UL50	39 125	43	8	44 816	BLLF2	30 952		
DNA helicase-primase	UL52	114 416	32 – 34	6	122 541	BSLF1	98 040		
Protein kinase	US3	52 831	44, 45	66	43 677				
Thymidylate synthetase				13	34 531				

[a] Subunit.

and on their probable counterparts in VZV, EBV and HCMV; the abbreviations used for the gene designation are taken from the original reports.

Significant homology between HSV-1 and VZV was found in all polypeptides indicated in Table III concerning amino acid sequences and localization of individual genes[21]. Thymidylate synthetase which is encoded by VZV gene 13 contains sequence motifs similar to thymidylate synthetases of other organisms[46]; however, HSV-1 or EBV do not encode this enzyme.

The degree of homology between proteins from alphaherpesviruses and EBV is less pronounced. The comparison of amino acid sequences of VZV and EBV (ref.[47]) revealed that EBV does not possess any gene corresponding to the U_S region of VZV. Considerable homology has been found in DNA polymerase and ribonucleotide reductase; on the contrary it is completely absent in gene products UL 8, UL 9 and UL 42 from HSV-1 with which genes BBLF 3, BBLF 2 and BMRF 1 have been matched only on the basis of similar localization in EBV genome. Their functional relationship is thus very uncertain.

Similarities between HCMV and HSV-1 polypeptides cannot be adequately specified. However, recent data indicate significant amino acid sequence analogy of DNA polymerases[48,49], major DNA binding proteins[50] and of that protein kinase[37], which corresponds to HSV-1 UL 13 protein kinase.

4. HSV DNA REPLICATION COMPLEX

Major progress in the identification of genes which are necessary for HSV-1 DNA replication has been achieved on an introduction of a new method which enables testing the ability of a set-up of cloned DNA segments to induce the replication of the plasmid containing HSV replication origin, ori_S (ref.[51]). Seven essential genes are sufficient for ori_S-dependent DNA synthesis[32,33]. Their localization in HSV genome is indicated in Fig. 3 and respective polypeptides are mentioned in Table III. For the replication of SV 40 DNA (simian virus 40) which does not contain the ori HSV only 6 genes are suffi-

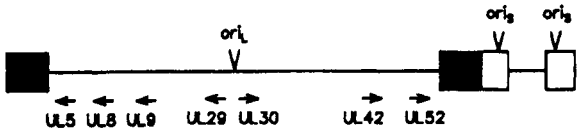

FIG. 3
Localization and orientation of open reading frames encoding proteins which participate in HSV DNA replication (from Challberg and Kelly[52]). Ori_L, ori_S origins of HSV DNA replication

cient and non-essential is only the origin binding protein which is the product of UL 9 gene[53].

For the biochemical characterization of HSV replication proteins either functional or immunological approach can be used. In the first case some functional analogy with proteins from other better known systems may be presumed and the proteins with expected function are subjected to further purification. Because of the identification of individual gene products essential for replication using specific antibodies[54] it is now possible to analyse these proteins more in detail without taking in account their possible function.

4.1. *Origin Binding Protein UL 9*

This protein was found in HSV-1 infected cells[55] and was purified almost completely by affinity chromatography[56]. The expression of UL 9 gene in insect cells with baculovirus vector yielded a product which integrated with ori_S in the same way as the polypeptide isolated from HSV infected cells[54]. Molecular weight of both proteins (84 kDa) contrasts with the values derived from their amino acid sequence (94 kDa). The purified protein binds to nearly identical sites of both arms of palindrome ori_S (refs[56,57]). Its role in HSV DNA replication is not clear; it is presumed that it may initiate the assembly of a multiprotein replication complex, or participate in the unwinding of the double strand at the beginning of replication, an introductory step of daughter strands synthesis[58].

4.2. *Single Stranded DNA Binding Protein UL 29*

UL 29 has been found several years ago in HSV-1 infected cells and designated as ICP 8 (infected cell protein 8) (ref.[59]). Its actual molecular weight (130 kDa) is in agreement with the calculated value (124 kDa). This protein binds more tightly to single stranded than to double stranded DNA (refs[60,61]), its binding does not depend on DNA nucleotide sequence[61] and in the absence of a single stranded DNA the protein forms long thick filaments[62]. It stimulates considerably the activity of HSV DNA polymerase with activated DNA as template[63] and enhances the replication of circular DNA double strand in the presence of nuclear extract from infected cells[62]; the protein has also a positive effect on the processivity of HSV DNA polymerase with a single stranded template and a single primer[64].

Presently it is believed that UL 29 fixes the single stranded DNA formed in the replication fork thus facilitating its use as a DNA polymerase template. It is probably due to its ability to unwind hairpin structures[64].

4.3. *Helicase-Primase Complex (Proteins UL 5, UL 8, UL 52)*

Procaryotic DNA helicases are remarkable by their DNA-dependent ATPase activity: they are able to combine ATP hydrolysis with unwinding of double-stranded DNA (ref.[65]). In HSV-1 infected cells this activity was described for the first time in 1988. However, the properties of the partially purified enzyme differed from those of its cellular counterpart especially by its chromatographic behavior and its ability to use besides ATP also GTP as a cofactor. The enzyme is composed of 3 polypeptides which were purified near to homogeneity and immunochemically identified as products of genes UL 5 (97 kDa), UL 8 (70 kDa) and UL 52 (120 kDa) (ref.[34]). Their molecular weights correspond approximately to the calculated values (Table III). The quantity of these proteins in infected cells is minimal and therefore their expression in insect cells using baculovirus recombinants offers the possibility to obtain sufficient amounts of material for more detailed analysis[67]. None of the activities of the enzyme complex created by recombinant proteins (270 kDa), DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase, differs from those of the enzyme complex isolated from infected Vero cells[67].

DNA primase with considerably different properties was isolated from infected HeLa cells[68]. It differs from the cellular primase both physico-chemically and immunochemically; it has a similar slightly alkaline pH optimum as HSV helicase-primase activity in Vero cells but completely different optimum concentration of magnesium ions, and, most importantly, different molecular weight (40 kDa). Recently, a new DNA primase with molecular weight of about 100 kDa, resembling to mitochondrial RNA polymerase[69], was detected in HSV infected cells. Since viral infection sometimes changes permeability of mitochondrial membrane and releases the enzyme into cytosol, it is possible that previously identified HeLa cell DNA primase in fact originates from mitochondrial RNA polymerase[69].

The function of individual components of the enzyme complex is not known. The activity of helicase using model substrates indicates that the enzyme is located on the lagging strand during unwinding of the replication fork and its movement orientation is from 5′- to 3′- end[66].

4.4. *DNA Polymerase Complex (Proteins UL 30 and UL 42)*

1. Subunit structure. The occurrence of distinct DNA polymerase (2′-deoxynucleoside 5′-triphosphate: DNA 2′-deoxynucleotidyl transferase, EC. 2.7.7.7) in HSV infected cells was observed for the first time in 1963 (ref.[70]); later on it was demonstrated that the enzyme differs from its cellular counterpart[71]. New approaches and methods of isolation were developed in numerous laboratories which afforded more homogenous enzyme preparations[72 - 75]. Besides the main component (140 – 150 kDa)

with which all of the determined properties were supposed to be connected, the enzyme preparations contained additional polypeptides whose contribution to total enzyme activity could not be elucidated. The finding that the gene UL 30 coded for DNA polymerase with molecular weight of 136 kDa (ref.[40]) supported the hypothesis that the enzyme consisted of a single polypeptide chain. During the attemps to isolate 54 kDa polypeptide which was present in the preparation of HSV-2 DNA polymerase it was found that this polypeptide was tightly bound to the major 150 kDa subunit and that it could be separated only partially[76]. During the isolation of one of many proteins capable to bind double stranded DNA in HSV infected cells, it could be shown that monoclonal antibodies against this protein were also inhibiting DNA polymerase activity. The polypeptide designated originally as K_{DBP} was shown to be the product of gene UL 42 (ref.[78]). The idea that DNA polymerase includes two subunits – protein UL 30 and UL 42 – occurred when a particularly elaborate isolation method provided specimen with molecular weight of 190 kDa. This molecular weight corresponds to the equivalent stoichiometric ratio of both subunits[79]. However, their separation was not feasible because of an easy denaturation.

The purification of subunits to homogeneity is required for the determination of their functional dependence. It can be achieved with the use of in vitro systems that utilize isolated genes. Three different systems were developed so far for the expression of HSV DNA polymerase:

– in vitro transcription with T7 RNA polymerase and in vitro translation of isolated RNA in rabbit reticulocyte lysate[80];

– expression in yeast vector with fused galactosidase promoter in *Saccharomyces cerevisiae*[81];

– expression in insect cells infected with recombinant baculovirus[82].

The enzyme expression in *Escherichia coli* was unsuccessful[83].

A 4- to 10-fold stimulation of DNA polymerase activity by protein UL 42 was obtained when subunits formed in an in vitro transcription–translation system were used with different templates; the cause of this phenomenon was not elucidated[84]. Possible function of protein UL 42 was uncovered quite recently during the experiments with the baculovirus system. The addition of this protein in the presence of single stranded DNA binding protein enhanced twice the processivity of DNA polymerase, i.e., its ability to synthesize the complementary strand without primer dissociation even on a very long single stranded template.

HSV-1 DNA polymerase was earlier considered highly processive[85] in comparison to DNA polymerase α; it has also been shown that this feature is accounted for by the presence of the single stranded DNA binding protein which is the product of gene UL 29 (ref.[62]). It may therefore be presumed that while the protein UL 29 destabilizes the secondary structure of a single stranded template, the subunit UL 42 participates in an extremely tight binding of HSV-1 DNA polymerase to the end of primer.

2. Properties. HSV-1 DNA polymerase isolated from infected cells[72-75] has the following properties:

- molecular weight of 140 – 150 kDa;
- reaction optimum within the range of pH 8.0 – 8.5;
- it is stimulated by high ionic strength, magnesium ions and dithiothreitol;
- it is inhibited by diphosphate analogues (phosphonoacetic and phosphonoformic acid), nucleotide analogues, N-ethylmaleinimide and Zn^{2+} ions;
- it possesses 5'-3' polymerase and 3'-5' exonuclease and RNase H activity.

All these properties are due to the single large polypeptide present in the polymerase preparations isolated from infected cells because identical properties were found in HSV-1 DNA polymerase prepared by in vitro systems (refs[80-82]).

3. 3'-5' Exonuclease activity (EC. 3.1.4.–). This activity was originally thought to be a deoxyribonuclease activity of HSV-1 DNA polymerase[72]; later on it was identified as 3'-5' exonuclease[74]. It is localized in a single polypeptide together with the DNA polymerase. This conclusion is sustained by the following evidence:

- both activities function under identical conditions[74,86];
- they are inhibited likewise by phosphonoacetic[74,86] and phosphonoformic[87] acid;
- their thermal denaturation profile is identical[75,86];
- both of them are inhibited by antibodies directed against a certain region of the polypeptide chain[88,89].

3'-5' Exonuclease activity of procaryotic DNA polymerase is believed to be the main reason for the high fidelity of DNA replication[60] since the inhibition of this repair activity lowers the preciseness considerably[91]. On the contrary, the replication fidelity of eucaryotic DNA polymerases α and β that do not possess 3'-5' exonuclease activity is low[91]; their replication fidelity is probably due to 3'-5' exonuclease activity of DNA polymerase δ (refs[92,93]).

HSV DNA polymerase has a higher relative exonuclease activity than procaryotic DNA polymerase and is able to remove the incorrect nucleotide from the primer end before its elongation. This suggests that the 3'-5' exonuclease activity may contribute to the considerable fidelity of herpes simplex virus DNA replication. On the other hand, the conditions blocking the repair activity of procaryotic polymerases do not affect HSV DNA polymerase; it is thus possible that the enzyme itself replicates DNA quite reliably[94].

4. Activity of ribonuclease H (5'-3' exonuclease activity, EC. 3.1.27.5). The ribonuclease H activity was discovered in HSV DNA polymerase preparation quite recently[79]. It degrades RNA in hybrid RNA-DNA double strands and DNA double strands in the 5'-3' direction under conditions different from the optimum for polymerase reaction, and it is not able to cleave either single or double stranded RNA. The degradation of hybrid RNA-DNA double strand was described also by other workers, but in 3'-5' direction[86].

The role of this enzyme activity is probably removal of RNA primers which have served for the initiation of Okazaki fragments synthesis on the lagging strand of the replication fork during the synthesis of viral DNA.

5. Structural and functional studies of HSV-1 DNA polymerase. HSV DNA polymerase is conceived as a heterodimer containing a trifunctional polypeptide (136 kDa) with DNA polymerase, 3'-5' exonuclease and 5'-3' RNase H activities and a product (62 kDa) of gene UL 42 (ref.[79]).

The major polypeptide contains 1 235 amino acid residues[40,95] with sequence in different regions similar to that of other procaryotic and eucaryotic polymerases belonging to the group of human DNA polymerase α (refs[96,97,109]). The regions with most significant sequence homology are concentrated in six clusters designated with numbers I – VI according to decreasing homology (Fig. 4).

Presuming that to retain a sequence during phylogeny depends on its functional importance, Fig. 4 suggests that the decisive role in the polymerase reaction is played by domains I, II and III. Different approaches have been used to elucidate in more detail the function of these domains:

a) Sequencing of DNA polymerase gene of viral mutants resistent or hypersensitive towards polymerase reaction inhibitors[99 - 104], analogues of natural substrates. It may be expected that the mutations involve amino acids which participate in the formation of substrate binding site.

b) The production of antibodies against defined enzyme regions which would neutralize its activity on interaction with functionally important sites[86,88,89,105].

c) Site directed mutagenesis and analysis of the products formed in vitro systems[80,106 - 108].

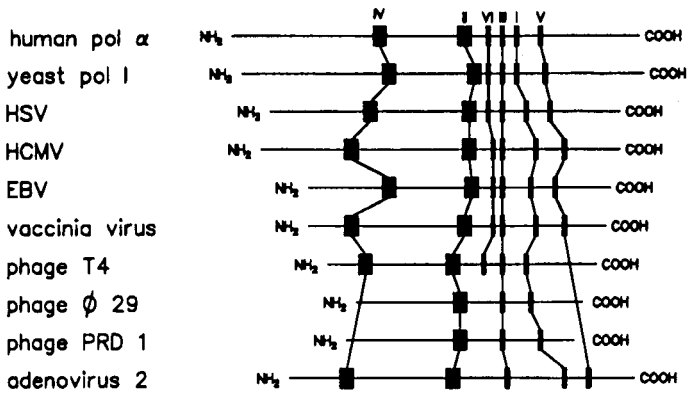

FIG. 4
Structural homology in human DNA polymerase group (according to Wang et al.[98])

Data obtained with the use of different approaches are summarized in Fig. 5.

A number of 14 different amino acid substitutions in HSV DNA polymerase chain of viral mutants resistent to nucleotide analogues, diphosphate and aphidicolin have been identified so far; the data indicate that:

- 4 mutations are mapped to region II [position 700, 724 (ref.[102]), 696 (ref.[100]), 719 (refs[99,101])];
- 6 mutations are mapped to region III [position 813, 821, 842 (ref.[102]), 815 (ref.[101]), 818 (ref.[104]), 841(refs[101,103])];
- 2 mutations are mapped to region "a" [position 597 (refs[101,102]), 605 (ref.[102])];
- one mutation is observed in the region V [position 961 (ref.[102])];
- one mutation is identified in nonconservative region [position 797 (ref.[102])];
- no mutation has been detected in domains I, IV, and V.

There seems to be no region which would specifically bind any particular substrate or inhibitor. Different parts of polypeptide chain evidently contribute to the formation of a catalytic center simultaneously with domains II and III playing an important role during substrate binding.

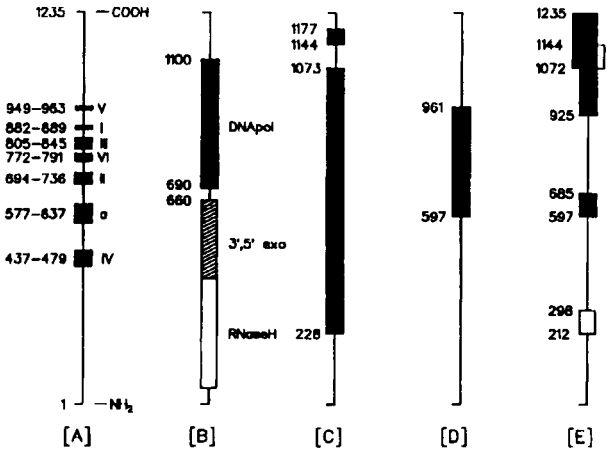

FIG. 5

Functionally important regions in HSV-1 DNA polymerase: [A] domains with high degree of amino acid homology with other DNA polymerases; region "a" is homologous only to viral polymerases sensitive towards different nucleotide analogues[102]; [B] hypothetic organisation of the polypeptide arranged according to structural similarities to DNA polymerase I from *E. coli*[108]; [C] regions required for maintaining polymerase activity[80,108]; [D] region including all sites of all known point mutations indispensable for enzyme resistance towards some inhibitors[99 - 104]; [E] regions tested with specific antisera; full rectangles represent those parts of chain whose immunochemical inhibition results in inactivation of polymerase and 3'-5' exonuclease activity, empty rectangles those parts whose inhibition does not affect the activity of enzyme[86,88,89,105]

The importance of region I for activity of HSV DNA polymerase was investigated using site-directed mutagenesis and subsequent analysis of enzyme products by in vitro transcription–translation system[107] (Fig. 6).

5. HSV THYMIDINE KINASE (ATP: Thymidine 5'-Phosphotransferase, EC. 2.7.1.21)

This enzyme was found in HSV infected cells in 1963 (ref.[110]). Its viral origin was later demonstrated beyond all doubt using isolated thymidine kinase deficient mutant[111] as well as by an immunological technique[112]. Functional enzyme was prepared by expressing the respective gene in *Escherichia coli* (refs[113,114]) and subsequently crystallized[115].

5.1. *Enzyme Characteristics*

HSV-1 thymidine kinase is composed of two identical subunits[118], each of molecular weight 43 kDa, containing 376 amino acids[38,39]. However, even smaller subunits have

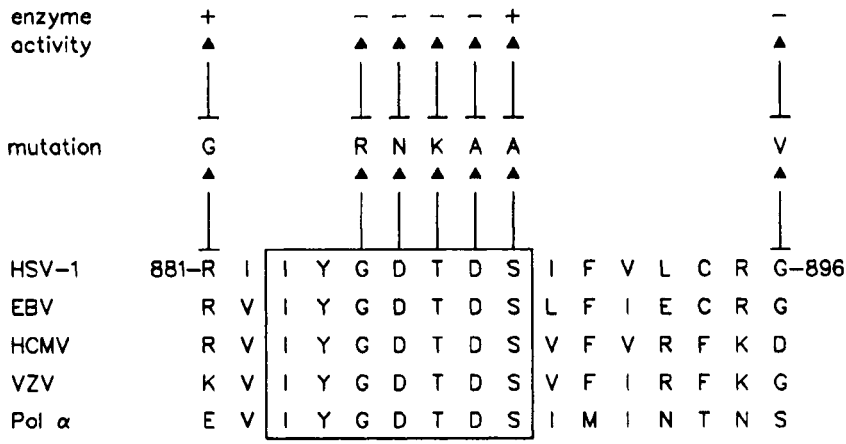

FIG. 6
Effect of single amino acids substitutions of the most conserved region of HSV-1 DNA polymerase chain on enzyme activity (according to Dorsky and Crumpacker[107])

been isolated (39 kDa and 38 kDa) corresponding probably to a polypeptide whose translation initiation starts on the second and third AUG codon of mRNA chain[128,132].

Due to its polyfunctional character the enzyme is designated as thymidine kinase complex[116]; it possesses following catalytic functions:

- ATP: thymidine (deoxycytidine) kinase[117 - 121];
- ATP: thymidine 5'-phosphate kinase[122 - 124];
- ADP: thymidine 5'-phosphotransferase[125];
- AMP: thymidine 5'-phosphotransferase[126,127].

Actually, it is a deoxypyrimidine kinase which phosphorylates deoxypyrimidine nucleosides to their monophosphates and 2'-deoxythymidine 5'-phosphate (but not 2'-deoxycytidine 5'-phosphate)[124] to its diphosphate. Phosphorylation of different nucleoside analogues has also been observed. The phosphate group donor is ATP, ADP and AMP. The above diverse enzyme activities have:

- different sensitivity towards dTMP, dTDP and dTTP (ref.[128]);
- different pH optimum[125];
- different sensitivity towards EDTA, divalent cations, thiol-alkylating agents and *o*-phenanthroline[125].

Earlier it was thought that thymidine kinase plays a certain "luxury" role in viral replication since TK^- strain grows in cell culture equally well as the wild one. However, it has been later observed that in cells growing under deficient nutritional conditions the TK^- virus mutants replicate at a lower rate compared to their wild-type counterparts[120]. Also in animal models the HSV TK^- strains exhibit decreased pathogenicity[130,131]. Thymidine kinase is indispensable for infection of resting cells which lack sufficient apparatus for DNA synthesis[132].

5.2. *Structural and Functional Studies*

Herpesvirus encoded thymidine kinases play a key role in the transformation of majority of nucleoside analogues to their activated forms; from the point of view of antiviral drugs development and action thymidine kinase is of crucial importance. Similary as DNA polymerase, the structure, especially the architecture of the active site of thymidine kinase is extensively investigated.

The effect of several regions (Fig. 7) on the properties of HSV-1 thymidine kinase dimer containing 376 amino acids has been studied with the use of different approaches. The comparison of HSV-1 thymidine kinase polypeptide amino acid sequence with that of counterparts encoded by other herpesviruses indicated two significant homology regions: segments 49 – 66 and 161 – 193 (ref.[134]). The first region probably participates in the ATP binding. In the region 51 – 63 there is a significant homology with sequences of a number of completely different enzymes which also bind ATP (ref.[135]). Moreover, the replacement of various amino acids in the region 56 – 64 resulted regu-

larly in a decreased affinity of the enzyme towards ATP (ref.[136]). The second region participates in nucleoside binding. It is supported by the sequence analysis of HSV-1 thymidine kinase mutant genes with modified enzyme–substrate affinity. These mutations were localized to positions 168, 176 and 336 (ref.[137]). The cysteine residue in position 336 was thought to be bound to cysteine 171 in conserved region; nevertheless, the presumed interaction which might lead to a disulfide bond formation was not confirmed: the substitution of cysteine 171 by serine or glycine did not result in any loss of enzyme activity[138]. There is 97% sequence homology between HSV-1 and HSV-2 thymidine kinases in region 161 – 193 (ref.[134]). The enzyme from HSV-1 strain resistant towards 5-bromovinyl 2'-deoxyuridine (BVdU) differs from its HSV-1 wild type counterpart by a single amino acid in position 168 (Ala-Thr) (ref.[218]). HSV-2 thymidine kinase which is much more resistant to BVdU (ref.[219]) differs within highly homologous region (161 – 193) from HSV-1 enzyme also by a single amino acid in position 168 (Ala-Ser). It is therefore possible that this particular substitution is responsible for different sensitivity of HSV-1 and HSV-2 towards BVdU. The deletion of 45 amino acid residues from the N-terminus affected the thermal stability of the enzyme and resulted in a loss of thymidylate kinase activity[141,142], but it did not affect the thymidine kinase activity[139,140]. HSV-1 and HSV-2 thymidine kinases differ considerably in the sequence of first 45 amino acids. N-terminus of VZV thymidine kinase begins with a sequence which is almost the same to compare with the segment from thirty eighth amino acid in HSV-1 thymidine kinase. HSV-2 thymidine kinase possesses a much lower thymidylate kinase activity than HSV-1 and also VZV enzyme; for this reason it was proposed that the decisive segment for thymidylate kinase activity of HSV-1 thymidine kinase is the region of amino acids 38 – 45 (ref.[141]).

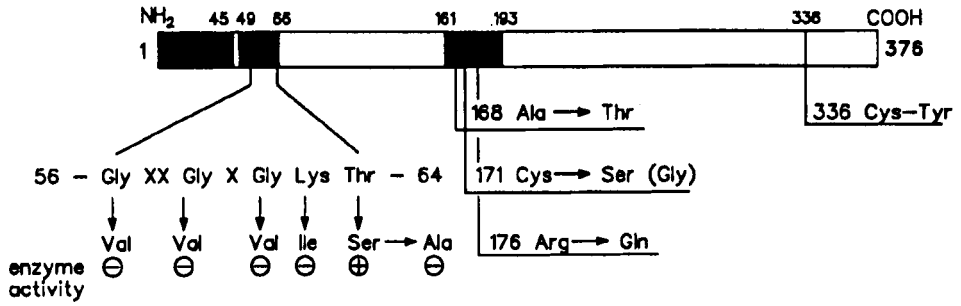

FIG. 7
Mutations in HSV-1 thymidine kinase polypeptide chain

6. HSV RIBONUCLEOTIDE REDUCTASE (Ribonucleoside 5'-Diphosphate Reductase, EC. 1.17.4.1)

The existence of HSV-1 specific ribonucleotide reductase was based on the observation that the virus can replicate in the presence of high concentrations of 2'-deoxythymidine which usually interfere with host cell metabolism[143]. Ribonucleotide reductase which catalyzes the reduction of all natural ribonucleoside 5'-diphosphates is the key enzyme of the synthesis of DNA precursors in eucaryotes and procaryotes[144]. Partially purified enzyme preparations have been isolated from virus-infected cell cultures; their characteristics differ from those of their host cell counterparts[145 - 148]. It was observed that the temperature sensitive HSV-1 mutant formed a thermolabile ribonucleotide reductase both in vivo and in vitro. Therefore, this enzyme is at least partially virus-encoded[149]. The mutation was mapped to 136 kDa polypeptide coding fragment[41] and immunoprecipitation with monoclonal antibodies uncovered a second polypeptide with molecular weight of 38 kDa (refs[150 - 152]). Homogenous and biologically fully active preparation of smaller HSV-1 ribonucleotide reductase was obtained by expression of a respective gene in *Escherichia coli*[153]. Moreover, the active enzyme was obtained in small yield from cultured human cells which expressed a small subunit of HSV-2 ribonucleotide reductase after infection with adenovirus bearing the built-in gene of the large subunit of HSV-2 ribonucleotide reductase[154].

6.1. *Enzyme Characteristics*

HSV ribonucleotide reductase is formed by two tightly bound subunits[155,156] of different size which lose their enzyme activity on separation[157]. Each subunit is a homodimer composed of polypeptides designated also as V_{mw136} (large subunit, a product of UL 39 gene) and V_{mw38} (small subunit, a product of UL 40 gene) (ref.[156]). The analysis of properties of the enzyme isolated from the cells infected by HSV-1 (ref.[148]) and HSV-2 (ref.[158]) indicated a considerable degree of similarity but a striking difference to ribonucleotide reductases from other sources[159].

The activity of mammalian and bacterial enzymes is allosterically regulated by the presence of 2'-deoxyribonucleoside 5'-triphosphates and ribonucleoside 5'-triphosphates[144]. Neither any inhibitory effect of dTTP and dATP on the reduction of CDP and ADP (refs[146 - 148,158]) nor any stimulation by ATP were observed in the viral enzyme. The K_m values for CDP, GDP and ADP reduction are much lower than those for ribonucleotide reductase from eucaryotes; each ribonucleoside 5'-diphosphate inhibits competitively the reduction of its three counterparts (the K_i values are comparable to the K_m values). The K_i values of 2'-deoxyribonucleoside 5'-diphosphates are approximately 10-fold higher than the K_m values of the respective ribonucleoside 5'-diphosphates[148]. The non-allosteric nature of the viral enzyme is supported by the fact that during the cell infection by HSV-1 the pool of 2'-deoxynucleoside 5'-triphosphates

is increasing[160]. The reduction of ADP, GDP, CDP and UDP evidently takes place only at single active site of the enzyme.

The study on proteolytic degradation of the large subunit suggests that the ribonucleotide reductase activity relies upon approximately two thirds of C-end of the polypeptide[156,161]. In this region, 90% homology of amino acid sequence was observed in HSV-1 and HSV-2 large subunit while their N-end domains differ considerably[162]. Interestingly, there was observed a protein kinase structural motif situated in the region of first 411 amino acids of large subunit of HSV-2 enzyme; the protein kinase activity was detected and neutralized by antibodies against synthetic polypeptide including amino acids 355-369 (ref.[163]). HSV-1 ribonucleotide reductase does not possess this structural motif of protein kinase.

It has been reported that the synthetic nonapeptide corresponding to 9 amino acids of the C-end of small enzyme subunit inhibits specifically HSV-1 ribonucleotide reductase activity probably by hindering the association of both subunits[164 - 167]. Quite recently it has been established that in the temperature sensitive HSV-2 mutant *ts 1207* the exchange of a single amino acid (Ser-Asn) in 961 position of large subunit results in the loss of subunit association thus preventing the formation of a functional complex[168].

It is questionable whether viral ribonucleotide reductase is indispensable for virus replication; in positive case it could be a target enzyme for development of chemotherapeutics. Various investigators who studied its role in the viral infection of cell cultures came mostly to a negative conclusion for the following reasons:

- the concentration of hydroxyurea which inhibits HSV-2 reductase by more than 95% decreases the production of the virus only 6-fold[169];
- HSV-1 mutant with insertion of lac Z segment into the region coding for reductase large subunit replicates only 4 – 5 times more slowly than wild-type strain[170,171];
- temperature sensitive HSV-1 mutant *ts 1222* with a defect in small subunit does not produce the functional enzyme, and at lower temperature (31 °C) it replicates at the same rate as wild type strain[172].

All the data available so far suggest that in the proliferating cells the HSV-viral reductase can be substituted by its cellular counterpart. On the other side, the HSV-1 mutant with major deletion of the gene coding for large subunit is unable to cause ocular and trigeminus infection in mice[173] suggesting the importance of virus encoded ribonucleotide reductase in the virus life-cycle. Moreover, a more than 106-fold decrease in the virulence of the mutant *ts 1222* in mouse cerebral tissue[174] may also indicate the relevance of ribonucleotide reductase in antiviral chemotherapy.

7. HSV DEOXYURIDINE TRIPHOSPHATASE (Deoxyuridine Triphosphate Nucleotidohydrolase, dUTPase, EC. 3.6.1.23)

This enzyme splits dUTP to dUMP and pyrophosphate in procaryotes[175] as well as in eucaryotes[176] and prevents thus the use of dUTP as a substrate for DNA polymerase[177]; simultaneously it may provide dUMP for dTMP synthesis.

The initial work with extracts from virus infected cells demonstrated the induced activity degrading dUTP (ref.[178]) and also, though to a lesser extent, dTTP and dCTP (refs[179,180]). However, purified preparations of HSV-1 (refs[181,182]) and HSV-2 (refs[183,184]) which have been obtained later were completely specific for dUTP. The identification of HSV-1 dUTPase gene[43] rendered the construction of mutants feasible; it consisted in inserting a small linker[185] or fragment coding for bacterial chloramphenicol resistance in dUTPase gene coding region of HSV-1. The resulting mutants do not induce active enzyme and may be used for dUTPase studies during viral infection.

7.1. *Enzyme Characteristics*

dUTPase isolated from KB cells infected with HSV-1 (ref.[181]) or HSV-2 (refs[183,184]) has the following characteristics:

- monomeric with the molecular weight 53 kDa;
- specific exclusively for dUTP;
- isoelectric points of 5.8 (HSV-1) and 5.9 (HSV-2);
- inhibited by EDTA; this inhibition can be reverted by addition of Co^{2+}, Mg^{2+} (HSV-2) and partially also by Mn^{2+} ions.

The host cell dUTPase differs from the viral enzyme by:

- different behavior in various chromatographic systems;
- lower molecular weight (46 kDa);
- different isoelectric point (6.3);
- higher thermostability;
- more extensive spectrum of divalent ions preventing inhibition by EDTA (Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}).

Surprisingly, dUTPase isolated from HeLa S3 cells infected with HSV-1 (ref.[182]) has also been described as a monomer, but with the molecular weight of 35 kDa and isoelectric point 8.0; the host enzyme is a dimer, with the molecular weight of 22.5 kDa (one subunit) and isoelectric point 5.7 – 6.5.

Since the HSV-1 mutant which does not induce dUTPase activity is virulent in BHK-21 cells it was thought that the enzyme was not a useful target for chemotherapeutic attack[185]. However, later on it was observed that the mutant virus which was employed originally did not express a functional dUTPase and simultaneously did not suppress the synthesis of cellular isoenzyme. The mutant which is able to switch off the synthe-

sis of host cell enzyme and at the same time does not produce its own dUTPase is much more sensitive towards 5-chloro and 5-fluoro 5'-deoxyuridine than the wild type strain or the strain mutated in dUTPase gene but not suppressing the cellular isoenzyme[187]. These results suggest a possible substitution of the viral enzyme by its cellular counterpart. Enhanced incorporation of the analogues or dUTP into HSV DNA may have a lethal effect due to fragmentation of DNA by viral or cellular uracil-DNA-glycosylase[187]. Thus the idea of dispensability of dUTPase activity for HSV infection is apparently doubtful.

8. HSV URACIL-DNA-GLYCOSYLASE

This enzyme belongs among antimutational enzymes. It cuts off uracil bases from DNA where they may occur either due to dUTP incorporation catalyzed by of DNA polymerase[188] or due to deamination of cytosine residues in DNA (ref.[189]).

Uracil-DNA-glycosylase cleaves N-glycosidic bond of uracil and 2'-deoxyribose-5'-phosphate residue in DNA resulting in DNA with apyrimidine site which is subsequently repaired[190]. This prevents the transition mutation of G–C to A–T pair.

The high content of G–C pairs in HSV genome and the fact that the enzyme was found in almost all procaryotic and eucaryotic organisms led to the presumption that its coding sequence existed also in HSV. Following the infection of cells by HSV-1 and HSV-2 the activity of uracil-DNA-glycosylase has increased twice and twenty times, respectively[178]. In contrast to the cellular enzyme, its viral counterpart was activated by high KCl concentration and neutralized by rabbit antiserum against HSV.

The evidence that the enzyme is virus-encoded appeared later on. The preparation of HSV-2 uracil-DNA-glycosylase coding cDNA has been described, and subsequently the corresponding mRNA was also isolated. Finally, a functional enzyme preparation has been obtained in vitro using this mRNA in a suitable translation system[192]. However, the SDS-polyacrylamide electrophoresis revealed 3 polypeptides (28, 32 and 38 kDa). It is possible that various initiation and termination sites of translation are located in a single mRNA or that there may be a number of overlapping mRNAs whose translation results in the formation of different polypeptides. The coding sequence of HSV-2 uracil-DNA-glycosylase was identified by an in vitro transcription of UL 2 region and by subsequent translation in vitro of the transcription product; in HSV-1 a mutant virus has been constructed which does not express uracil-DNA-glycosylase activity since it contains the UL 2 defective gene with lac Z *Escherichia coli* insertion[30]. Thereby the viral origin of the enzyme was unequivocally confirmed and, its dispensability for HSV-1 replication was demonstrated.

However, the enzyme has not been isolated and characterized so far and its usefulness for antiviral chemotherapy remains undecided. The virus displays considerable dUTPase activity which largely prevents the incorporation of dUMP into viral DNA. It is possible that uracil-DNA-glycosylase largely contributes to the removal of

uracil residues arising by cytosine deamination. However, the enzyme is most active during the DNA replication; it is probable that besides its known function the enzyme may have additional activities which are closely connected with DNA replication[192].

9. HSV DEOXYRIBONUCLEASE (DNase, EC. 3.1.21.2)

The enzyme was found in HSV-1 (ref.[70]) and HSV-2 (ref.[193]) infected cells many years ago; on account of its alkaline optimum it has been designated as alkaline nuclease. Purified enzyme preparations which were obtained by different laboratories possessed endo- and exonuclease activity; nevertheless, they differ from each other by the number and size of their polypeptide chains. Hoffman and Cheng[194,195] found a single polypeptide with molecular weight of 68 kDa, while other authors isolated a mixture of 4 polypeptides with molecular weight within the limits of 70 – 90 kDa (ref.[196]). The homogenous HSV-2 alkaline nuclease (85 kDa) was used for the production of monoclonal antibody[197]. The alkaline nuclease of the same molecular weight is formed in *Xenopus laevis* oocytes following an injection of total mRNA from virus-infected human cells[35]. A surprising result was obtained while detecting the nuclease activity of HSV-1 infected cell lysate on SDS-polyacrylamide electrophoresis gels with built-in DNA: After renaturation of separated enzymes, the nuclease activity has been determined by staining gels with ethidium bromide and the viral origin of separated proteins has been confirmed by immunological tests. By this method 6 virus specific nucleases (90, 85, 80, 76, 71 and 65 kDa) were detected and their different levels during the course of infection observed[198].

9.1. *Enzyme Characteristics*

Of the group "early" HSV proteins, deoxyribonuclease is produced in the virus-infected cells in most considerable quantities[199]. At present it is regarded as a polypeptide (85 kDa) with endonuclease and 5′-3′ exonuclease activity[86]. Recent studies with DNA templates differentially labelled at 3′- and 5′-end did not confirm[86] the previous report on the existence of 3′-5′ exonuclease activity[200]. Optimum pH value is between 9 and 10 (refs[194,195,197]) and the enzyme was found to be considerably resistent towards alkaline pH. However, Knopf and Weisshart[86] reported optimum activity of HSV-1 deoxyribonuclease at pH 8. The enzyme is activated exclusively by Mg^{2+}-ions and inhibited by Ca^{2+}-, Zn^{2+}- and Mn^{2+}-ions as well as by the presence of high ionic strength[86,197].

Alkaline nuclease is indispensable for HSV-2 replication as indicated by the studies of heat-sensitive HSV-2 mutant[201]. Following infection of cells by this mutant at a temperature of 39.2 °C almost no nuclease activity was detected, DNA synthesis was reduced and the titre of virus was more than 3 000-fold lower than at 31 °C or with a wild-type virus infection. In parallel it has been found that the revertant restores the

level of DNase activity, DNA synthesis as well as the virus replication at 39.2 °C to levels of wild-type strain[201]. However, a similar HSV-1 mutant has not yet been observed.

Several hypotheses have been proposed in order to elucidate the function of HSV deoxyribonucleases:

a) The enzyme may participate in the degradation of host DNA. This would explain a considerable reduction of cell chromosome fragmentation following infection with heat-sensitive alkaline nuclease HSV-2 mutant compared to the wild-type virus infection[202]. Also the recent model of the mechanism of deoxyribonuclease action takes into account[86] its degradative role, i.e. DNA cleavage by endonuclease followed by 5'-end attack by 5'-3' exonuclease activity which finally results in the formation of different length oligonucleotides with overlapping 3'-ends which can neither be effectively ligated nor can they serve as DNA polymerase templates.

b) The enzyme may participate in viral DNA processing during the course of replication and also in splitting its concatameres to units corresponding to the length of viral genome. The sequence specificity of the enzyme has not been established; it cannot be excluded that it may associate with some sequence specific polypeptides[203]. It has further been shown that the viral enzyme together with one of three cellular deoxyribonucleases constitute a part of viral nucleoprotein[204].

c) In combination with DNA ligase the enzyme may function also as topoisomerase[205,206].

10. HSV PROTEIN KINASE (ATP: Protein Phosphotransferase, EC. 2.7.1.37)

It is well known that the phosphorylation of proteins is an important regulatory element of different normal cell metabolic processes, and it is probably of significance for the herpes virus life cycle[207,208]. In infected cells it may be catalyzed by host or virus protein kinases. It has been observed that HSV virions exhibit protein kinase activity[209-211] although it has not been excluded that a cell enzyme is involved[44]. The isolation of protein kinase from cytosol of hamster fibroblasts infected with porcine herpes virus (pseudorabies virus) yielded a preparation with properties entirely different from those of cell isoenzymes[212]. An analogous procedure has been used to isolate the enzyme from the same cells infected with HSV-1 (refs[44,213]). In contrast to its cellular counterpart, the viral enzyme is active in an extremely high-ionic strength medium (1M KCl), it phosphorylates exclusively serine and threonine residues of basic proteins utilizing only ATP as a cofactor[215]. The natural substrate has not been identified yet.

The assumption that the HSV-1 protein kinase is a virus-encoded protein is supported by the following observations:

a) The tentative amino acid sequence of the product of HSV-1 gene US 3 includes motifs which are regarded as highly conserved in a number of eucaryots and also in retroviral protein kinases[45].

b) HSV-1 mutant with an extensive deletion in US 3 gene does not form a functional protein kinase in infected cells[214].

c) The antiserum against synthetic octapeptide which stands for eight C-end amino acids of US 3 protein reacts with highly purified protein kinase isolated from HSV-1 infected cells (ref.[44]).

With the exception of different oncogenic retroviruses, herpesviruses are presently the only family of eucaryote viruses whose genomes contain structural motifs of cellular protein kinases. The original idea that the occurrence of protein kinase is limited only to alphaherpesviruses is rather questionable since analogous structural motifs were found also in human cytomegaloviruses and Epstein–Baar virus genomes[36,37].

The significance of this enzyme for the virus life-cycle is not evident. The majority of genes in the region of HSV-1 US genome are dispensable for virus propagation in vitro[216]. However, as the HSV-1 mutant with US 3 gene deletion is considerably less virulent in mice[217], protein kinase may be also regarded as a target for chemotherapeutics.

11. ENZYMES OF OTHER HUMAN HERPESVIRUSES

The structural and sequence similarities of individual regions of VZV, EBV and HCMV to HSV genome indicate that these viruses probably encode for a range of enzymes analogous to HSV enzymes but in comparison to HSV the knowledge of these enzymes is limited or even missing.

11.1. *VZV DNA Polymerase*

Only few papers published some years ago report different data on biochemical characteristics of this enzyme. The existence of virus-induced DNA polymerase has been proposed in 1977 (ref.[220]) on account of the inhibition of VZV replication by phosphonoacetic acid in tissue culture. The enzyme has been partially purified from nuclei of VZV infected human embryonic cells[221] and later on characterized[222]. In distinction to cellular alpha and beta polymerases it behaves differently on chromatographic carriers, has different template specificity, is activated by higher ionic strength and is more sensitive towards phosphonoacetic acid. The comparison of a number of viral DNA polymerases revealed a lower degree of VZV DNA polymerase stimulation in the presence of ammonium sulfate and also lower sensitivity to phosphonoacetic acid than in HSV-1 and HSV-2 DNA polymerases[223]. Later on different reports analysing the enzyme inhibition by various nucleoside analogs appeared[224,225].

11.2. *EBV DNA Polymerase*

The lack of a simple permissive tissue system for EBV makes it difficult to obtain large amounts of virus. However, lytic virus cycle may be induced in cell lines derived from Burkitt's lymphoma explants or EBV-infected B-lymphocytes. This procedure results in the formation of large amount of infectious particles in producer cells (P3HR1, D98HR1, B95-8), whereas in non-producer cells (Raji) only early events of lytic viral cycle occur. The inducers of lytic viral cycle are chemical reagents (12-O-tetradecanoyl-phorbol-acetate(TPA), sodium butyrate, iododeoxyuridine) or the superinfection of non-producer cells by virus from producer cells.

A surprisingly large number of papers appeared which report EBV DNA polymerase activities and their purification to different degree making use of various cell line strains and ways of inducing lytic virus cycle. The enzyme induction has been described after treatment of producer cells by iododeoxyuridine[226,227], TPA(refs[228 - 231]), n-butyrate[232] and of non-producer Raji cells by TPA and n-butyrate[233,234] and by superinfection[235]. Even though chemicals may induce also unexpected reaction of host cells, and the events in virus producer and non-producer differ considerably, it has been shown that the properties of enzyme isolated from both systems are essentially similar. The enzyme is activated by high ionic strength, inhibited by phosphonoacetic and phosphonoformic acid and is more resistant towards the effect of N-ethylmaleimide and aphidicolin than its cellular counterpart.

Molecular weight of EBV DNA polymerase using SDS-PAGE is 110 kDa; however, with gel chromatography the respective value was 185 kDa. It is probable that the native enzyme is composed of numerous proteins since it has been observed that 4 polypeptides (110, 100, 55 and 49 kDa) are connected with polymerase activity[234]. With the exception of 100 kDa protein all of them are distinguished by monoclonal antibody against early EBV antigens which neutralizes EBV DNA polymerase[234]. Moreover, 45 kDa viral protein stimulating specifically EBV DNA polymerase has been found[231]. The evidence for two different DNA polymerases has never been confirmed[228].

The comparison of EBV and HSV-1 DNA polymerase indicates that EBV enzyme is 10 – 20 fold less sensitive towards different analogues of deoxynucleotide triphosphates, it is inhibited to a lesser degree by phosphonoacetic and phosphonoformic acids, aphidicolin and by N-ethylmaleimide and it is more thermolabile[236].

Although many years ago ORF BALF 5 region of EBV genome was proposed to encode for DNA polymerase on the basis of its sequence homology to encoding region of HSV-1 (ref.[40]) and HCMV DNA polymerase[48], its identification was reported only recently[237]. The approach consists in transcription and translation of ORF BALF 5 in vitro in rabbit reticulocyte lysate, and in the analysis of the product – 110 kDa polypeptide possessing DNA polymerase activity which can be neutralized either by serum

from patients with nasopharyngeal carcinoma or by rabbit antiserum against synthetic polypeptide derived from part of BALF 5 sequence. This system will probably be valid for the identification of functional enzyme domain.

11.3. *HCMV DNA Polymerase*

HCMV DNA polymerase is biochemically well characterized. Enzyme preparations were obtained from HCMV-infected human fibroblasts differing from cellular DNA polymerases by their chromatographic behaviour on various carriers, and by their specificity towards template-primer as well as by stimulation in the presence of high ionic strength[238 - 240]. In comparison to DNA polymerase alpha the enzyme is inhibited by a number of deoxynucleoside triphosphate analogues[241,242] as well as by phosphonoacetic and phosphonoformic acids[243,244]; however, its inhibition profile by aphidicolin is similar, and like cellular enzyme it is not inhibited by ddTTP (ref.[239]). In analogy to HSV DNA polymerase[74,75] HCMV DNA polymerase is equipped with 3'-5' exonuclease activity which probably plays a proof-reading role during polymerization. Highly purified enzyme preparation is composed of two polypeptides with molecular weight of 140 and 58 kDa (ref.[242]).

The genomic localization, gene sequence and transcription analysis of the HCMV DNA polymerase have been published[48,49]. The functional evidence that ORF HFLF 2 encodes for the DNA polymerase was furnished by cotransfection of cloned DNA fragments from HCMV mutants resistant to phosphonoacetic acid with HCMV wild-type strain DNA (ref.[245]).

11.4. *VZV Thymidine (Deoxypyrimidine) Kinase*

The induction of new deoxypyrimidine kinase activity in human embryonal cells was observed in 1977 (ref.[246]) and its approximate molecular weight (72 kDa) was determined[247]. Later on it was found that the enzyme is homodimeric (70 kDa); in contrast to HSV thymidine kinase it phosphorylates deoxycytidine preferentially to thymidine and is more thermostable than HSV-1 and HSV-2 isoenzymes[248]. All ribonucleoside- and deoxyribonucleoside triphosphates, with the exception dTTP, may serve as phosphate donors; moreover the enzyme phosphorylates series of nucleosides including some analogues[248]. Using antibodies against purified deoxypyrimidine kinase it has been shown that the enzyme is localized in nuclei of infected cells[249]. The encoding region of the enzyme was identified by transfecting cloned viral DNA fragments in TK^- murine cells followed by their transformation to TK^+ phenotype[250] and subsequent comparison of sequence homology with HSV thymidine kinase gene[25]. The enzyme subunit is composed of 341 amino acids[25]. On the basis of amino acid homology with HSV

thymidine kinase it has been found that the ATP-binding domain corresponds to region 12 – 29 while nucleoside-binding domain to region 129 – 145 (refs[251,252]). The mutation map of deoxypyrimidine kinase gene in VZV strains resistant to acyclovir (ACV)[253] and to 5-bromovinyldeoxyuridine (BVdU)[254] revealed that the enzymes from ACV-resistant and wild-type strains differed by a single amino acid substitution : Pro-Leu (position 154) or Gln-Arg (position 130) or stop codon in position 225. All of these strains induce an enzyme with very low deoxycytidine kinase activity of deoxypyrimidine kinase[253]. Enzymes from two BVdU-resistant strains have their polypeptide chain prematurely terminated in position 69 and 162; this results in the deletion of deoxypyrimidine kinase activity[254].

11.5. *EBV Thymidine (Deoxypyrimidine) Kinase*

It is well known that after chemical activation of EBV from Burkitt's lymphoma cells new deoxypyrimidine kinase activity appears[255]. The presence of EBV-associated deoxypyrimidine kinase has been reported after superinfection and chemical activation of non-producer Raji cells[256 - 260] and notwithstanding low levels of viral proteins it has been purified[259,260]. The following data support the virus-encoded character of the enzyme[261]:

a) Mouse leukemic cell line TK^- is transformed to TK^+ phenotype by transfection of a discrete region of EBV genome and newly formed protein exhibits antigenic cross-reactivity with HSV-1 thymidine kinase.

b) Plasmid expressing ORF BXLF 1 of EBV genome can complement the defect in TK^- deficient strains of *E. coli*.

Furthermore, a significant amino acid homology of the product of BXLF 1 gene which encodes for 607 amino acids to HSV thymidine kinase was observed especially in positions 284 – 301 (ATP-binding domain) and 292 – 408 (nucleoside-binding domain)[35]. However, the first 243 amino acids from N-terminus of EBV polypeptide chain have no counterparts in HSV TK and their role is unknown.

The expressed enzyme was isolated[261] and characterized[262]. Similarly to VZV and HSV thymidine (deoxypyrimidine) kinase it utilizes all ribonucleoside triphosphates and with the exception of dTTP also deoxyribonucleoside triphosphates as phosphate donors. It phosphorylates a broad range of substrates, including some antiviral nucleoside analogues, but in contrast to VZV and HSV enzyme it has a much lower deoxycytidine kinase activity. This may explain the relative inefficiency of acyclovir phosphorylation in EBV-infected cells since it has been claimed that the ability to phosphorylate deoxycytidine correlates with the ability to accept acyclovir as a substrate[263].

Human cytomegalovirus does not encode for its own deoxypyrimidine kinase[29], but it can induce high level of cellular deoxyguanosine kinase[264 - 266] which may participate

in the phosphorylation of certain HCMV inhibitors, e.g., Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine][267].

11.6. *Ribonucleotide Reductase*

This enzyme was isolated and characterized from VZV-infected cells[268]. Similarly to HSV ribonucleotide reductase the induction of all four substrates is obviously realised at a single site. Similarly to its HSV counterpart the enzyme is inhibited in the presence of ATP or magnesium ions but in contrast to HSV the ATP–Mg^{2+} complex is also inhibitory. Isolated VZV ribonucleotide reductase differs immunochemically from the cellular and HSV enzyme; nevertheless, it has been observed that the antibody against 9 C-terminal amino acids of a small subunit of HSV-1 ribonucleotide reductase precipitates from extracts of VZV-infected cells a protein with similar molecular weight[164]. On the basis of amino acid sequence homology of ORF potentially encoding for ribonucleotide reductase in VZV and EBV genomes, the molecular weight for large and small subunit of VZV enzyme appears to be 87 and 35 kDa (ref.[162]), for EBV enzyme 93 and 34 kDa (ref.[269]). The only evidence for the existence of EBV ribonucleotide reductase is the observation that in extracts from virus producer cells following chemical induction and in non-producer cells after superinfection ribonucleotide reductase activity appears to be much more resistant to hydroxyurea than in case of non-induced cells[270].

11.7. *dUTPase*

This enzyme has been found up to now to be virus specific only in EBV transformed cells following superinfection and chemical induction[271].

11.8. *Deoxyribonuclease (DNase)*

Much information has been obtained in case of EBV DNase. The enzyme has been found in extracts from superinfected[272 - 274] and chemically induced[275 - 277] Raji cells and virus-producer cells[273,277 - 279]. Enzyme preparations of different purity have been isolated[272,275,277 - 279] and ORF encoding EBV DNase (BGLF 5) has been defined; in transcription–translation system its cDNA produces a protein with characteristics of virus enzyme[280,281]. EBV DNase was expressed in *E. coli*, the enzyme was purified to homogeneity and characterized[282,283]. The expressed enzyme does not differ in principle from enzyme isolated from infected cell cultures. Its molecular weight is 52 kDa, it possesses exo- and endonuclease activity, pH optimum is in alkaline range and it requires magnesium ions. It is relatively sensitive to high ionic strengh (50% inhi-

bition at 60 – 100 mM-KCl) (refs[272,279,282]), although residual activity has been observed at 300 mM-KCl (ref.[283]); at this concentration the enzyme from non-induced cells is completely inhibited[276]. No substrate specificity is known. It has been reported that the enzyme preferentially cleaves A–T rich DNA (ref.[278]).The role of EBV DNase is unknown. It is possible that it participates in degradation of cellular DNA (ref.[274]) or it may split concatemers of viral DNA to genomes of individual length[283]. In extracts from VZV infected cells DNase activity was found to be 7-fold increased in comparison with mock-infected cells; however, it is not known whether the enzyme is encoded by virus[248].

11.9. *Protein Kinase*

Protein kinase activity was found in EBV virions[284] and in EBV nuclear antigen (EBNA) (ref.[285]) phosphorylating preferentially serine and threonine residues. VZV induces protein kinase which phosphorylates serine and threonine from viral glycoprotein gp I (ref.[286]). In HCMV, protein kinase activity is associated with 68 kDa structural protein which preferentially phosphorylates serine and threonine but not tyrosine residues of virion proteins[287] and also acid proteins, e.g., casein[288 – 290].

11.10. *Thymidylate Synthetase (5,10-Methylenetetrahydrofolate: dUMP-Methyltransferase, EC. 2.1.1.45)*

The enzyme catalyses reductive methylation of dUMP to dTMP. The only human herpesvirus which encodes for this enzyme is VZV. A computer-assisted homology search of human herpesvirus genomes revealed that only VZV gene 13 is similar to other eucaryotic and procaryotic thymidylate synthetase genes[46]. The expression of VZV gene 13 in *E. coli* mutant unable to grow in minimal agar without thymine led to the formation of protein with thymidylate synthetase activity possessing the same molecular weight (32.5 kDa) as its counterpart from VZV infected cells[291].

12. CONCLUSIONS

The progress in our knowledge of the function of virus-encoded proteins including the detailed protein stereochemistry of most promising drug targets might appear decisive for the development of new antiherpetic drugs. The beginning of this period is marked by studies of DNA polymerase and thymidine kinase which are and probably in the near future will remain main targets of antiherpetic chemotherapy. Similar development can be awaited in other herpesvirus enzymes, and in not too distant future the detailed information about their structure and function may lead to the synthesis of specific inhibitors.

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