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DNA Replication Fidelity of Herpes Simplex Virus

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1. Introduction

The role of DNA replication is to successfully transfer all genetic information from parental to daughter cells. While this process requires faithful DNA duplication to maintain genome integrity, certain errors during DNA replication, coupled with selection and fitness, are critical for evolutionary events. Therefore, regulatory mechanisms are involved in achieving a certain degree of DNA replication fidelity and satisfying these diverse needs. It is reasonable to predict that microorganisms also replicate under tight regulations to accomplish the required DNA replication fidelity for selection and fitness. This notion also applies to herpes simplex virus type 1 (HSV-1), which replicates DNA with a genomic mutation rate similar to those of other DNA-based microbes examined (Drake and Hwang, 2005). Since the HSV-1 genome can be easily manipulated, it offers a good model for a variety of studies, including the study of DNA replication (reviewed in (Coen, 1996)). This review discusses the current knowledge of HSV-1 DNA replication fidelity with an emphasis on the role of DNA polymerase and other replicative proteins in regulating replication fidelity.

2. Antiviral therapy and emergence of resistant mutants

HSVs are ubiquitous human pathogens, causing significant public health issues. Although current antiviral drugs can effectively inhibit HSV replication, the increasing number of drug-resistance mutants is becoming a major problem in successful treatment of HSV diseases. Resistance to antiviral drugs arises from the development of mutations in viral genes that encode the antiviral target proteins. Antiviral drug treatment may provide an environment suitable for the emergence of resistant mutants, especially in immunocompromised patients, which confer the advantage to survive under antiviral drug pressure. Alternatively, spontaneous mutations may arise during drug treatment (reviewed in Kimberlin and Whitley, 1996). Although it can be debated that antiviral drugs may induce and/or enhance the selection and growth of drug-resistant mutants, it is generally believed that replication errors (spontaneous mutations) play a role in the development of mutations. Therefore, it is important to understand the regulatory mechanisms, including the replication errors and the subsequent repair processes, leading to the development of mutations during HSV DNA replication. This information can also be applied to understand the general mechanisms of how a cell controls and maintains certain degrees of DNA replication fidelity.

3. HSV-1 as a model for studying DNA replication fidelity

HSV-1 contains a linear double-stranded DNA genome with a size of 152 kbp, coding for approximately 80 open reading frames. HSV can be amended to delete or modify the genetic components, regardless of whether the gene is essential or non-essential for growth in cultured cells. This offers the advantage that the virus can be modified for a variety of biological, biochemical and genetic studies of each gene. For example, recombinant virus HP66, which is a HSV-1 polymerase catalytic subunit (*pol*) null mutant and contains a reporter gene, β-galactosidase, replaced parts of the *pol* sequences, had been constructed (Marcy, Yager, and Coen, 1990). Since Pol is essential for viral replication, such a recombinant virus can only be propagated in cells containing the integrated *pol* gene and expressing the Pol proteins, such as DP6 or Pol A5 cells (Hwang et al., 1997; Marcy, Yager, and Coen, 1990). Using this approach various *pol* mutant viruses, including the lethal mutants (Gibbs et al., 1991; Marcy, Yager, and Coen, 1990), can be constructed, propagated, and characterized.

3.1 HSV-1 for the study of DNA replication fidelity

While biochemical assays can measure the enzyme kinetics and the fidelity of the Pol *in vitro*, examination of the *in vivo* fidelity of DNA replication is more complicated than the *in vitro* enzymatic studies. Nonetheless molecular biological assays have been developed to examine DNA replication fidelity of HSV-1 in infected cells. The information obtained by the *in vivo* experiments together with kinetic data is invaluable for understanding the mechanisms regulating the fidelity of DNA replication. Three *in vivo* mutagenesis assays are discussed below.

3.1.1 TK mutagenesis assay

The TK (thymidine kinase) mutagenesis assay applies the principle that HSV-1 encoded TK enzyme is not essential for virus growth in proliferating cells in cultures and that it can specifically activate certain antiviral drugs, leading to the inhibition of viral growth. In another words, *tk* mutants that do not express the TK enzyme or express TK with altered substrate specificity can grow and form plaques in the presence of antiviral drug while those with functional TK that can activate the drugs fail to form plaques in plaque assay. Based on the unique property of the TK enzyme, Hall et al. (Hall et al., 1984) devised the TK mutagenesis assay to examine the mutation frequency of HSV-1.

For example, HSV-1 TK can induce the phosphorylation of the antiviral drug acyclovir (ACV), a guanosine analog. Following phosphorylation by TK enzyme, the mono-phosphate form of ACV is converted to di- and tri-phosphate ACV by cellular enzymes, allowing its incorporation into newly synthesized DNA by HSV Pol. Since ACV lacks the 3′-OH group on the acyclic ribose structure, its incorporation into nascent DNA blocks further extension from the 3′-terminus and inhibits viral DNA replication. Thus, HSV TK plays an essential role in activation of the antiviral drug, and HSV TK mutants that fail to activate ACV will become resistance and form plaques on cultured cells in the presence of ACV.

It is worth noting that the inhibitory effect of ACV triphosphate (ACV-TP) on viral Pol is mediated by a three-step mechanism (Reardon and Spector, 1989). In the first step, ACV-TP binds to Pol and acts as a competitive inhibitor of dGTP. The interaction between Pol and ACV-TP results in the incorporation of ACV monophosphate (ACV-MP) into the growing DNA chain, leading to chain termination. However, the inhibitory effect on Pol is dependent

on the presence of the next nucleotide complementary to the next template base, which freezes the ACV-MP on the primer/template to form the dead-end complex (Reardon and Spector, 1989). Examination of certain Pol mutants containing mutations within the conserved Pol domain further demonstrate that a Pol resistance to ACV-TP can be due to the altered enzyme kinetics of K_m or K_{cat}, or both. Further information is discussed in (Huang et al., 1999). Furthermore, HSV Pol exhibits a much greater affinity for ACV-TP than does cellular Pol, which offers the specificity of inhibiting viral DNA replication with the very low toxicity of normal host cells (Furman et al., 1979).

Although *tk* mutants are easily identified in a simple plaque assay, this method may not be sensitive enough to detect all mutants, such as silent mutations and mutations that maintain enzyme activity. It is estimated that the TK mutagenesis assay can detect less than 50% of all possible substitutions of *tk* mutations (Hwang, Liu, and Hwang, 2002). Despite this, an extensive study (Lu, Hwang, and Hwang, 2002a) of *tk* mutants derived from wild-type HSV-1 strain KOS found that HSV replicates DNA with a genomic mutation rate similar to those of other DNA-based microbes (Drake and Hwang, 2005). However, earlier studies (Hall et al., 1984; Hall et al., 1985; Parris and Harrington, 1982; Hwang and Chen, 1995) demonstrated a roughly 10-fold higher mutation frequency than those (Hwang et al., 1997; Lu, Hwang, and Hwang, 2002a) used to calculate the mutation rate. Whether the selection methods (the use of antiviral drugs), experimental bias, or other unknown factors, such as the virus stocks and/or host cells used, could lead to the differences require further studies. Perhaps it is important to examine clinical isolates with limited passages in cell cultures, which may avoid the viral adaptation. Nevertheless, the study by Lu et al. (Lu, Hwang, and Hwang, 2002a) demonstrates that HSV-1 *pol* mutants can replicate the *tk* gene with different mutation spectra (see below).

3.1.2 *Lac Z* mutagenesis assay

The *lacZ* mutagenesis assay has also been applied to examine the mutation frequency of the *lacZ* gene when it is inserted into the viral genome (Hwang et al., 2003; Pyles and Thompson, 1994). This assay is based on observing the relative ratio of the number of white/light blue plaques over the total number of plaques examined by plaque assay after X-Gal staining. The advantage of this assay is its simple procedure of X-Gal staining of the plaques formed on cultured monolayers. However, identifying plaques with mutated *lacZ* genes may not be as simple as expected since bias could be derived from cells infected with a mixture of viruses containing wild-type and mutated *lacZ* genes; a similar bias also can be observed in the TK mutagenesis assay. This method may not be sensitive enough to detect all mutations, similar to the sensitivity issue of the TK mutagenesis assay. Therefore, we developed a more sensitive mutagenesis assay, the *supF* mutagenesis assay to study the replication fidelity of HSV.

3.1.3 *SupF* mutagenesis assay

The *supF* mutagenesis assay (Fig. 1) was first introduced by Seidman and colleagues (Seidman et al., 1985) to examine the mutagenic effect of carcinogens in mammalian cells using a shuttle plasmid containing SV40 DNA sequences for its replication in SV40-permissive cells and an *E. coli* amplicon for recovering DNA from *E. coli*. The shuttle plasmid contains the *supF* gene, which is a bacterial suppressor tRNA gene that can serves as a mutagenesis marker. The *supF* gene can suppress the amber codon present in the β-

galactosidase (*lacZ*) gene, forming blue colonies in the presence of X-gal and IPTG in *E. coli* host that harbor the amber mutation in the *lacZ* gene. Mutated and inactivated *supF* genes, on the other hand, fail to suppress the amber mutation and are unable to express the β-galactosidase enzyme, and thus, produce white colonies. Measuring the ratio of the number of white/light blue colonies over the total number of colonies reveals the mutation frequency. Furthermore, the short coding sequences (~90 bp) can be easily sequenced to identify the mutation. Other advantages of the *supF* mutagenesis assay include the low spontaneous mutation background and that more than 96% of possible changes in the *supF* gene have been demonstrated to become inactive and fail to express blue colonies. Therefore, it is a very sensitive assay (reviewed in (Canella and Seidman, 2000)).

We have modified and inserted the *supF* amplicon into the *tk* locus, allowing measurement of the *supF* mutation frequency in the context of the viral genome during viral replication (Hwang and Hwang, 2003; Hwang, Liu, and Hwang, 2002; Hwang et al., 2004). Furthermore, the *supF* amplicon can be modified to contain the origin (*ori*) sequences

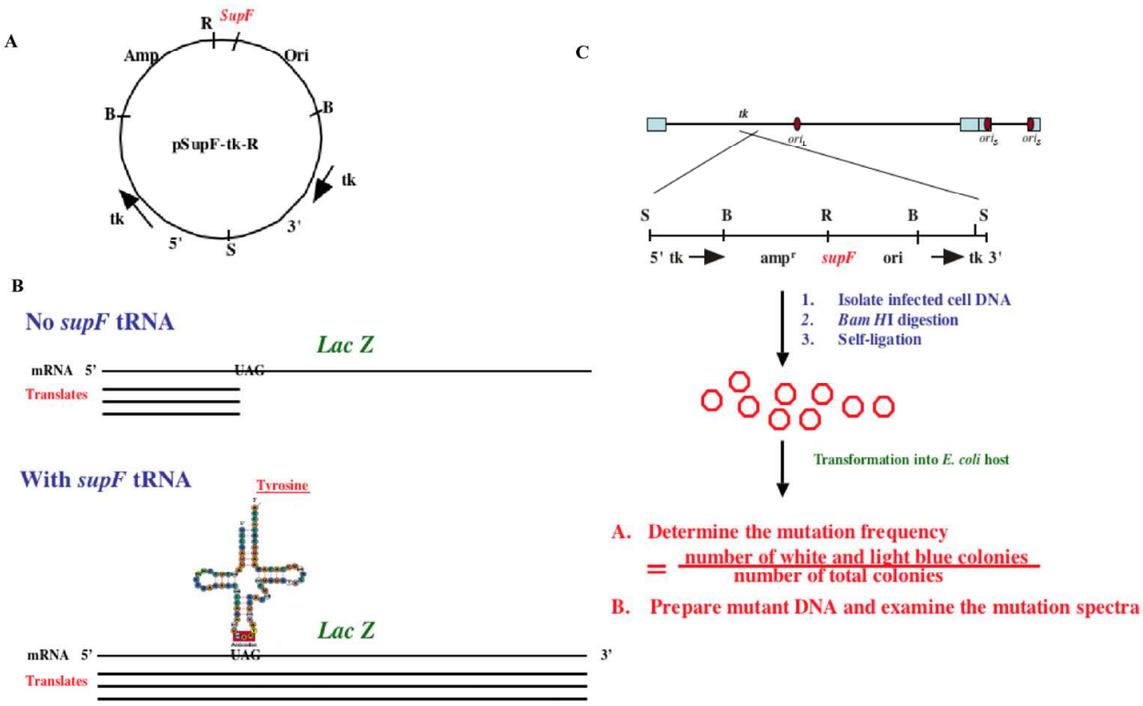

Fig. 1. *SupF* mutagenesis assay used in HSV-1 DNA replication fidelity. (A). Map of the plasmid DNA used for the construction of recombinant virus. (B). The action of *supF* tRNA in suppression of the amber codon in the *lacZ* gene in *E. coli*. In the absence of the *supF* tRNA only the truncated β-galatosidase proteins are translated due to the presence of an amber codon in the *lacZ* gene. In the presence of the *supF* tRNA the amber codon is suppressed by the insertion of a tyrosine residue and leads to the continuous synthesis of the full-length β-galatosidase protein. (C). Experimental procedures of the *supF* mutagenesis assay. The top line denotes the linear DNA of HSV-1. The relative locations of the thymidine kinase (*tk*) locus and origins of DNA replication (*oriL* and *oriS*) are shown. The components of an integrated *supF* amplicon within the *tk* locus are shown. The procedures of the mutagenesis assay are briefly illustrated.

required for HSV-1 DNA replication and applied as a shuttle plasmid for transient DNA replication in HSV-infected cells for the mutagenesis assay (Hwang et al., 1999), an assay similar to that applied by Seidman et al. (Seidman et al., 1985). The assay requires a minimal manipulation of DNA samples, which includes isolation and purification of total infected cell DNA, enzyme digestion, and ligation of the *supF*-containing amplicon. The *supF*-containing amplicon can then be recovered in *E. coli* for the blue/white selection and the measurement of the mutation frequency, again represented as the ratio of the number of white/light blue colonies to total number of colonies recovered. Figure 1 illustrates the *supF* mutagenesis assays used for the study of HSV-1 replication fidelity. Studies of recombinant viruses derived from the HSV-1 KOS strain and the transient replication assay using the modified shuttle plasmid have revealed that the KOS virus replicates the *supF* genes at a mutation frequency (Hwang, Liu, and Hwang, 2002; Hwang et al., 1999), which is consistent with that observed for the *supF* amplicon propagated in mammalian cells (Seidman et al., 1985).

4. Polymerase activity and DNA replication fidelity

4.1 Multiple activities of HSV-1 polymerase

DNA polymerase is the pivotal enzyme involved in DNA replication and fidelity. HSV-1 DNA polymerase is composed of the catalytic subunit encoded by the polymerase gene (*pol*) and the accessory protein processivity factor (or UL42) encoded by the *UL42* gene. The Pol subunit is a 1,235 amino acid polypeptide (Gibbs et al., 1988). It shares sequence homology with α-, δ-, and prokaryotic DNA Pols (Bernad et al., 1989; Brown, 2004) that contribute to the polymerization activity (the polymerase domain) and contains conserved motifs, namely Exo I, II, and III, common to other Pols possessing exonuclease activity (Bernad et al., 1989). Although a structural study reveals that HSV-1 Pol contains separated polymerase and exonuclease domains (Liu et al., 2006), the conserved δ-region C overlaps with the Exo III motif and the conserved region IV with the Exo II motif, suggesting the interdependence of DNA polymerase and its associated 3'-exonuclease proofreading activity (reviewed in (Coen, 1996)). In addition to these two functional components, HSV-1 Pol also contains a C-terminal domain that is required for Pol's interaction with the accessory protein UL42 (Digard and Coen, 1990). Fig. 2 depicts the regions of Pol possessing these functional domains or activities.

Fig. 2. Map of HSV-1 Pol polypeptide. The Pol protein is composed of 1,235 amino acids (a.a.). The labels above the line depict the relative locations of conserved regions I –VII and δ-region C, which are conserved among the polymerase domains of other Pol proteins. The labels below the line indicate the relative locations of conserved exonuclease motifs (Exo I, II, and III) and the UL42 binding domain located at the C terminus. The overlapping regions between the conserved polymerase and exonuclease sequences argue for the structural and functional interdependence of two activities.

Genetic and biochemical studies of mutations within the conserved regions of the polymerase domains confirm that these sequences are critical for the polymerase activity as

mutations within these conserved regions confer altered sensitivity to certain antiviral drugs, which are nucleoside or pyrophosphate analogs (Gibbs et al., 1988; Hall et al., 1984; Hall et al., 1985). Regarding viral DNA replication fidelity, the effect of mutations in conserved regions of the polymerase domain, including those in both the polymerase and exonuclease domains, have been studied and summarized below.

4.2 Polymerase mutation affects the replication fidelity

The TK mutagenesis assay demonstrates that mutations in Pol can result in anti-mutator or mutator phenotypes (Hall et al., 1984; Hall et al., 1985). Such studies have provided the first evidence that HSV-1 Pol can regulate the replication fidelity of viral DNA and that TK mutagenesis is a useful means to measure the mutation frequency of HSV-1. Biochemical study has also demonstrated that mutant Pol can have the anti-mutator phenotype (PAA^{r}5) and that the improved fidelity can be attributed to the improved nucleotide selectivity (Hall et al., 1985). Sequencing has subsequently demonstrated that such a *pol* mutant contains a mutation within the conserved region of the polymerase domain (Gibbs et al., 1988). Examination of several *tk* mutants also demonstrates that the mutant Pol (PAA^{r}5) replicates the *tk* genes with different mutation types compared with those mediated by wild-type Pol (Hwang and Chen, 1995). PAA^{r}5 mutant Pol generates only frameshift changes, whereas the wild-type Pol generates tk mutants of both base substitutions and frameshift mutations at 1:1 ratio. Therefore, HSV-1 Pol is critical for regulating replication fidelity.

Enzymatic studies of wild-type and PAA^{r}5 Pols demonstrate that the mutant PAA^{r}5 Pol has higher K_m of nucleoside triphosphates (dNTPs) relative to wild-type Pol (Hall et al., 1985; Huang et al., 1999), suggesting that the mutant Pol may have the better selectivity of incoming dNTPs. Study of the HSV-1 Pol model, based on the known structure of RB69 Pol, also suggests that arginine residue 842 of HSV-1 Pol is in close proximity to the sugar ring of dNTPs and that the mutation of arginine to serine (R842S; conserved region III; Fig. 2) in PAA^{r}5 Pol may weaken the interaction of Pol with dNTPs.

The *supF* mutagenesis assay further demonstrates that the PAA^{r}5 mutant replicates DNA with an altered fidelity relative to the parental strain KOS virus. This mutant Pol induces differences in the type and distribution of base substitutions relative to those by wild-type Pol (Hwang, Liu, and Hwang, 2002), suggesting that the amino acid change in PAA^{r}5 Pol results in alterations in its replication fidelity. However, this mutant Pol has a modest mutator activity in replicating the *supF* gene (Hwang, Liu, and Hwang, 2002), which is in contrast to the anti-mutator activity observed by the TK mutagenesis assay (Hall et al., 1984; Hall et al., 1985; Hwang and Chen, 1995). Perhaps the sequence contents of the target genes contribute to the differences, similar to those observed for other Pols (reviewed in (Goodman et al., 1993)). Alternatively, the assay method could influence the outcomes of the studies (see below).

The *supF* mutagenesis study also revealed that another Pol mutant (L774F) has an anti-mutator phenotype (Hwang et al., 2004). This mutant contains a mutation in conserved region VI of the polymerase domain and confers altered drug sensitivities, supporting that this conserved region plays a role in forming the structural and functional polymerase active site. Studies of mutations in this conserved region of other Pols, together with the structural information of RB69 and HSV-1 Pols (Wang et al., 1997; Liu et al., 2006), suggest that conserved region VI has a functional role in the HSV-1 Pol interaction with incoming dNTPs. In relation to the thumb, palm and finger subdomains of Pol, the L774F mutation, with a hydrophobic phenylalanine replacing the leucine residue, may induce a local

structure change of the finger subdomain and cause the finger subdomain to adapt a different orientation relative to other polymerase subdomains. The slight structural change of L774F may affect the relative position of the finger residues and subsequently lead to an altered catalytic reaction, binding affinity of dNTP and rate of polymerization, which may result in higher DNA replication fidelity (Hwang et al., 2004). Further enzymatic and structural studies of the mutant Pol will be necessary to demonstrate the proposed mechanism leading to the higher replication fidelity.

5. Pol's exonuclease activity and DNA replication fidelity

The 3′-5′ exonucleolytic proofreading activity plays a significant role in maintaining DNA replication fidelity. A Pol lacking the proofreading activity replicates DNA with a 10- to 100-fold less fidelity than that with such activity (reviewed in (Kunkel, 1988)).

5.1 Mutator phenotype of HSV-1 Pol with defective exonuclease activity

HSV-1 Pol has an intrinsic exonuclease activity that is mapped to the N-terminal half of the protein (Weisshart et al., 1994) and contains three conserved Exo motifs found in all Pols with proofreading activity. Mutations of the highly conserved residues within these Exo motifs result in the loss of exonuclease activity accompanied with the altered polymerase activity *in vitro* (Baker and Hall, 1998; Hall et al., 1995; Hwang et al., 1997; Kuhn and Knopf, 1996). However, the degree and extent of the altered polymerase activity due to the mutations within the Exo motifs vary from study to study. For example, the Exo I mutant of D368A was found to retain wild-type levels of polymerase activity and to extend from a mismatched base at the 3′-end of the primer (Kuhn and Knopf, 1996; Song et al., 2004), while another study demonstrated the failure of extension from a mismatched base (Baker and Hall, 1998). It is possible that the differences in assay conditions and the primer-templates used could lead to such differences. Nevertheless, it is evidenced that a Pol with defective exonuclease activity is highly mutagenic (Baker and Hall, 1998).

Studies of Exo III mutant Pols also demonstrate the roles of conserved residues Y577 and D581 on exonuclease activities. Mutants of Y577A, Y577H, D581A, and the double substitutions Y577H/D581A are defective in exonuclease activity and impaired in polymerase activity (Hwang et al., 1997; Kuhn and Knopf, 1996). The TK mutagenesis study demonstrates that Y577H and Y577H/D581A recombinant viruses are highly mutagenic relative to the parental wild-type virus (Hwang et al., 1997). On the other hand, the *supF* mutagenesis assay reveals the modest effects of the exonuclease activity on replication fidelity (Hwang, Liu, and Hwang, 2002). It is possible that different sequence content of the target genes could contribute to the difference of mutation frequency. Alternatively the assay method could play a key factor affecting the detection and measurement of mutations. For example, the TK mutagenesis assay can only identify the mutants among viable viruses, whereas the *supF* mutagenesis assay examines total DNA being replicated regardless of whether the mutations (in essential genes) are lethal. The higher magnitude of the mutation frequencies observed in the *tk* mutagenesis study may be theoretically explained by the fact that the *tk* mutation frequency could be amplified due to the increases of lethal mutations that reduces the total number of progeny viruses been analyzed on cell cultures. Nevertheless, the exonuclease-deficient Pol mutants replicate both target genes with significant difference of mutation spectra compared to those induced by the wild-type Pol (Hwang and Hwang, 2003; Lu, Hwang, and Hwang, 2002b).

5.2 Exonuclease deficient polymerase induces altered mutation spectra

Examining *tk* mutants derived from wild-type strain KOS demonstrates that the majority of drug-resistant *tk* mutants are frameshift mutations (45 out of 66 mutants examined; 67%), containing insertion or deletion of one or two nucleotides in the regions containing homonucleotide runs. In contrast, the Y7 recombinant virus, which contains the Y577H mutation in the Exo III motif, replicates the *tk* gene with significantly less frameshift mutations (21%; $p < 0.005$) relative to wild-type virus (Lu, Hwang, and Hwang, 2002b). Furthermore, although the majority of substitutions are transitional changes in mutants derived from both wild-type and exonuclease-deficient viruses, they distribute differently (Lu, Hwang, and Hwang, 2002b). These findings imply that HSV Pol can incorporate mispaired nucleotides, and most of the misinsertions are corrected by the proofreading activity during DNA synthesis. Furthermore, the Y577H mutation not only loses exonuclease activity but also alters the polymerase activity as it replicates misinsertions at positions distinct from those of wild-type Pol. Only 3 identical base substitutions are found among 15 and 52 *tk* mutations replicated by wild-type and exonuclease negative Pol, respectively (Lu, Hwang, and Hwang, 2002b). Theoretically, one would expect to observe more identical substitutions in both groups if the exonuclease Pol does not have altered polymerase activity. The observation that recombinant Y7 (Y577H) virus also exhibits altered drug sensitivities relative to wild-type virus further supports that the Exo III motif has a role on the polymerization activity (Hwang et al., 1998) and that the polymerase and exonuclease domains of HSV-1 Pol are interdependent structurally and functionally, also as suggested by the overlap between conserved δ-region C and Exo III motif (Fig. 2).

Not surprisingly, *supF* mutagenesis studies of the Exo III mutant demonstrate modest increases of the *supF* mutation frequency (Hwang and Hwang, 2003). This is sharply different from the several hundred fold increases of *tk* mutation frequency replicated by such mutant viruses (Hwang et al., 1997). Presumably the sequence content between two target genes and the assay methods, as discussed above, contribute to these differences. In agreement with these assumptions, the *in vitro* mutagenesis study of an Exo I mutant Pol using the *lacZ*α reversion mutagenesis assay demonstrates increases of 3- to 18-fold in the reversion frequencies, depending on the dNTPs concentrations (Baker and Hall, 1998).

5.3 Enzyme kinetics of exonuclease deficient polymerase

Enzymatic studies of steady (and perhaps pre-steady) state kinetics of the Pol protein will provide information to illustrate the affinity of Pol on dNTPs within the context of a matched or mismatched 3′-terminus and the relative catalytic activity of Pol's incorporation of incoming dNTPs. Based on the kinetic data the extension efficiency and misinsertion efficiency of the Pol from a primer-template (p/t) can be obtained to indicate a possible mechanism of how a Pol discriminates between correct and incorrect nucleotide during the polymerization reaction. Although little information of enzyme kinetics is available, Baker and Hall (Baker and Hall, 1998) have demonstrated that both wild-type and the Exo I mutant Pol D368A have high affinity (low K_m) for correct nucleotide incorporation from a matched p/t, and these Pols exhibit extremely high K_m (in the order of 3 magnitudes) for nucleotide incorporation from a mismatched p/t. Furthermore, the kinetic data demonstrate that exonuclease activity is required for removal of the misinserted nucleotides by slowing the primer extension (low V_{max} for nucleotide incorporation at the mispaired 3′-termini) and reinsertion of the correct base to resume the efficient extension. Thus an enzyme lacking the ability to remove the mispaired base significantly reduces the extension efficiency from the

mispaired 3′-termini. Consistent with the results discussed above, we observed that both Y577H and Y577H/D581A mutant Pols had altered K_m, V_{max}, and extension efficiency from mispaired 3′-termini dependent on the mismatched 3′-base. Furthermore, both mutant Pols exhibited increases in infidelity (f_{inc}), determined as the ratio of $(K_m/V_{max})_{incorrect}/(K_m/V_{max})_{correct}$ (unpublished data). Additional information of the kinetic approach addressing the mechanism affecting the fidelity of HSV-1 Pol can be found in a recent review (Zhu et al., 2010).

6. Processivity factor and DNA replication fidelity

6.1 UL42 functions as a processivity factor

The UL42 protein was originally identified as a non-specific double-stranded DNA (dsDNA) binding protein (Marsden et al., 1987; Powell and Purifoy, 1976) and was defined as a processivity factor for Pol (Gottlieb et al., 1990). Unlike other known processivity factors, UL42 binds DNA directly. It also directly interacts with the Pol subunit to form the Pol/UL42 heterodimer, which binds more tightly to the p/t-DNA than to single-stranded DNA (ssDNA). On the other hand, Pol alone binds more tightly to ssDNA than to p/t-DNA (Weisshart, Chow, and Coen, 1999). Therefore, the Pol/UL42 heterodimer exhibits a ~2-fold faster association rate and a ~10-fold slower dissociation rate to p/t-DNA than does Pol alone. However, the increased affinity and stability of the Pol/UL42 complex to p/t-DNA does not alter the elongation rate relative to Pol alone (Weisshart, Chow, and Coen, 1999). The increased half-life, but not the elongation rate, of UL42/Pol on p/t-DNA compared with the Pol subunit alone could afford a greater ability for the excision of a misincorporated base by the exonuclease activity intrinsic to wild-type Pol (Chaudhuri, Song, and Parris, 2003). This suggests that, in addition to its processivity, UL42 may enhance the fidelity of wild-type Pol in viral DNA replication via the exonuclease activity.

6.2 Processivity factor and replication fidelity

Although the effect of UL42 on DNA replication fidelity at the nucleotide level has not been examined, studies from other systems demonstrate that the processivity factor can influence replication fidelity. For example, the T7 Pol processivity factor thioredoxin can increase the fidelity of single-nucleotide insertions by an exonuclease-deficient T7 Pol. It can also increase the frequencies of certain base substitutions and deletions (Kunkel, Patel., and Johnson, 1994). These findings imply that slippage may occur during enzyme dissociation or reassociation and that thioredoxin enhances the processivity of Pol by preventing it from dissociating from the p/t, thereby reducing the slippage-mediated insertion mutations. Paradoxically, the reduced accuracy of certain base substitutions synthesized by T7 Pol in the presence of thioredoxin also implies that it promotes the extension of mismatches and misalignments. Therefore, the processivity factor can act as an anti-mutator factor in the slippage-mediated insertion mutations and as a mutator factor in base substitutions and deletions. Recent studies also demonstrate that the accessory protein gp45 of bacteriophage BR69 Pol can modulate site-specific mutation rates in the target gene compared to those by the Pol alone, although the overall mutation rate increases only modestly (Bebenek et al., 2002; Bebenek et al., 2005). The sliding clamp protein PCNA that enhances the processivity of replicative DNA Pol in eukaryotes can promote the bypass of DNA lesions (Mozzherin et al., 1997; O'Day, Burgers, and Taylor, 1992) and acts as a mutator factor. Studies have also demonstrated that mutations in genes encoding PCNA or subunits of replication factor C

(RFC) increase mutation frequency *in vivo* (Chen et al., 1999; Johnson et al., 1996; McAlear et al., 1994; McAlear, Tuffo, and Holm, 1996; Umar et al., 1996).

Several UL42 mutations that increase or decrease DNA binding without affecting its interaction with Pol have been constructed (Komazin-Meredith et al., 2008; Randell et al., 2005). These studies suggest that UL42 interacts with DNA via interaction between the basic charged residues located on the surface of the molecule and the negatively charged DNA. Recombinant viruses expressing UL42 with increased DNA binding exhibits impaired phenotypes, including the formation of smaller plaques, decrease replication of progeny and synthesis of DNA with higher ratio of DNA copies per plaque forming unit (PFU) (Jiang et al., 2009). As expected the mutants are more mutagenic than the control virus expressing wild type UL42 (Jiang et al., 2009). Similarly mutants with decreased DNA binding also have defective phenotypes and are highly mutagenic (Jiang et al., 2007a), and that mutants with no detectable DNA binding are deleterious in viral growth and DNA synthesis (Jiang et al., 2007b). These studies provide information that UL42 cannot bind DNA too tightly or too weakly and that any perturbation of DNA binding can impact virus production and replication fidelity. Future studies of the effects of mutation on the enzyme kinetics and on the mutation spectra at the nucleotide level will be invaluable for understanding the mechanisms attributed to UL42 in regulating DNA replication fidelity.

7. Nucleotide metabolism enzymes and DNA replication fidelity

In addition to the TK enzyme, HSV-1 also encodes several genes expressing nucleotide metabolism enzymes, including deoxyuridine triphosphatease (dUTPase), uracil-N-glycosidase (UNG), and ribonucleotide redutase (RR). The dUTPase can convert dUTP to dUMP, which can subsequently lead to an increased dTTP pool and a decreased dUTP pool. The UNG enzyme can remove uracil bases, which are resulted from deamination of cytosines, on DNA. The activity of RR is to convert ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates. Theoretically, these enzymes may act to maintain an optimal condition favoring HSV-1 DNA replication. The study of Pyles and Thompson (Pyles and Thompson, 1994), however, demonstrates intriguing results of how viruses lacking the expression of these enzymes affect the replication fidelity in infected cells. Using recombinant viruses containing a deletion of tested genes and the *lacZ* reporter system the viral dUTPase is shown to have an antimutator activity, whereas the viral TK has the mutator activity. While the UNG^- viruses replicated the *lacZ* gene with no significant difference of the mutation frequency relative to that of UNG^+ recombinants, continuous passage of UNG^- viruses lead an increased mutation frequency, suggesting its antimutator activity. Furthermore, the interaction between UNG and Pol and the association of UNG with viral replisome also suggest a role UNG in regulating DNA replication fidelity (Bogani et al., 2010). There is no information whether HSV-1 encoded ribonucleotide redutase plays a role on replication fidelity.

In contrast to the observed mutator activity of the TK enzyme (Pyles and Thompson, 1994), we demonstrated that the TK enzyme of strain KOS does not possess the mutator activity; recombinant viruses harboring either the *lacZ* or *supF* gene replicate both genes with equal fidelity in HSV-1 TK expressing or non-expressing cells (Hwang et al., 2003). Although it is not clear what the factor leads to the difference, one may wonder why the virus were

encoding a mutagenic gene. However, it is also important to note that each recombinant virus, including those used to establish the baseline relative mutation frequency, constructed by Pyles and Thompson (Pyles and Thompson, 1994) harbors the integrated *lacZ* gene at different location in the viral genome, which may affect the replication fidelity (see section 8.2). Further studies using recombinant viruses containing the reporter gene integrated at the defined location and with or without the expression of gene to be examined are necessary to demonstrate the effects of these nucleotide metabolism enzymes on replication fidelity.

8. Other factors may affect the replication fidelity

8.1 Single-stranded DNA binding protein and replication fidelity

Little information is available regarding the contribution of other accessory proteins in DNA replication fidelity. However, a recent *in vitro* study of DNA replication fidelity using RB69 Pol with and without accessory proteins, including single-stranded DNA binding protein (SSB), the processivity clamp, and the clamp-loading proteins, demonstrated that these proteins induced altered mutation rates in a site-specific manner, although the overall mutation rate of the target gene did not differ significantly relative to that by Pol alone (Bebenek et al., 2002; Bebenek et al., 2005). Thus, SSB protein and the other accessory proteins also play a role on replication fidelity.

Our previous study examining the effect of an exonuclease-deficient Pol mutant, Y7(Y577H), on replication fidelity suggests the possibility that other DNA replicative proteins may have effects on regulating the replication fidelity (Hwang and Hwang, 2003). The mutagenic ability of the exonuclease-deficient Y7 Pol is expected to generate various mutations in the entire genome, including genes coding for Pol and other replicative proteins, and result in heterogeneous populations in viral stocks. Examining the *pol* gene sequences confirms the presence of heterogeneous *pol* sequences in a single viral stock. Marker rescue experiments in which the mutated *pol* is rescued to the wild-type sequences demonstrate that several rescued recombinants exhibit higher fidelity than those with wild-type Pol. These allow one to test the hypotheses that there are mutations in other replicative genes, which may contribute to the higher replication fidelity of the *supF* gene. It is also important to explore the mechanisms by which SSB may regulate replication fidelity in infected cells since this protein associates and colocalizes with cellular DNA repair and recombination proteins (Taylor and Knipe, 2004; Wilkinson and Weller, 2004) and the SSB protein is able to promote recombination *in vitro* (Nimonkar and Boehmer, 2002; Nimonkar and Boehmer, 2003; Nimonkar and Boehmer, 2004; Reuven et al., 2004). Along this line the contributions of other cellular replicative proteins in HSV DNA replication warrant for future studies.

8.2 Can the position of target gene influence replication fidelity?

The genomes of all alpha-herpesviruses, including HSV-1, are composed of unique long (UL) and short (US) sequences and repeated sequences that segment the unique regions. Study of proteins conserved among six alpha-herpesviruses reveals that US proteins are more divergent than those encoded by UL sequences (Brown, 2004). The study further indicates that proteins encoded by sequences at the repeat regions, UL1, UL56, US1, and US12, are highly divergent than those of other regions (Brown, 2004). This observation suggests that selective sequences within the viral genome have a comparatively rapid evolutionary rate or high mutation rate. It is possible that the virus has evolved to arrange

the sequences in clusters that can be the targets of different mutation rates. If this is the case, then one may speculate that HSV may have position-dependent DNA replication fidelity, which we have described as the position effect (Hwang et al., 2003). This may also explain that the proteins clustered in the middle of the UL regions (between UL27 and UL33) are highly conserved as they are essential for viral replication. However, two proteins required for viral DNA replication, UL42 and UL8, that encode a helicase/primase subunit are highly divergent. Perhaps, these essential proteins quickly evolved to have multiple functions to include differential activity critical for each virus's life cycle.

8.3 Sequence context, replication mode and replication fidelity

Consistent with other studies (reviewed in (Goodman et al., 1993), *tk* and *supF* mutagenesis assays also demonstrate that the sequence content affects HSV-1 replication fidelity. We demonstrate that the *supF* mutation spectra observed in the plasmid-borne replication assay differ from those replicated as a part of viral genome (Hwang, Liu, and Hwang, 2002; Hwang et al., 1999). The differences could be related to the mechanism of how DNA is replicated in infected cells. For example, viral genome replication involves the formation of head to tail arranged DNA concatemers and branched structure, presumably mediated by a recombination mechanism; the processing and cleavage of complex DNA molecules are required for DNA packaging and maturation of viral particles (Bataille and Epstein, 1997; Slobedman, Zhang, and Simmons, 1999). On the other hand, the *ori*-based plasmid may not replicate by the same mechanisms. It is reasonable to hypothesize that the lack of viral repeat sequences in the *ori*-containing plasmid DNA may not form isomers similar to those observed in genomic DNA replication, and that the mechanisms involved in branch DNA formation may not be obvious. Further studies are required to determine whether the plasmid-borne DNA is replicated with a different mechanism relative to viral genomic DNA replication.

9. Conclusions

A large body of literature discusses DNA replication fidelity in both prokaryotic and eukaryotic cells, yet limited information is available regarding how HSV DNA replication is regulated to maintain a certain degree of fidelity. Understanding the mechanisms regulating HSV DNA replication fidelity may lead to comprehension of how drug resistant mutants develop. The proteins that regulate replication fidelity definitely play roles in HSV DNA replication. In the aspect of antiviral therapy, the knowledge gained from such studies may lead to the design of new strategies to combat HSV diseases.

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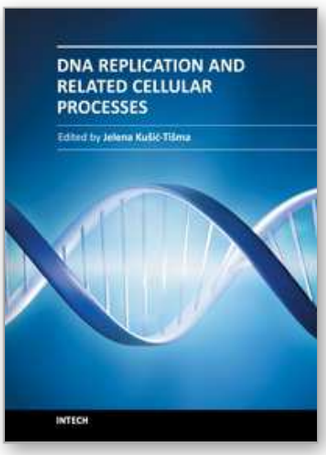

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