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Mini-Review

The structure and function of the HSV DNA replication proteins: defining novel antiviral targets

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Summary

The absolute dependence of herpes simplex virus (HSV) replication on HSV DNA polymerase and six other viral-encoded replication proteins implies that specific inhibitors of these proteins' functions would be potent antiviral agents. The only currently licensed anti-herpes simplex drug, acyclovir, is an inhibitor of HSV DNA polymerase and is widely held to block viral replication primarily by specifically inhibiting viral DNA replication.

In spite of the substantial advance in HSV therapy in recent years through the introduction of acyclovir, this anti-HSV compound and most of the other compounds under pharmaceutical development are substrate analogs. Since antiviral drug resistance has become an issue of increasing clinical importance, the need for structurally unrelated agents which incorporate novel mechanisms of viral inhibition is apparent.

Understanding the structure and function of herpesvirus DNA polymerase and its interaction with the other six essential replication proteins at the replication origin should assist us in designing the next generation of therapeutic agents. The sequences of these proteins have been deduced and the proteins themselves have been expressed and purified in a variety of systems. The current challenge, therefore, is to use the available information about these proteins to identify and develop new, exquisitely specific antiviral therapeutics.

In this review, we have summarized the current approaches and the results of structure/function studies of the herpes virus proteins essential for DNA replication, with the goal of more precisely defining novel antiviral targets.

HSV replication protein; HSV DNA polymerase; Antiviral

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Introduction

Herpes simplex virus (HSV) normally replicates by productive lytic infection (Challberg and Kelly, 1989). In contrast to genetically simpler viruses such as simian virus 40 (SV40), HSV has the potential to encode at least 72 distinct proteins including many, if not all of the proteins that are involved in the expression, replication and packaging of its genome (McGeoch et al., 1988). In developing novel therapeutics, therefore, HSV would seem to offer a wide variety of unique, viral proteins as potential drug targets ranging from encapsidated transcriptional trans-activators (Olivio et al., 1989; O'Hare, 1991) to proteins which participate late in viral assembly (Liu and Roizman, 1991; Baines et al., 1991).

Selective interference with HSV DNA replication has already been demonstrated clinically as an effective and selective antiviral strategy (Keating, 1992), and all seven essential viral-encoded replication proteins are attractive targets for antiviral intervention. To date, however, only the viral-encoded DNA polymerase (pol) has been exploited as an antiviral target (Davey, 1990). Over the past three decades, a number of anti-pol agents have emerged for preclinical evaluation (Scheffer, 1988). A troubling observation, however, is that we are increasingly encountering HSV virus strains that are resistant to acyclovir, particularly in immunocompromised individuals (Hirsch and Schooley, 1989; Englund et al., 1990; Freifeld and Ostrove, 1991). The essential problem is that there are only two major structural classes of anti-herpes drugs (Coen, 1991): a wide variety of nucleoside analogs, such as acyclovir (Elion, 1982; Matthews and Boehme, 1988), or analogs of pyrophosphate, such as foscarnet (Oberg, 1989). An example of the potential scope of this resistance is illustrated with acyclovir. Four distinct groups of single mutations that confer resistance to acyclovir exist and appear to emerge at a rapid rate (Coen, 1991). While mutations in three of these groups affect the viral-encoded thymidine kinase (TK) required to activate this prodrug, the fourth group of mutations includes viruses with alterations within the HSV pol gene. Double mutants with alterations in both TK and pol have also been isolated. Many acyclovir-resistant polymerase mutants are also resistant to foscarnet (foscavir, PFA). Additional results suggest that drug-sensitive viruses can complement drug-resistant viruses resulting in enhanced pathogenicity. The emergence of an HSV virus with mutations in both TK and pol that could be resistant to all clinically useful anti-HSV drugs and retain considerable pathogenicity is possible.

In view of the impending clinical inadequacies of the available compounds, how should we search for more potent and specific HSV inhibitors of HSV DNA replication? Traditionally, drug screens have been brute force exercises, until a useful lead compound is identified. Unfortunately, probably less than 1 in 10 000 compounds synthesized from such a lead compound becomes a clinically useful drug (Prusoff et al., 1990). This is largely because the refinement of lead compounds is initially a random process and quantitative

structure–activity relationships (QSAR) can be derived and applied only after establishing a large database of compounds. However, tremendous progress in molecular genetics, immunology and molecular modelling have now been applied to herpes virology. Applying these technologies to explore the structures and mechanisms of action of the HSV replication proteins should help identify novel screens which can rapidly identify new pharmacophores and enhance the selectivity of drug action. As we gain more knowledge of the replication protein structures, we may be able to design compounds that are less likely to rapidly develop drug-resistant viruses.

Herpes Simplex Virus DNA and Genetics of HSV DNA Replication Proteins

Herpes simplex virus type 1 DNA is a linear double-stranded molecule of approximately 153 kilobase pairs in size (McGeoch et al., 1988). The genome consists of two components, designated L and S, each of which is flanked by inverted repeat (IR) sequences (Fig. 1). As a result, the HSV genome is terminally redundant (Sheldrick and Berthelot, 1974; Roizman, 1979). The HSV genome contains 3 replication origins designated ori_L and ori_S which are closely related in sequence (Vlazny and Frenkel, 1982; Lockshon and

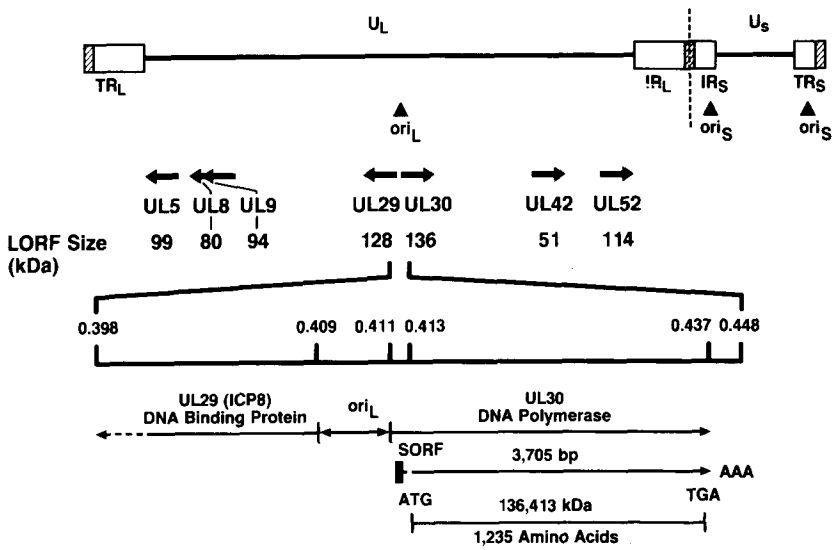

Fig. 1. HSV genome organization. A representation of the HSV-1 genome with the unique long (U_L) and the unique short (U_S) sequences is shown, together with the internal and terminal repeat elements (IR and TR). The location of the unique origin of replication within the long sequence (ori_L) and the diploid origins of replication in the short sequence (ori_S) are indicated. The relative genome positions, direction of transcription and the size of polypeptides (kDa) predicted from the long open reading frame (LORF) of the seven essential DNA replication proteins are also shown (adapted from Challberg and Kelly, 1988). A more precise positioning of UL29 (ICP8) and the short (SORF) and long (LORF) open reading frames of HSV DNA polymerase (UL30) relative to ori_L is provided (adapted from Yager and Coen, 1988).

TABLE 1

Seven *trans*-acting replication proteins of HSV-1

LORF	Putative function(s) in DNA replication
UL5	Helicase-primase activity in association with UL52
UL8	Co-purifies with UL5/UL52 complex Primer stabilization
UL9	Site-specific origin binding protein Origin specific helicase
UL29	Non-specific binding to single stranded DNA Co-localizes with pol within discrete nuclear areas
UL30	5′-3′ DNA polymerase activity 5′-3′ Exonuclease (RNase H-like) activity 3′-5′ Exonuclease (proofreading) activity Binds to UL42
UL42	Binds stoichiometrically to polymerase (UL30) Increases polymerase processivity
UL52	Helicase-primase activity in association with UL5

(Adapted from Hammerschmidt and Mankertz, 1991.)

Galloway, 1988). These origins may be functionally equivalent, although at least one copy of ori_S can be deleted without apparent affect (Polvino-Bodnar et al., 1987).

Many conditional lethal mutants of HSV DNA replication in cell culture have been isolated and characterized (Schaffer et al., 1987). Several of these mutations affect viral DNA synthesis indirectly, either by altering proteins that are trans-activators or enzymes which are involved in DNA metabolism. Other mutations, however, directly affect HSV DNA replication, and seven complementation groups of such mutants are known (Challberg and Kelly, 1989; Weller, 1990). Through the application of a transient origin-dependent plasmid amplification assay, seven viral genes that are required for HSV DNA replication have been independently identified (Challberg, 1986; Wu et al., 1988). The positions within the genome, direction of transcription and predicted molecular weights (kDa) of the proteins potentially encoded by the long open reading frames (LORF) of the seven replication genes (UL5, 8, 9, 29, 30, 42 and 52) identified in these assays are shown in Fig. 1. The putative functions of these seven *trans*-acting replication proteins are summarized in Table 1.

One of the most effective means for studying the effects of defects in these seven genes on virus replication has been through the construction of targeted gene-deleted null-mutants. This approach has been used to create mutants with deletions in each of the seven genes required for HSV-1 DNA replication (Orberg and Schaffer, 1987; Goldstein and Weller, 1988; De Bruyn Kops and

Knipe, 1988; Carmichael et al., 1988; Marcy et al., 1990c; Carmichael and Weller, 1991; Johnson et al., 1991; Zhu et al., 1992a). There is a rigid correlation between the genes required for DNA synthesis, deduced from the transient plasmid amplification system, and the genes required for viral DNA replication, identified by mutational analysis. Use of these mutants has already begun to facilitate the characterization of important functional domains within specific proteins (Marcy et al., 1990a). The availability of these mutants should be useful in clarifying both the importance of domains within individual proteins on viral pathogenicity and drug sensitivity and the interrelationships between the replication proteins.

Regulation of the Expression of HSV Replication Proteins

Transcription and accumulation of transcripts encoding the replication proteins display delayed-early kinetics, consistent with proteins involved in viral DNA replication (Zhang and Wagner, 1987). As with the majority of HSV primary precursors, the mRNAs encoding the replication proteins are unspliced (Roizman and Sears, 1990). In perhaps the best studied of these transcripts, the majority of mature pol transcripts are co-linear with the pol gene (Holland et al., 1984; Yager and Coen, 1988); however, minor species which could generate a new spliced variant of pol with a modified N-terminus have been described (Bludau and Freese, 1991). As a result, cell-type-specific HSV-1 pol gene products are possible (Bludau and Freese, 1991). The function, if any, of the modified N-terminal pol polypeptides is currently unknown, although they could be important if two distinct HSV pol molecules synthesize different DNA strands.

Although pol mRNA is transcribed at a rate comparable to that of several other delayed early viral genes, pol protein is not as abundant as either thymidine kinase or ICP8 (UL29 gene product). In fact, four of the proteins (UL5, UL8, UL9 and UL52 gene products) are made in smaller amounts compared to the other three. Pol expression is apparently regulated by inefficient translation mediated by a sequence other than the short upstream open reading frame (SORF, Fig. 1), and this leads to an early shut-off of polymerase synthesis during HSV infection (Dorsky and Crumpacker, 1988; Yager et al., 1990). The role of the SORF in the regulation of pol expression is unclear at this time (Marcy et al., 1990c).

Two of the seven genes, UL29 (ICP8) and UL30 (POL) flank the unique ori_L palindrome of HSV-1 (Quinn and McGeoch, 1985; Fig. 1). The close localization of origins of replication and transcriptional control regions has been observed in cellular and especially viral genomes of SV40, polyomavirus, bovine papilloma virus 1, adenovirus and Epstein-Barr virus. Presumably, this arrangement is important for an interrelation of transcription and replication control, and these elements may increase the efficiency of DNA replication in vivo (DePamphilis, 1988; Virshup, 1990). Antiviral strategies to either restrict

the abundance or translation of the mRNA for the seven replication proteins or to relax the coordination of the transcription of pol and ICP8 during origin-dependent replication may best be addressed by anti-sense technology (James, 1991).

The seven essential replication proteins are not subject to extensive post-translational modification, although the UL42 gene product is modified by phosphorylation (Marsden et al., 1987; Thomas et al., 1988). Whether this phosphorylation is essential to UL42 function is uncertain. All seven replication proteins are localized to the nucleus, suggesting the presence of as yet unidentified localization signals (Olivio et al., 1989). Once they are synthesized, the seven replication proteins turn over slowly.

Biochemistry of HSV DNA Replication

UL29: The single-stranded DNA binding protein

The functions of the products of most of the seven essential replication genes have only recently been characterized, with the UL29 gene product (ICP8) among the more extensively studied. UL29 may function in both the regulation of viral gene expression (Godowski and Knipe, 1985; Gao and Knipe, 1989; Gao and Knipe, 1991) and in viral DNA replication (Bush et al., 1991; De Bruyn Kops and Knipe, 1988). UL29 may stimulate the replication of ssDNA templates by binding single-strand DNA and acting as a classical helix-destabilizing protein (Hernandez and Lehman, 1990). This function of UL29 would seem analogous to the single strand binding protein of phage T4 or perhaps more appropriately RF-A, which is required for in vitro SV40 replication (Hurwitz et al., 1990; Diffley and Stillman, 1991). HSV polymerase can localize to the cell nucleus without other viral proteins; but a functional UL29 is apparently required for polymerase to localize to pre-replicative sites (De Bruyn Kops and Knipe, 1988). An additional function of UL29, therefore, may be to organize the structure and composition of the nuclear replication compartments. It would be of considerable interest from an antiviral perspective to define the amino acid residues which participate in the interaction between the UL29 and pol proteins (Bush et al., 1991). The interesting prospect of interfering in the assembly of pre-replication complexes could result in replication proteins that would be inappropriately distributed within the nucleus and therefore, non-functional.

UL9: Origin of replication binding protein

The UL9 protein consists of an 83-kDA polypeptide which exists as a dimer. It binds specifically to the origins of DNA replication at three nearly identical sites (Elias and Lehman, 1988; Olivio et al., 1991). Although the role of UL9 in HSV replication is unresolved, by analogy to other eukaryotic origin binding

proteins, the binding of the UL9 to the origin sequences may initiate the assembly of the multiprotein replication complex. Consistent with this hypothesis, UL9 appears to contain an intrinsic helicase activity and a consensus ATP-binding domain, similar to that found in both the SV40 T-antigen and *E. coli* dna A proteins. If the simultaneous, coordinated occupation of multiple sites within the viral replication origins is essential for the initiation of replication, interference with either UL9-DNA binding or UL9 dimerization would suggest highly specific viral targets.

UL5, UL8 and UL52: Helicase-primase complex

HSV-infected cells induce novel helicase and primase activities (Crute et al., 1988). The helicase and primase are components of a heterotrimer consisting of the products of the UL5, UL8 and UL52 genes in a 1:1:1 ratio (Crute and Lehman, 1991). A stable sub-assembly consisting of only the UL5 and UL52 gene products has been purified to near-homogeneity from insect cells doubly-infected with baculovirus recombinants for these two genes. This purified complex has DNA-dependent ATPase, DNA dependent GTPase, DNA helicase and DNA primase activities that are characteristic of the three subunit holoenzyme (Dodson and Lehman, 1991). The purified UL8 gene product, although required for viral DNA replication, does not exhibit these enzymatic activities nor does it appear to stably associate with either the UL5 or UL52 gene products (Calder and Stow, 1990). The helicase uses either ATP or GTP as a substrate and moves in the 5′ to 3′ direction on the strand to which it binds (Crute and Lehman, 1989). UL5 appears to encode several sequence motifs that are shared by cellular helicases (Zhu and Weller, 1992). The primase activity associated with the enzyme complex catalyzes the synthesis of oligoribonucleotides 8-12 bases in length. The multi-enzyme (UL52/UL5) complex may prime lagging-strand synthesis, as it unwinds DNA at the replication fork; UL8 may be involved in transport of the complex into the nucleus (Crute and Lehman, 1991). The helicase-primase activity is an attractive antiviral target, since DNA replication would be blocked in the absence of activity. In spite of homology with cellular helicases, it may be possible, by analogy to DNA polymerase inhibitors, to identify novel nucleotide inhibitors of this complex. A more attractive strategy, however, may be interference with the coordinated action of the complex by directly interfering with subunit assembly.

UL42: Double-stranded DNA binding/polymerase accessory protein

The UL42 protein was initially identified as a 65-kDa double-stranded DNA binding protein which co-purified with the HSV-1 DNA polymerase (Ostrander and Cheng, 1980; Gallo et al., 1988; Parris et al., 1988). A highly purified preparation of the HSV-1 DNA polymerase consists of the UL42 protein in a 1:1 complex with the 136 kDa pol polypeptide (Crute and Lehman,

1989). The 140 kDa pol protein of HSV-2 also co-purifies with a 55 kDa polypeptide designated ICP34/35 serving as an HSV-2 analogue of the HSV-1 UL42 protein (Powell and Purifoy, 1977; Vaughan et al., 1985).

This pattern is repeated throughout the other human herpes viruses. For example, although the UL42 and Epstein Barr virus (EBV) BMRF-1 gene products do not share extensive homology, the BMRF-1 product may function as an EBV DNA polymerase accessory protein (Kiehl and Dorsky, 1991). Purified HCMV DNA polymerase also consists of two proteins of approximately 140 kDa (HCMV pol) and 58 kDa (ICP36); VZV polymerase interacts with a DNA binding protein (gene 16) and this gene 16 protein shows considerable sequence similarity to the HSV-1 polymerase associated DNA binding protein UL42.

Addition of purified UL42 protein to purified HSV polymerase results in a substantial increase in the polymerization of deoxynucleotides, resulting in higher molecular weight polynucleotides (Gallo et al., 1989; Hernandez and Lehman, 1990). The regions within UL42 which interact with HSV polymerase have been partially defined (Gallo et al., 1989), suggesting that the UL42 protein is an accessory factor that interacts functionally with the HSV DNA polymerase to enhance processivity. UL42 may act as a sliding clamp that tethers the polymerase to the primed template in a manner analogous to the beta subunit of the *E. coli* DNA polymerase III holoenzyme (Stukenberg et al., 1991). Whether the stimulation of processivity by UL42 defines its singular, essential role in HSV DNA replication has not yet been fully resolved.

UL30 (HSV DNA pol): General protein structure and enzymatic activities

In contrast to the other essential replication proteins, HSV-1 DNA polymerase has been extensively purified and studied in many laboratories. It was recognized very early in the studies of HSV replication that extracts of HSV-infected cells contain a novel DNA polymerase activity distinguishable from endogenous cellular polymerases by ionic strength and DNA template requirements (Weisbach et al., 1973; Powell and Purifoy, 1977; Knopf, 1979).

Catalytically active HSV pol, as measured by the incorporation of labelled nucleotides into exogenous templates, has been expressed in vitro (Dorsky and Crumpacker, 1988), in yeast (Haffey et al., 1988), and in SF9 insect cells using recombinant baculovirus virus (Marcy et al., 1990b). The enzyme consists of a monomer of a single 140 kDa polypeptide, in good agreement with the protein predicted from DNA sequence analysis of the long open reading frame (LORF). As shown in Fig. 1, the polymerase gene includes a 3705 bp major open reading frame capable of encoding a 136,314-kDa polypeptide. The deduced sequences of pols from several HSV-1 strains are known and the amino acid differences have been examined (Quinn and McGeoch, 1985; Gibbs et al., 1985; Knopf, 1986; Larder et al., 1987; Knopf and Weisshart, 1988). In comparison, the complete sequence of the HSV-2 polymerase gene includes a 3720 bp major open reading frame capable of encoding a 137,354-kDA (1240

amino acid) polypeptide and is 95% homologous with pol from HSV-1 (Tsurumi et al., 1987). The HSV DNA polymerase alone is moderately processive. The most highly purified preparations of polymerase have catalytic activity on simple primer templates such as poly(dC):oligo(dG) (Knopf, 1979). The HSV pol, like many other polymerases, contains an intrinsic 3′ to 5′ exonuclease activity (Kunkel, 1988; Bernad et al., 1989; Gibbs et al., 1991). As demonstrated by their nearly identical response to thermal inactivation, drug inhibition and neutralization by polyclonal antibodies, there is a tight linkage of the polymerizing and 3′ to 5′ exonuclease activities (Knopf and Weisshart, 1990).

HSV pol may also encode a 5′ to 3′ (RNase-like) exonuclease activity (Crute and Lehman, 1989). By contrast, none of the five cellular polymerases has an associated 5′ to 3′ exonuclease activity (Wang, 1991), and the presence of this unique activity remains controversial (Strick and Knopf, 1991). However, most recently, the 5′ to 3′ exonuclease activity was observed in HSV pol expressed and purified from baculovirus recombinant-infected cells, suggesting that both activities are associated with the same gene product (Marcy et al., 1990b).

Genetic structure and predicted functional domains

An extremely important approach to drug discovery is the combination of X-ray crystallography to define the target structure, the sites of interaction of antiviral compounds and/or substrates with the target, and computer-assisted drug design to model new compounds that would bind to those sites (Chapman et al., 1990; Otto and Eustice, 1991). Studies with rhinovirus 14 and the antiviral compound disoxaril provide an example of the use of X-ray crystallography in combination with a known inhibitor to develop and evaluate a family of new inhibitors (Smith et al., 1986; Diana et al., 1987).

In the absence of a crystallographic structure, conservation of sequences in similar enzymes implies that these regions are required for similar enzymatic function (Earl et al., 1986; Bernad et al.,1987; Wong et al., 1988; Becker, 1988; Gibbs et al., 1988). The isolation and sequencing of 40 different DNA pol genes (Ito and Braithwaite, 1991) has helped to further define conserved regions in these DNA polymerases and suggest their probable function in the HSV pol.

Putative catalytic/deoxynucleotide interaction domain

Sequence comparison of various prokaryotic and eukaryotic DNA polymerases has revealed a striking evolutionary conservation of several groups of amino acid sequences (Wang, 1991; Fig. 2), and suggested that these polymerases may all have derived from a common primordial gene. The regions shown in Fig. 2 are designated I to VI according to their extent of conservation, with region I the most conserved and region VI the least. A seventh region (VII, amino acids 938–946) has recently been identified (Hwang et al., 1992). The potential functional significance of these seven regions is underscored by their relative linear spatial relationship within each polymerase polypeptide; the relative order being IV-II-VI-III-I-V. The lengths of the amino and carboxy

termini and the distance between the segments is variable. Sequence comparison of human DNA pol α and HSV pol (amino acids 437 to 963) reveals homology in all seven conserved regions (Wong et al., 1988; Hwang et al., 1992).

The presence of the three consensus sequences, regions I, II and III in the DNA polymerases from bacteriophage to mammals suggests that these separate sequences serve in the most essential functions in polymerase catalysis. Region I contains the sequence YGDTDS which is most highly conserved among the α-like polymerases. Site-specific mutagenesis of region I resulted in non-functional enzyme in HSV-1 pol (Dorsky and Crumpacker, 1990), as well as several other polymerases (Joung et al., 1991) confirming that region I may be critical for the catalytic function of the α-like polymerases. A similar motif is also found in viral reverse transcriptases and RNA-directed RNA polymerases (Jablonski et al., 1991).

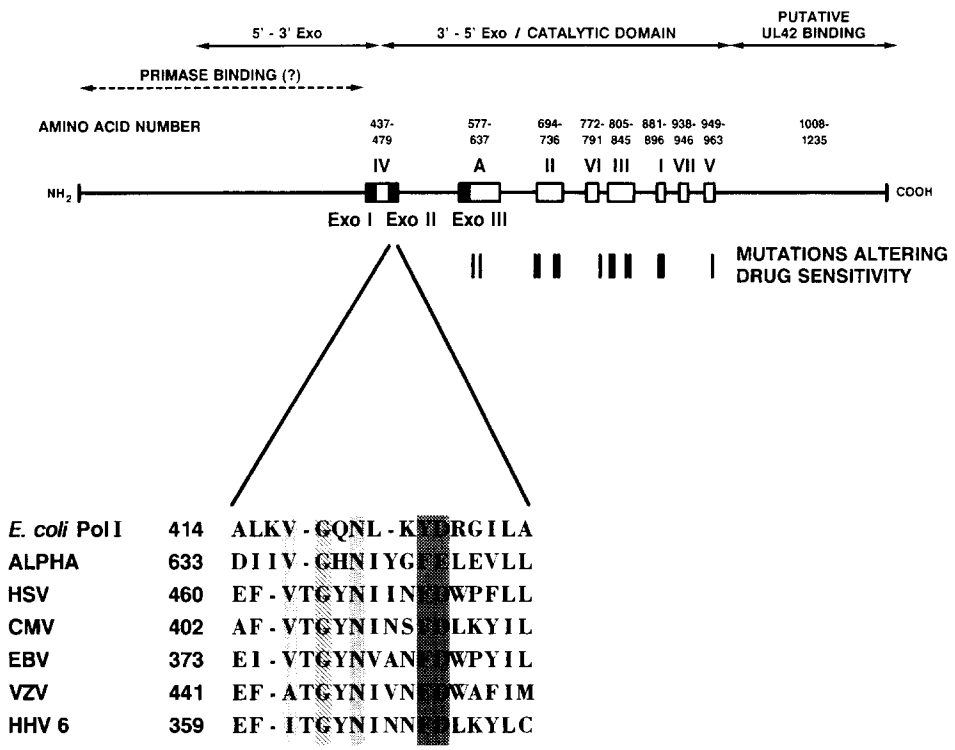

Fig. 2. Organization of the HSV-1 DNA polymerase polypeptide. The relative positions of the putative primase, 5′-3′ and 3′-5′ exonuclease, catalytic and UL42-binding domains within the HSV polymerase polypeptide are shown. The regions of sequence homology in both 3′-5′ exonuclease (EXO I–III) and catalytic (I–VII) domains which HSV polymerase shares with several other polymerases are indicated. A more detailed comparison of the amino acid homology of the herpes viruses within the EXO II region is shown. The approximate location of mutants in HSV pol which alter drug sensitivity are marked (adapted from Gibbs et al., 1988 and Gibbs et al., 1991).

Region I may be involved in substrate recognition, since several mutant constructs in this region show resistance to acyclovir triphosphate and phosphonoacetic acid (PAA) and hypersensitivity to aphidicolin (Marcy et al., 1990a). Argos has presented a hypothetical model for the sequence YGDTD as a loop structure (Argos, 1988) and Delarue and co-workers have suggested that region I in pol α-like enzymes is located within a loop at the end of a beta hairpin, equivalent to the beta hairpin formed by strand 12 and 13 of *E. coli* polymerase I (Delarue et al., 1990). The fact that Region I is critical for polymerase function makes it a vulnerable target for inhibition. However, its extreme conservation among polymerases makes selective inhibition difficult to attain. For example, anti-peptide sera to HSV Region I neutralized both viral and cellular (α) polymerase activities (unpublished results).

An additional method to obtain information on the structure of an enzyme's catalytic site, in the absence of direct crystallographic data, is to determine the sequence changes in mutants which alter enzymatic behavior towards substrate and substrate analogues. Since current antiviral drugs mimic and compete with natural deoxynucleotides (dNTPs) or pyrophosphate (PP_i) substrates for enzyme binding, mutations may identify amino acids that are involved in recognition of these substrates. In HSV pol, mutations conferring altered sensitivity to antiviral deoxynucleoside triphosphate analogs, pyrophosphate analogs or aphidicolin map within the region encoding the carboxy terminal portion of the polymerase (Knopf et al., 1981; Gibbs et al., 1985; Knopf, 1987; Tsurumi et al., 1987; Larder et al., 1987; Hall and Woodward, 1989; Hall et al., 1989; Hwang et al., 1992). Many of the mutants are single amino acid substitutions within consensus regions II and III (Fig. 2). These regions may be directly involved in dNTP binding or pyrophosphate hydrolysis. The identification of any single region as the sole binding site for dNTPs, PP_i or aphidicolin has not been possible, suggesting that the interaction of these compounds with polymerase requires the folding of several non-contiguous regions in the polymerase.

Much of the available research on HSV pol mutants has focused on mapping the positions of mutations to well-characterized antiviral agents. In an effort to characterize novel nucleoside analogs, we generated a variety of mutated HSV polymerases by site-directed single amino acid changes, each altered at amino acid residue 696 (Region II), 724 (Region II) and 815 (Region III) associated with antiviral phenotypes against standard drugs (Gibbs et al., 1985; Larder et al., 1987; Fig. 2). These mutations in HSV pol were expressed in *Saccharomyces cerevisiae* under the control of an inducible galactose promotor (Haffey et al., 1988; Matthews et al., 1989). Extracts were analyzed for HSV polymerase specific activity and for a minimum two-fold increase in resistance (R) or sensitivity (S) to antiviral compounds compared to both wild type HSV polymerase purified from infected HeLa S3 cells and expressed in yeast (pRC205, see Table 2). The antiviral phenotype of the parental mutants (pRC208, pRC207 and pRC206) which we generated were indistinguishable from those previously reported (Larder et al., 1987). Within this framework, we

TABLE 2

Sensitivity of HSV-1 DNA polymerase mutants to aphidicolin, phosphonoacetic acid, acyclovir triphosphate and ganciclovir triphosphate

Yeast lysate	(Mutation)*	APH	PAA	ACV-TP	GCV-TP
pRC205	(wild type)	S	S	S	S
pRC400	(G355D)	S	S	S	S
pRC208	(Y696H)	R(8–10×)	HS	S	HS
pRC207	(S724N)	S	HS	R(7–8×)	S
pRC240	(S724D)	S	HS	R(20–25×)	S
pRC241	(S724Q)	S	HS	R(25–30×)	S
pRC242	(S724E)	S	HS	R(15–20×)	S
pRC243	(S724A)	S	HS	R(10–15×)	S
pRC244	(S724K)	S	HS	R(30–35×)	S
pRC245	(S724T)	S	HS	R(30–35×)	S
pRC247	(S724H)	S	HS	R(25–30×)	S
pRC401	(G355D/S724N)	S	HS	R(7–8×)	S
pRC206	(N815S)	S	HS	R(40–60×)	S
pRC220	(N815D)	S	S	S	S
pRC222	(N815Q)	S	S	R(2–4×)	S
pRC223	(N815T)	S	HS	R(40–60×)	S
pRC225	(N815L)	S	HS	S	S
pRC226	(N815V)	S	HS	S	S
pRC227	(N815Y)	S	HS	S	S
pRC228	(N815G)	S	S	S	S
pRC229	(N815A)	S	S	S	S
pRC230	(N815E)	S	HS	S	S
WT IC_{50}, μM		0.3–0.8	180	0.8–1.5	6.5–7

*The position of the amino acid substitution and the Dayhoff single letter amino acid code is shown. Phenotype: S, sensitive; HS, hypersensitive, R, resistant (fold reduction).

then asked what other amino acid changes were acceptable at these positions for retention of polymerase activity; and whether novel, cyclobutyl nucleoside analogs under development (Field et al., 1990; Terry et al., 1990) could inhibit these altered polymerases. As shown in Table 2, an entire spectrum of mutations at amino acids 724 and 815 is tolerated. The extent of resistance and sensitivity clearly indicate that mutations at these residues can produce distinguishable phenotypes (compare pRC223 and pRC228). Furthermore, as shown in Table 3, pol mutations (e.g. pRC223) resulting in significant resistance to acyclovir triphosphate (ACV-TP) may remain sensitive to some novel nucleotide inhibitors such as [1*R*(1α,2β,3α)]-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine triphosphate ((*R,S*)-BHCG-TP) and 9-[2-(phosphonomethoxy)ethyl]guanine diphosphate (PMEGpp). This method of creating defined mutations in pol resulting in altered antiviral phenotypes, should be useful in characterizing the interaction of new classes of antiviral agents with the polymerase target.

In spite of the structural similarities among polymerases in the conserved regions, antiviral drugs that mimic natural polymerase substrates can

TABLE 3

Sensitivity of HSV-1 DNA polymerase mutants to nucleotide analogs

Yeast lysate	(Mutation)*	(*R*,*S*)-HHCG-TP	(*R*,*S*)-BHCG-TP	(*R*)-BHCG-TP	HPMPGPP	PMEGPP
pRC205	(wild type)	S	S	S	S	S
pRC400	(G355D)	S	S	S	S	S
pRC208	(Y696H)	S	S	S	HS	S
pRC207	(S724N)	R(3×)	S	S	R(3×)	S
pRC240	(S724D)	S	S	S	S	S
pRC241	(S724Q)	S	S	S	S	S
pRC242	(S724E)	S	S	S	S	S
pRC243	(S724A)	S	S	S	S	S
pRC244	(S724K)	S	S	S	S	S
pRC245	(S724T)	R(3×)	S	S	R(5×)	S
pRC247	(S724H)	R(3×)	S	S	S	S
pRC401	(G355D/S724N)	R(4×)	S	S	R(3×)	S
pRC206	(N815S)	R(6×)	S	S	R(4×)	S
pRC220	(N815D)	S	S	S	S	S
pRC222	(N815Q)	R(4×)	S	S	R(4×)	S
pRC223	(N815T)	R(3×)	S	S	R(5×)	S
pRC225	(N815L)	R(3×)	S	S	S	S
pRC226	(N815V)	R(5×)	S	S	S	S
pRC227	(N815Y)	R(4×)	S	S	S	S
pRC228	(N815G)	R(3×)	S	S	S	S
pRC229	(N815A)	R(4×)	S	S	S	S
pRC230	(N815E)	R(4×)	S	S	S	S
WT IC_{50}, μM		1–1.5	0.4	0.3	1.3–1.7	1.5–2

*The position of the amino acid substitution and the Dayhoff single letter amino acid code is shown. Phenotype: S, sensitive; HS, hypersensitive, R, resistant (fold reduction).

selectively inhibit HSV-1 DNA polymerase, indicating that small differences in the conserved regions are important. We also sought to examine amino acid differences in conserved Region V and ask whether some amino acid sequences may be more specific to viral polymerase function than others. To do this, we have created a library of chimeric mutations and expressed them in *ts* polymerase I yeast cells (Budd and Campbell, 1987). The essential feature of this system is the observation that the yeast cells (ts in pol I) cannot grow at the non-permissive temperature (37°C), unless a functional polymerase is provided in *trans* by the complementing plasmid. Thus, by investigating the effect of amino acid substitutions in selected regions of the pol I gene, we can determine critical regions which are uniquely required for HSV pol function, compared to regions which can be interchanged between HSV-1 and the yeast polymerase.

The map of the expression vector and the region V sequences we mutated in yeast pol I to resemble HSV pol region V are shown in Fig. 3. The results of the complementation assay indicated that the intact α pol segment (pJT13) but not the intact HSV-1 region V (pJT17) can complement the yeast *ts* pol I mutation. If the HSV-1 segment is broken into shorter intervals (pJT14, pJT22 and

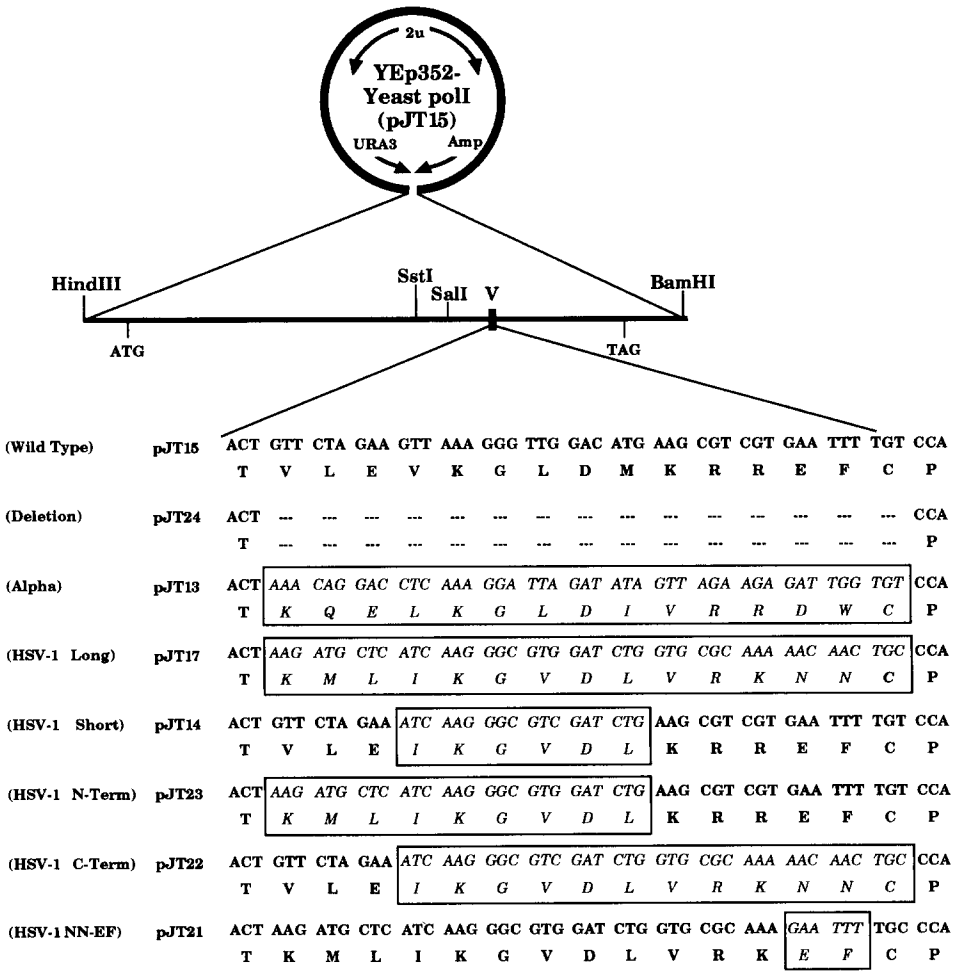

Fig. 3. Chimeric sequences within yeast polymerase I region V. A map of the yeast vector used to express wild type (YEp35-yeast pol I, pJT15) pol I and the position of region V in pol I is shown (black rectangle). The sequences deleted (pJT24, dashes) or mutated to express human KB DNA α polymerase or HSV-1 pol region V sequences (shown within the boxes) are illustrated.

pJT23), insertions similar to the carboxy segment of HSV-1 region V are the most disruptive. In yeast DNA pol I, the appearance of the small sub-motif EF is distinct from HSV-1 pol at these positions. If this dipeptide sequence is preserved in another HSV-1 region V sequence (pJT21), complementation is restored. Whether it is coincidental that an antiviral resistance mutant in HSV-1 pol (N961K) also occurs at these residues is presently uncertain. The results of this approach clearly indicate that subtle differences in region V can dramatically affect the ability of the *ts* DNA pol yeast cells to grow.

Although the extreme conservation of region I may limit chimeric

constructions within this sequence, this technique should be useful in dissecting any of the other moderately conserved pol regions and could form the basis of a genetic method for differentially screening polymerase inhibitors.

Putative primase domain

All DNA polymerases require a 3′ hydroxyl terminus of a pre-existing primer for addition of dNTPs. DNA polymerase α is the only known eukaryotic polymerase with a tightly associated primase (Burgers, 1989). Analysis of a temperature-sensitive mutant of yeast polymerase I identified a point mutation that confers a *ts* phenotype and maps to the consensus region IV as a single amino acid substitution of glycine (493) to arginine (Pizzagalli et al., 1988). This region of yeast pol I exhibits near perfect homology with human DNA pol α, suggesting a domain that is responsible for DNA primase/polymerase interaction; a comparable primase binding domain has not been identified in HSV pol by sequence alignment. Consistent with this prediction, HSV polymerase is not typically purified in association with the helicase-primase, suggesting the association, if any, during replication may be transient or of low affinity (Challberg, 1991).

Proposed exonuclease domains

The 5′ to 3′ exonuclease activity in HSV polymerase extracts is similar to that of *E. coli*. This exonuclease can apparently act on duplex DNA, as well as on RNA-DNA hybrids and may act to remove the RNA primers that initiate the synthesis of Okazaki fragments during lagging-strand synthesis (Crute and Lehman, 1989). Intragenic complementation suggests that the HSV pol contains a 5′ to 3′ exonuclease (RNase H) activity in a discrete N-terminal domain (see Fig. 2). Furthermore, sequence similarity among the N-termini of *E. coli* DNA polymerase I, T4 DNA polymerase and HSV DNA polymerase supports this assignment.

The 3′ to 5′ exonuclease active site of *E. coli* DNA polymerase has been defined by site-specific mutagenesis and X-ray crystallography (Ollis et al., 1985; Freemont et al., 1988; Derbyshire et al., 1988; Fig. 2). In an extensive analysis of pol sequences, Bernad et al. (1989) made the observation that three small, distinct regions of sequence are conserved among the α-like polymerases. In terms of the Klenow fragment structure, these regions are centered on the carboxylate metal ligands at the exonuclease active site (Joyce, 1991). As shown in Fig. 2, a similar sequence alignment has been suggested for HSV pol and other herpesviruses. An indirect test of the significance of the Exo I-III sequence alignments has been undertaken by introducing mutations in highly conserved amino acids in several of these regions (Gibbs et al., 1991). Several of the mutations resulted in a reduction in polymerase catalysis suggesting this region of HSV pol may be required for polymerase function.

Putative UL42 binding domain

Sequence comparison of human DNA polymerase α and yeast polymerase I

reveals a cysteine-rich region towards the carboxy terminus which can potentially form a DNA binding motif (Zn-finger) (Wong et al., 1988). A similar motif is absent in HSV pol; however, a series of carboxy terminal deletions in HSV pol has indicated that residues within the carboxy terminus could not be deleted without loss of polymerase activity. Anti-peptide sera to this region could also specifically neutralize HSV polymerase activity (Knopf and Weisshart, 1988; Weisshart and Knopf, 1988; Matthews et al., 1990). One interpretation is that an accessory DNA binding protein(s) may be involved in stabilizing the DNA-polymerase complex. Consistent with this, a discrete domain for the DNA binding protein, UL42 has been identified at the HSV pol carboxy terminus. The work from several laboratories suggests that a 227 amino acid region of the HSV polymerase carboxy terminus (1008-1235 amino acids) is both necessary and sufficient for a stable association with the accessory protein, UL42 and that this association stimulates polymerase processivity (Digard and Coen, 1991). In complementary experiments, several laboratories have defined residues within UL42 as dispensable in promoting complex formation with polymerase and stimulation (Gallo et al., 1989).

The possibility that peptides and non-peptide analogs which mimic the domains in either UL42 or the polymerase which promote complex formation could interrupt this interaction and interfere with HSV replication has been suggested (Digard and Coen, 1991). Marsden has presented evidence that peptide sequences corresponding to those in UL42 were inhibitory, as measured by a decrease in processivity in a primer-extension assay (Marsden, 1991). An analogy is the inhibition of the association of the two HSV ribonucleotide reductase subunits by peptide analogs (Gaudeau et al., 1992).

In identifying and developing peptide leads such as inhibitors to pol/UL42 complex formation, UL9 dimerization or UL5/UL8/UL52 complex formation, existing methods are limited both by their inability to generate and screen the requisite number of individual peptides and by the requirement of some knowledge of the structure of the interacting domains. The recent introduction of alternative methods of peptide synthesis (Lam et al., 1991; Houghton et al., 1991) should greatly accelerate the screening of very large numbers of short peptides in automated enzymatic or binding assays.

In SV40 viral DNA replication, it is reasonable to assume that there are protein-protein interactions between the T antigen and cellular replication proteins during the initiation of SV40 DNA replication. The results strongly suggest that initiation of DNA replication in vitro from the SV40 origin occurs by a sequential ordered protein-protein interaction and that there is a high stringency in the protein–protein interactions during the synthesis of the nascent DNA fragment at the origin of the replication fork (Hurwitz et al., 1990).

In the HSV system, which will presumably demonstrate the same stringency, only the contacts between the polymerase and UL42 proteins have been examined as sites for antiviral intervention. It should be clear, however, that the pairwise interaction of any of the seven essential replication proteins are

attractive antiviral targets. Conventionally, protein–protein interaction between two proteins have been studied using biochemical techniques such as cross-linking, co-immunoprecipitation and co-chromatography. However, a novel genetic system may be applicable as a general method to identify proteins that interact with a known protein by the use of a simple galactose selection (Fields and Song, 1989). In this system, the two interacting domains of the target proteins are converted to fusion proteins of the GAL4 DNA binding and activating domains. Interaction of the target proteins reconstitutes the GAL4 and results in expression of β-galactosidase. The clear advantage of this approach is that it cannot only function as a research tool to define the residues which contribute to protein–protein interactions, but could be adapted for large through-put antiviral screening. Moreover, the recent introduction of simplified technology (BIACore, Pharmacia) to measure directly the interaction of proteins should help to quantitate the effect of inhibitors on these complexes, as well as provide insights into their mechanism of action.

Three-dimensional structure of HSV polymerase: molecular modelling

Progress has recently been made in rational drug screening based on knowledge of the structure of the target enzyme. A computer search based on the three-dimensional structure of the active site of HIV-1 protease and chemical structures identified haloperidol as a weak inhibitor of the protease (DesJarlais et al., 1990). Other modelling approaches have led to the identification of other potent lead compounds (Blundell et al., 1990).

In view of amino acid sequence conservation between *E. coli* pol I and HSV pol, the important question arises whether HSV polymerase may fold into a pol I-like structure and whether this information, although of low resolution, may be useful in drug targeting. Similarity of folding among polymerases is at present unanswered, as the three-dimensional structure of no other DNA polymerase is available for a direct three-dimensional determination. However, based on the apparent similarities of important residues at the polymerizing and 3′ to 5′ exonuclease active sites, and similarities between the 5′ to 3′ exonuclease portions of other polymerases, it appears that molecular modelling may be appropriate (Joyce, 1991). In a parallel example, predictions about the HIV-1 RNase H domain based on *E. coli* pol I have proved to be quite accurate (Yang et al., 1990; Hughes, 1991). We have attempted to follow this strategy to develop a first generation molecular model of the HSV DNA pol catalytic core based on the available structure of the *E. coli* polymerase I Klenow fragment.

The structure of the Klenow fragment of *E. coli* DNA polymerase I has been refined to 2.75 Å (Ollis et al., 1985). The three-dimensional structure of the Klenow fragment reveals that the 605 amino acid polypeptide is folded into two structural domains of approximately 200 and 400 amino acids. The discrete, smaller N-terminal domain contains the 3′-5′ exonuclease activity (Derbyshire et al., 1988). As shown in Fig. 4A, the larger C-terminal domain contains a deep cleft that binds duplex DNA and contains the active site of polymerization. Mutations that change polymerase I V_{max} and K_m values are

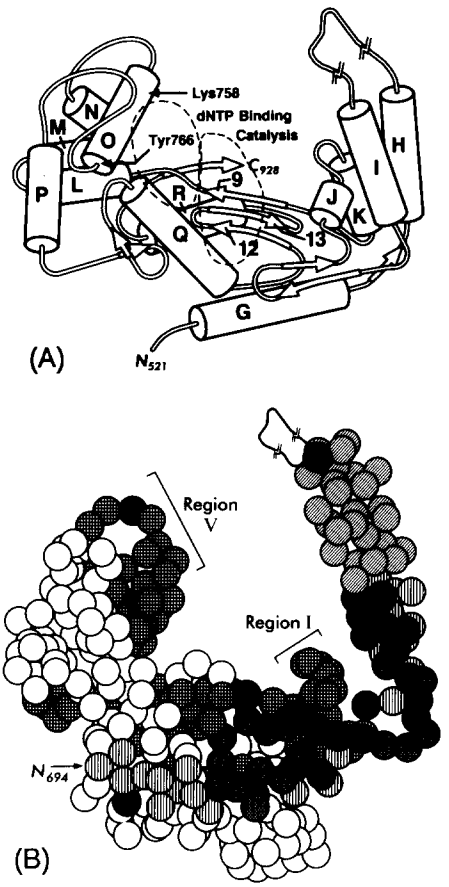

Fig. 4. The catalytic domains of *E. coli* polymerase I and HSV-1 polymerase. (**A**) The three-dimensional structure of the *E. coli* polymerase I catalytic domain (Klenow fragment) as determined by X-ray crystallography is shown (Joyce and Steitz, 1987). The N-terminal 3′-5′ exonuclease domain of the Klenow fragment is not shown. The structure includes the prominent cleft consisting of helical walls with a predominantly beta-sheet canyon. The disordered transition of the H and I helices and the residues of the O helix (Lys 758 and Tyr 766) which can be specifically labelled with affinity-labelled nucleotide analogues are indicated. The approximate areas participating in dNTP binding and catalysis are highlighted (adapted from Joyce, 1991). (**B**) A first-generation molecular model of the HSV-1 polymerase generated by amino acid sequence alignment with *E. coli* polymerase I and successive cycles of refinement using PEER and COGEN is shown (Haffey et al., 1990). Individual amino acids are displayed as spheres; the first residue (N694) of the putative HSV pol catalytic domain is shown. In this model, the approximate positions of regions I and V are marked and regions II and III are highlighted as vertically shaded and diagonally cross-hatched spheres, respectively. The remaining amino acids constituting the catalytic cleft are shown as textured spheres. The prominent cleft and putative disordered loop as well as the positions of amino acids (solid spheres) which are changed in HSV mutants with altered drug sensitivity are also shown. With the exception of PFA^{r}5 (Y696H), many of these antiviral mutations (Fig. 2) occupy the inner surface of the putative DNA/nucleotide cleft.

clustered in this region of the enzyme and may represent the substrate binding and active site region (Polesky et al., 1990). One area is unresolved in the crystal structure, suggesting that this segment may be a flexible subdomain that could fold over a DNA molecule located within the proposed cleft. Processivity would be enhanced by the movement of this disordered subdomain over the DNA substrate by serving to block dissociation during translocation.

We have aligned the amino acid sequence of the HSV pol with the Klenow fragment and constructed a 3-D tracing of the polypeptide chain on the basis of the known alpha-carbon coordinates of the catalytic domain of the Klenow fragment (Haffey et al., 1990). After completion of this step, the interactive graphic programs PEER and COGEN (Bruccoleri and Karplus, 1987) were used to model the rest of the HSV polymerase backbone (insertions and deletions). The final approximate model of the HSV pol catalytic domain is displayed in Fig. 4B.

In this model, most pol mutations which alter antiviral sensitivity map to the putative cleft region with an increased concentration at the base of the canyon. For these mutations, the model helps to reconcile the observation that these mutations are widely separated in the primary sequence. In terms of drug targeting, this type of model also has predictive value. For example, this model identifies residues 749 through 794 as the segment in HSV polymerase which most likely constitute the disordered loop. This region does not share extensive primary sequence homology with other polymerases. If, by analogy to *E. coli* pol I, this loop does move over the DNA/nucleotide binding cleft during polymerization, agents which bind to this loop or bind the opposing face of the cleft may interfere selectively with HSV DNA replication.

An equally compelling question from a drug discovery perspective is whether all DNA polymerases share the same molecular mechanism and whether structural and mechanistic conclusions derived from work on Klenow can be extrapolated to other polymerases (Joyce, 1991; Caroll and Benkovic, 1990). There may be fundamental differences between α-like DNA polymerases (e.g. HSV pol) and *E. coli* DNA polymerase I, with less distinction between the 3′ to 5′ exonuclease and polymerase functions within the α-like DNA polymerases (Gibbs et al., 1991). For example, mutations in the Exo II domain of HSV DNA pol (Fig. 2) destroy enzyme activity and thus region IV is essential for viral growth. Since the mapping of drug-resistant mutations indicates that region IV does not appear to interact with dNTP or pyrophosphate analogs, it might also serve as a target for novel antiviral drugs.

The relationship of the putative RNase H domain to the polymerase domain, the structure of the polymerization domain itself, and the complexities of the interactions with the various substrates and inhibitors all remain to be solved. Future X-ray crystallographic and extensive mutational analyses of the α-like family of DNA polymerases are necessary to confirm the structural and functional relationships of these proposed exonuclease domains. Recent progress in obtaining milligram quantities of the HSV pol protein from baculovirus expression systems may result in the crystalline HSV polymerase

needed to directly answer these structural questions.

Conclusions and Future Directions

The need for new classes of anti-HSV compounds is becoming increasingly acute as mutants resistant to conventional substrate analogs emerge, especially in the vulnerable immunocompromised patient. All of the essential virally-encoded HSV DNA replication proteins have now been identified. In spite of the lack of 3-dimensional structures for these proteins which precludes rational drug design, many of the reagents and methods to define novel structural targets are currently available. In addition, several of these replication proteins have intrinsic properties which can be assayed in very specific large through-put screening systems. Equally important, counter screens of cellular or related viral enzymes are available to provide the necessary secondary counter-screens for selectivity. The determined application of these methods should lead to the identification of new structural classes of compounds as anti-HSV therapeutics.

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