CHAPTER III

REVIEW OF LITERATURE

1. History

Herpesviruses are virus of eukaryotes (67-71). More than 100 herpesviruses have been isolated from a wide range of vertebrates, including fish, amphibians, reptiles, birds, marsupials, and placental mammals. While herpesviruses are ubiquitious in vertebrates, each is usually restricted to a single specie in natural infection. Inter species transmission of herpesvirus, which may be rare in nature, can cause severe disease (e.g. infection in humans with B virus of world monkeys). The high degree of host specificity of herpesviruses suggests that herpesviruses have evolved in closed associated with their hosts. With rapid replication and selection by the host, genetic variation of virus is common. Study of virus genome variation by tracking it's sequence has been referred as molecular epidemiology (72). These studies facilitate differentiation and classification of strains and examination of evolution of viruses, mode of transmission close contact between individuals tend to be divided to geographically isolated genotypes (73-75). More readily transmitted viruses may become rapidly distributed over wide geographic region (76).

HSV is the first of the human herpesviruses to be discovered and is among the most intensively investigated of all viruses. Its attraction is biologic properties and, in particular, ability to cause a variety of infections, to remain latent in their host life, and to be reactivated to cause lesions at or near the site of initial infection. It serves as a model and tool for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, and a myriad of other biological problems, both general to viruses and specific to HSV. For year, their size and complexity served as a formidable obstacle to intensive research. More than 40 years passed from the time of their isolation until Schneweiss (77) demonstrated that there were, in fact, two serotypes, HSV-1 and HSV-2, whose formal designations under International Conference for Taxonomy of Virus (ICTV)

rules are now human herpesviruses 1 and 2 (78). Not until 1961 were plaque assay published (79), and only much later were the genome sizes and the extent of homology between these two viruses reported. In 1736, Astruc J. (80), found the correlation established between herpetic lesion and genital infection. In 1883, Unna PG. (81), studied the recognition of human transmission of HSV infection between individual. In 1930, Andrews CH, and Carmichael EA (82), initial studied of host immune response to HSV. In 1939, Barnett FH, and Williams SW. (83), first accurate description of the biology of HSV infections in human. In 1962, Schneweiss KE (84), demonstrated of antigenic biologic differences between HSV-1 and HSV-2. In 1967, Dowdle *et al.* (85), studied association of antigenic type of herpesvirus with site of viral recovery. In 1975, Hayward *et al.* (86), first studied determination of organization of HSV genome. In 1978, Bachman *et al.* (87), demonstrated of DNA polymorphism suitable for use in molecular epidemiology. In 1981, Post L and Boizman B (88), developed the technology for genetic engineering of HSV-1 genome and in 1988, McGeoh *et al.* (89), determined the sequence of HSV unique long region.

Infections caused by HSV have been described from ancient Greek and roman times. The word herpes means "to creep" and probably described cutaneous skin lesions. The vesicles associated with herpetic infection. Studies at this time also demonstrated the infections nature of material obtained from ocular or skin lesions by transmission to rabbit eye (90). In 1930, tests for neutralizing antibodies to HSV were described. By the year 1950, most of the syndromes now known to be caused by HSV had been documented. The current era of HSV research and diagnosis was ushered in when two biologically distinct subtypes, HSV-1 and HSV-2, were associated with different clinical syndromes (91). Subtyping tests and type-specific antibody assays were developed during the year 1980, leading to increase understanding of the natural history of HSV-1 and HSV-2 infections. Effective antiviral drug was also developed and are now widely used. In the past two decades, impressive advances have been made in our understanding of HSV replication and the molecular basis of neurovirulence and latency. Finally, the ability to sequence and manipulate HSV genes and gene products has led to the current era of vaccine development.

The family Herpesviridae is divided into three subfamilies of Alpha-, Beta-, and Gammaherpesvirinae, based on biological properties (92). Eight human herpesviruses have been identified to date, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) (93,94), varicella-zoster virus (VZV) (95), Epstein-Barr virus (EBV) (96), human cytomegalovirus (HCMV) (97), human herpesvirus 6 (HHV-6) (98), human herpesvirus 7 (HHV-7) (99,100), and human herpesvirus 8 (HHV-8) corresponding to kaposi's sarcoma-associated herpesvirus (KSHV) (101). HSV-1, HSV-2 and VZV are members of Alphaherpesvirinae, HCMV, HHV-6 and HHV-7 are of Betaherpesvirinae, EBV and HHV-8 are of Gammaherpesvirinae. (Table 1.)

Table 1. Human herpesviruses of the family Herpesviridae

Subfamily	Genus	Virus
Alphaherpesvirinae	Simplexvirus	Human herpesvirus-1 (HSV-1)
		Human herpesvirus-2 (HSV-2)
	Varicellavirus	Human herpesvirus-3 (VZV)
Bataherpesvirinae	Cytomegalovirus	Human herpesvirus-5 (CMV)
	Roseolovirus	Human herpesvirus-6 (HHV-6)
		Human herpesvirus-7 (HHV-7)
Gammaherpesvirinae	Lymphocryptovirus	Human herpesvirus-4 (EBV)
	Rhadinovirus	Human herpesvirus-8 (HHV-8)

HSV is divided into two types, HSV-1 and HSV-2 , on the basics of antigenic differences detected by neutralization assays (102). These two antigenic types exhibit a number of biological differences, e.g. site of recovery, plaque formation, etc (33). Hybridization study has revealed that both have 50% homology in their DNA sequences. In 1968, Nahmias AT and Dowdle WR (91), demonstrated that HSV-1 was more frequently associated with nongenital (above the waist) infection, which HSV-2 has associated with genital infection (below the waist). Whereas prior studies have indicated that the HSV-1 is an infrequent cause of infections "below the waist", recent

studies suggest an increasing prevalence of genital HSV-1 infection (21-23). Similarly, HSV-2 pharyngitis appears to be increasingly common. The subsequent recurrence rate of genital HSV-1 infection appears to differ from that of genital HSV-2 disease.

Historically, the indication that strains may vary considerably emerged many years ago (103,104), the studies on the variability of HSV began with a chance isolation of a mutant that fused cells into polykaryocytes from a wild type virus which causes cells to round and clump (105). Subsequent studies show that the variation differed with respect to buoyant density in CsCl gradients (91), affinity chromatography (106), neutralization with specific antisera (25), the ability to accumulate glycoprotein VP8 (gC) (107), and ultimately, which respect to several genetic loci concerned with cell fusion in addition to accumulation of glycoprotein VP8 (gC) (77). The discovery that HSV represents two viruses, HSV-1 and HSV-2 (108) and studies on isolated and mutant of each (77,109), reinforced the hypothesis that HSV strains vary in number of characteristics.

Our interest in the diversity of HSV stemmed from the realization that these viruses are responsible for a wide repertoire of diseases. In 1976, Pereira *et al.* (110), in an attempt to determine whether HSV strain differing with respect to site of localization in the human body or pattern of disease. Pereira, who discovered that although a correlation between clinical manifestations and structural proteins could not be demonstrated, the variability in the electrophoresis mobility of several of structural proteins was beyond to expectation. The study made apparent the now established fact that the HSV genome are variable, perhaps more important, the identity of virus isolate from epidemiologically related individuals suggested that the electrophoresis pattern of the structural proteins could be used to identify viruses and follow their spread in the human population.

2. General Characteristics of Herpes Simplex Virus

Membership in family Herpesviridae is based on the architecture of the virion (Figure 1). A typical herpesvirion consists of (i) a core containing a linear, double-stranded DNA, (ii) an icosadeltahedral capsid, approximately 100-

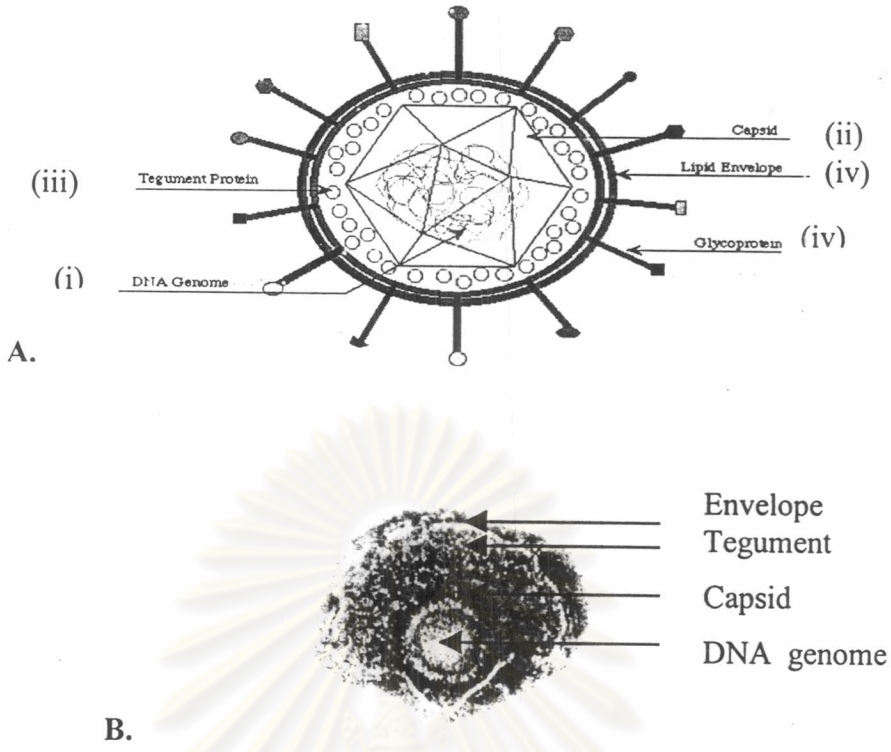


Figure 1. The morphology of herpesviruses. A. Schematic representation of the herpesvirion seen through a cross section, (i) core containing DNA genome, (ii) an icosadeltahedral capsid, (iii) tegument, and (iv) an envelope containing viral glycoprotein spikes on its surface. B. Electron micrograph of thin section of HSV virion.

110 nm in diameter, containing 162 capsomers with a hole running down the long axis, (iii) an amorphous tegument surrounding the capsid, and (iv) an outer envelope exhibiting spikes on its surface (Figure 1). The core of the mature virion contains the viral DNA in the form of the torus (111,112). The torus appears to be suspensed by a proteinaceous spindle consisting of fibrils embedded in the underside of the capsid and passing through the hole of the torus. The precise arrangement of the DNA in the toroid is not known. The structural features of the capsid (100 nm diameter and 162 capsomers) are characteristic of all herpesviruses. The pentameric capsomers at the vertices have not been well characterized. The hexameric capsomers are 9.5 x 12.5 nm in longitudinal section; a channel is 4 nm in diameter part way from the surface along the long axis (113). The tegument is a term introduced by Roizman and Furlong (114) to describe the structures between the capsid and envelope (113,115,116). The thickness of tegument may vary, depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space (117). The available evidence suggests that the amount of tegument is more likely to be determined by the virus than by the host (118). The tegument is frequently distributed asymmetrically. Electron-microscopic studies on thin sections have shown that outer covering, the envelope, of the virus has a typical trilaminar appearance (119); the envelope appears to be divided from patches of altered cellular membranes (116,120,121). The presence of lipids was demonstrated by analyses of virions (122,123) and by sensitivity of the virions to lipid solvents and detergents (124-126). The herpesvirus envelope contains numerous protrusions of spike, which are more numerous and shorter than those appearing on the surface of many other enveloped viruses. Wildy and Watson (113) estimated that the spikes on HSV virions are approximately 8 nm long. The spikes consist of glycoproteins (127). The number and relative amounts of viral glycoproteins vary; there are 11 known viral glycoproteins (B-M) and a 12th (gN) is predicted (128). The variation is, in part, due to variability in the thickness of tegument. Another major source of variability is the state of the envelope. Intact envelopes are impermeable and generally retain the quasi-spherical shape of the virion. Damaged envelopes are permeable to negative

stains; permeated virions have a sunnyside-up egg appearance, with a diameter much larger than that of an intact virion. The precise number of polypeptide species contained in the herpesvirions is not known and may vary from one virus to another. They estimated generally range from 30 to 35 polypeptides.

3.Viral DNAs

The herpesvirus DNAs extracted from virions and characterized to date are linear and double-stranded, but they circularize immediately upon release from capsids into the nuclei of infected cells. The variable features of the herpesvirus DNAs are their molecular weight and base composition. The molecular weight of herpesvirus DNAs varies from approximately 80 to 150 million, or the genome range from approximately 120 to 230 kilobase pairs. The variability in the size of herpesvirus DNAs does not reflect a polymorphism in the size of DNAs of individual viruses. The variation in the size of the genome of any one herpesvirus to be minimal, but not insignificant. Thus, many viral DNAs contain terminal and internal reiterated sequences. Because of variability in the number of these reiterations, the size of individual genomes may vary by >10 kilobase pairs. The base composition of herpesvirus DNAs varies from 31 to 75 G + C moles percent. Furthermore, herpesvirus DNAs vary respect to the extent of homogeneity of base sequence distribution across the length of the genome. The extent of inhomogeneity in the base composition varies from minimal (e.g. HSV) to very extensive (130).

HSVs are members of a family of large DNA viruses. HSV DNA is linear and double-stranded (131-133). In the virion, HSV DNA is packgaged in the form of a toroid (134). The end of genome are probably help together or are in close proximity in as much as the DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells (135). DNA extracted from virions contains nick and gaps (136-139). The HSV genome is approximately 152 kilobase pairs, molecular weights approximately 96 x 10^6 daltons, with a G + C content of 68% (HSV-1) or 69% (HSV-2) and the DNAs encodes over 70 polypeptides (131,132,138). It consists of two covalently linked components, designated as L (long) and S (short) components comprise 82% amd 18% of DNA, respectively. Each component consists of unique sequences bracketed by inverted repeats (139,140). The repeats of the

L component are designated *ab* and *a'b'*, while those of the S component are designated *a'c'* and *ca* (141). The number of *a* sequence repeats at the L-S junction and at the L terminus is variable; the HSV genome can then be represent as "$a_L a_n b$ —U_L—$b'a'_m c'$—U_S—ca_S where a_L and a_S are terminal sequences with unique properties described below, and a_n and a_m are terminal *a* sequences directly repeated zero or more time (n) or present in one to many copies (m) (141-146) (Figure 2). The structure of the *a* sequence is highly conserved but consists of a variable number of repeat elements.

The L and S components of HSV can invert relative to one another, yielding four linear isomers (147,148). The isomers have been designated as P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both S and L components) (149-150). The evidence for the repetition of terminal sequences in inverted orientation was based on electronmicroscopic studies of denatured HSV-1 DNA allowed to self-anneal (151). These studies, as well as partial denaturation profiles of HSV DNA, revealed that the terminal repeats are repeated internally and that the repeats of each end differ in size and sequence arrangements (147,140). The isomerization of HSV DNA resulting from the inversion of L and S components relative to each other is an intriguing, tantalizing feature of the HSV genome shares with only a few other herpesviruses. In the circular form, the HSV genome from two isomers, each containing two L-S component junctions. Cleavage of one circular isomeric at the two junctions would yield the P and I_{SL} arrangements, whereas the corresponding cleavages of the other circular isomer would yield the I_S and I_L isomers. Generation of I_S and I_L arrangements from the first circular isomeric form would require inversion of either the S or the L components through the inverted repeat sequence. Fundamentally, there are several issues. First, inversion of covalently linked components is not a property of all herpesvirus genome. Second, the physiologic function of the inversions is not clear in as much as genomes frozen in one orientation as a consequence of deletion of internal inverted repeats are viable (135,152).

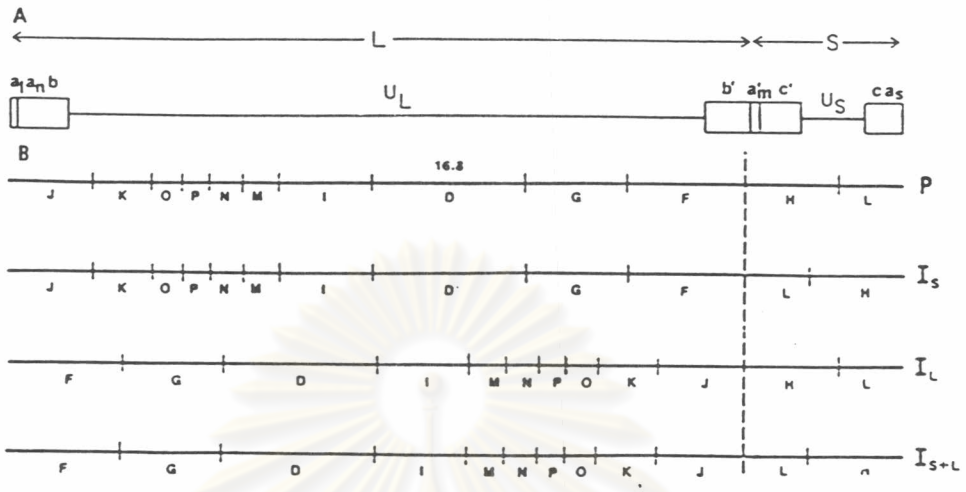


Figure 2. Schematic representation of the arrangement of DNA sequence in the HSV genome. A: the domains of the L and S complements are denoted by the arrows. The second line shown the unique sequences (thin lines) flanked by the inverted repeats (boxes). The letters above the second line designate the following: the terminal *a* sequence of the L component (a_L); a variable (n) number of additional *a* sequences; the *b* sequence of the L component (U_L); the repetition of the *b* of *a* variable (m) number of a sequences (a_m); the inverted *c* sequence (*c'*); the unique sequence of the S component (U_S); and, finally, the terminal *a* sequence (a_S) of the S component. B: the *Bgl*II restriction endonuclease map of HSV-1(F) strain for the P, I_S, I_L, and I_{SL} isomers and variability of HSV-genomes. Note that because *Bgl*II does not cleave within the inverted repeated sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

However, all wild-type isolates examined to date do contain the inverted repeat sequences, and viruses lacking internal inverted repeats have a reduced capacity for growth in animal tissue. Third, insertion of the junction between the L and S components, and especially of the 500-base-pair *a* sequence, results in additional inversions of DNA segments contained between inverted repeats of *a* sequences (149,153-155). The internal inverted repeat sequences are not essential for growth of the viral in cell culture; mutants, from which portions of unique sequences and most of internal inverted repeats have been deleted, have been obtained in all four arrangements of HSV DNA (156). The genomes of these mutants do not invert; each is frozen in one arrangement of the L and S components, but all retain their viability in cell culture.

4. HSV Replication Cycle

HSV is a large DNA virus that uses cellular transcriptional and translational machinery for the expression of its genes. HSV genes expression is regulated as an ordered cascade (157-159). HSV-1 and HSV-2 each comprises at least 77 genes whose expression is tightly regulated (160). HSV has two distinct lifestyles, productive infection and latent infection. During productive infection, three major kinetic classes of viral genes, the first genes expressed are the α (immediate-early, IE) genes, which encode regulatory proteins required for the efficient expression of later genes. Next, the β (early, E) genes, which largely encode DNA replication proteins, are expressed. The levels of early mRNAs and the rates of early protein synthesis peak at about four to seven hours postinfection and then decline. Early gene expression is relatively insensitive to inhibition of viral DNA synthesis. In contrast, the expression of γ (late, L) genes, which largely encode virion proteins, continues to increase until very late in infection and is relatively sensitive to inhibition of viral DNA synthesis. These genes are assigned to four kenetic classes, designated as α, β, γ1, and γ2 on the basis of the timing of and requirement for their abundantly expression in an ordered cascade (161). The five of α (IE) genes [infected-cell proteins (ICP) 0, 4, 22, 27, and 47] are expressed first in the absence of viral protein synthesis and are responsible for regulated expression of other viral genes, which encode regulators of viral gene expression during productive infection. Of these IE proteins, ICP4 and ICP27

are essential for viral replication. Although ICP0 is not essential, it is critical for efficient viral replication and for the full expression of all for three gene classes, especially at low multiplicity of infection (MOI) (162-167). The β (E) genes require functional of α (IE) gene products their expression and encode proteins and enzyme that are directly involved in DNA synthesis and nucleotide metabolism. The γ genes are expressed from the last set of viral genes with the γ2 class having viral DNA replication as a strict requirement for their expression. To initiate infection the virus must attach to cell surface receptors, it's envelope to the plasma membrane, and allow the de-enveloped capsid transport to the nuclear pores. DNA is released into the nucleus of the cell, where transcription, replication of viral DNA, and assembly take place.

Viral DNA is transcribed throughout the reproductive cycle by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated (Figure 3). Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion, with the approximately 70 gene products forming at least four groups on the basis of both transcriptional and posttranscriptional regulation. The five α mRNA are transported into the cytoplasm and translated, postranslational the proteins are transported into the nucleus. A new round of transcription results in the synthesis of β proteins. At this stage in the infection, the chromatin is degraded and displaced toward the nuclear membrane, where the nucleol (round, hatched structures) become disaggregated. Viral DNA is replicated by a rolling circle mechanism that yeilds head-to-tail concatamers viral DNA. A new round of transcription and translation yields the γ proteins consisting primarily of structural proteins of the virus. The capsid proteins form empty capsids.

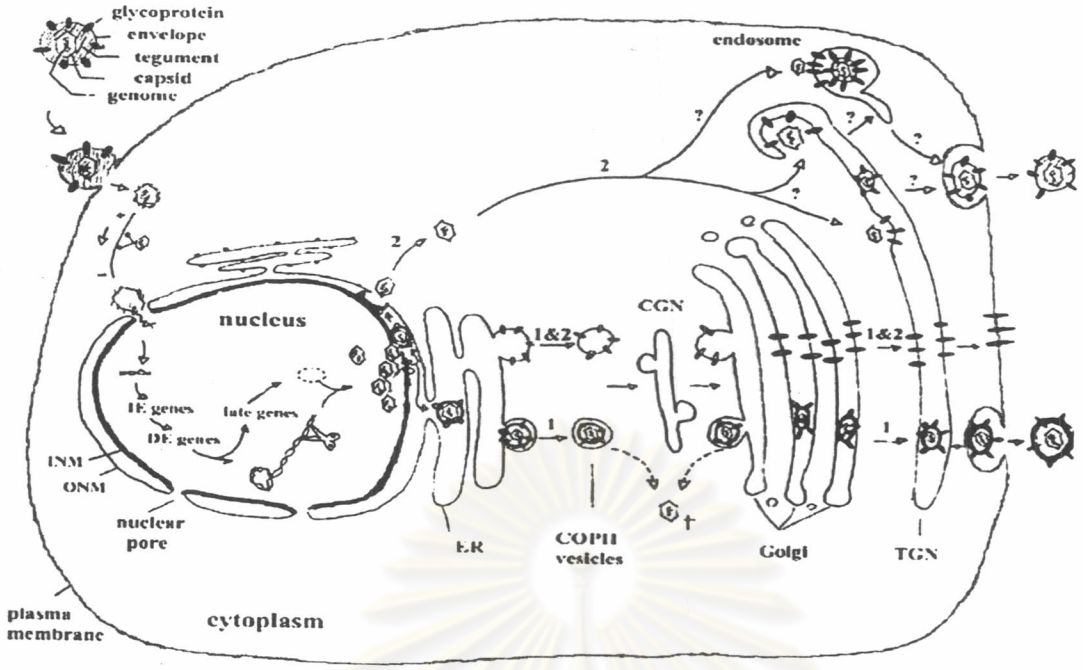


Figure. 3 Sequence of events in the multiplication of herpes simplex virus from entry of the virus into cell by fusion of the virion envelope with the membrane of the endocytic vacuole to assembly of virions and their exit from the cell through the endoplasmic reticulum. Also illustrated are transcription and coordinated sequential processing of mRNA and synthesis of set of protein (α, β, γ) required from DNA replication and virion structures. Assembly of the virus begins in the nucleus, with acquisition of the envelpe as the capsid buds through the inter lamella of the nuclear membrane, virus is transported through the cytoplasm to the plasma membrane, where release of progeny virions occurs. (Modified a diagram kindly supplied by R. Lippe', 2000, www.google.com).

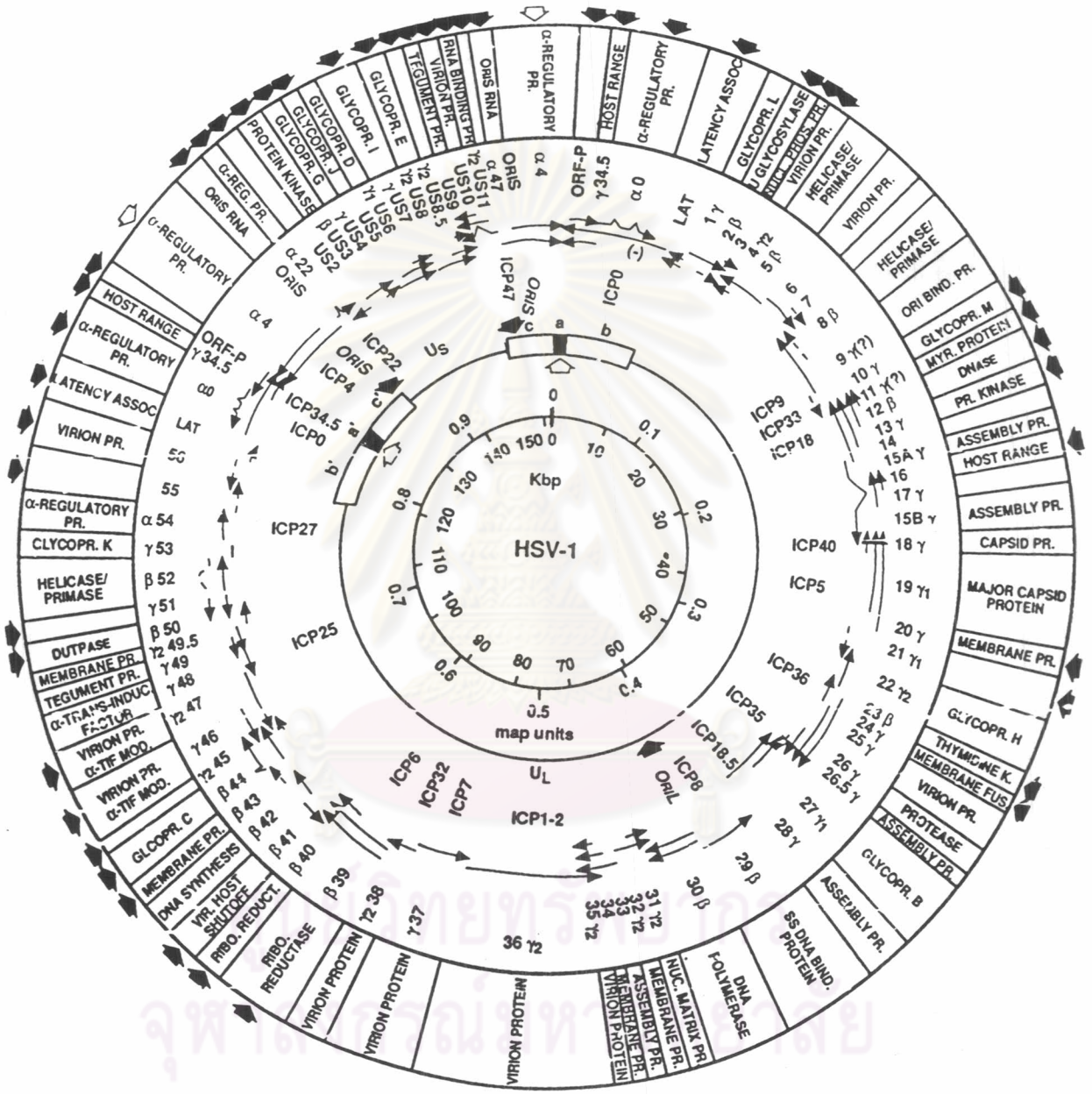
Unit-length viral DNA is cleaved from concatemers and packed into the preformed capsids. Capsids containing viral DNA acquire a new protein. Viral glycoproteins and tegument proteins accumulated and formed patches in cellular membranes. The capsid containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. The enveloped capsids accumulate in the endoplasmic reticulum and are transported into the extracellular space. Assembly occurs in stages, after packaging of DNA into preassembled capsids, the virus matures and acquires infectivity by budding through the inner lamellae of the nuclear membrane. In fully permissive tissue culture cells, the process takes approximately 18-20 hours.

5. HSV Proteins

The HSV-1 and HSV-2 genome containing 1.6 x 10^5 base pairs, 10% of which is reiterated twice (140,168) which has capacity to code for approximately 100 proteins (169), polypeptides , containing a total of approximately 44,000 amino acids. Investigators designate these HSV proteins as either infected cell specific polypeptides (ICSPs) or infected cell polypeptides (ICPs). Some 50 ICPs have been characterized by high resolution polyacrylaminde gel electrophoresis (PAGE) (170,171). Some of these proteins are described below.

Three groups of HSV proteins i.e. α, β, and γ, were synthesized from genes, designated as α, β, γ1 and γ2 based on the timing of and requirements for their expression (172). The five α genes are expressed first in the absence of viral protein synthesis and are responsible for the regulated expression of the other viral genes. The β genes require functional α gene products for their expression and encode proteins and enzymes that are directly involved in DNA synthesis and nucleotide metabolism. The γ genes are expressed from the last set of viral genes to be expressed, with the γ2 class having viral DNA replication as a strict requirement for their expression. The source of the data for the functional organization of the HSV genome was shown in Figure 4.

The eleven glycoproteins (g) were designated as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM. Six of these glycoproteins (gC, gE, gG, gI, gJ, and gM) appear unnecessary for entry and egress in most infected cells. The biologic properties of some of these glycoproteins have been identified (173,174). Glycoproteins are the major viral antigens expressed on the surface of both HSV virions and infected cells. Several potential biological functions have already been ascribed to various HSV glycoproteins (173). Glycoprotein B (gB) is required for infectivity, which has been implicated in viral penetration and fusion (175-178), glycoprotein C (gC) bind to the component of complement which has a receptor for the C3b component of complement (78) and confers type-specific antigen (80). Glycoprotein D (gD) is likely related to viral infectivity and is most potent inducer of neutralizing antibodies which is a major target for virus-neutralizing antibodies (181), composed of both type-common and type-specific determinants (182) and also implicated in cell adsorption (183), whereas glycoprotein E (gE) contains a receptor for the Fc protein of immunoglobulins (Ig) (184-186), binds to the Fc portion of IgG and necessary for viral infectivity (187). Glycoprotein F (gF) is identified only in HSV-2 infected cells and suggested to be type-specific (188,189). Later Zweig *et al.* (190), showed that HSV-1 gC and HSV-2 gF had related antigenic determinants since a monoclonal antibody specific for HSV-2 gF could also weakly precipitate gC in HSV-1 infected cell extract. Glycoprotein G (gG) is the target for antibody-mediated, complement-dependent viral neutralization (191), provides antigenic specificity to HSV and, therefore, results in an antibody response that allows for the distinction between HSV-1 (gG-1) and HSV-2 (gG-2). Glycoprotein H (gH) has been reported to have fusion activity (192). Furthermore, glycoprotein I (gI), has biologic properties that are thought to be involved with gE at the Fc receptor, which has been mentioned recently (193). Thus, Fc receptors may be composed of gE and gI and both may be required for Fc receptor activity (194). The role of gJ, gK, gL, and gM are not well characterized.


HSV-1
Kbp
map units
Us
U_L
ORIS
ORIS RNA
ORF-P
LAT
ICP0
ICP4
ICP22
ICP47
ICP34.5
ICP27
ICP25
ICP6
ICP32
ICP7
ICP1-2
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α-REGULATORY PR.
HOST RANGE
LATENCY ASSOC
GLYCOPR. L
GLYCOSYLASE
NUCL. PHOSPH.
VIRION PR.
HELICASE/ PRIMASE
ORI BIND. PR.
GLYCOPR. M
MYR. PROTEIN
DNASE
PR. KINASE
ASSEMBLY PR.
CAPSID PR.
MAJOR CAPSID PROTEIN
MEMBRANE PR.
GLYCOPR. H
THYMIDINE K.
MEMBRANE FUS
PROTEASE
GLYCOPR. B
SS DNA BIND. PROTEIN
DNA POLYMERASE
NUC. MATRIX PR.
MEMBRANE PR.
ASSEMBLY PR.
VIRION PROTEIN
RIBO. REDUCTASE
VIR. HOST SHUTOFF
DNA SYNTHESIS
GLCOPR. C
VIRION PR. α-TIF MOD.
α-TRANS-INDUC. FACTOR
TEGUMENT PR.
OUTPASE
CLYCOPR. K
PROTEIN KINASE
GLYCOPR. G
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GLYCOPR. I
GLYCOPR. E
TEGUMENT PR.
VIRION PR.
RNA BINDING PR.

Figure 4. Functional organization of the HSV-1 genome. The circles are described from inside out. **Circle 1**: Map unit are kilobase pair. **Circle 2**: Sequence arrangement of HSV genome. The letters *ab*, U_L, and *b'a'* identify the long (L) component consisting of the unique sequences U_L flanked by the inverted repeats. The letters *a'c'*, U_S, and *ca* identify the corresponding sequences of the short (S) component. The *open arrow* shows the sites of cleavage of concatemeric or circular DNA to yield linear DNA. Since the L and S components can invert relative to each other, the arrangement shown is that which would yield the "prototype arrangement" if linearization were to occur by cleavage of the DNA between map units 0 and 100. the *filled* arrows represent were the three originals of viral DNA synthesis, one in the middle of U_L (OriL) and two (both designated OriS) within the inverted repeats flanking the S component. **Circle 3**: The transcriptional map of the HSV-1 genome. The map serves the purpose of identifying the direction of teranscription, the approximate initiation and termination sites, and the families of 3' coterminal transcripts. *Dashed lines* identify transcripts mapped imprecisely. The designation between the second and third ring identify proteins encoded by the transcripts according to their ICP number. The designations outside this ring identify the open reading frame (those mapping in U_S have the prefix US) number and the kinetic class (α, β, γ) to which they belong. **Circle 4**: The known functions of the proteins specified by the open reading frames. The *filled arrows* identify open reading frames which can be detected without affecting the ability of the virus to multiply in cells in culture. The open *arrows* identify the two copies of the α4 gene; only one copy the this gene can be deleted without affecting the capacity of the virus to multiply (221).

6. Pathogenesis, Latency and Immune Response

The pathogenesis of HSV infections can be understood through knowledge of the events of replication and establishment of latency in both animal models and humans. The pathogenesis of human disease is dependent upon the intimate, personal contact of a susceptible individual (namely, one who is seronegative). Virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. With viral replication at the site of infection, either an intact virion or, more simply, the nucleocapsid is transported by neurons to the dorsal root ganglia, where latency is established. These events were demonstrated in a variety of animal models (165). Transport of the virion is by retrograde axonal flow (196). The fundamental principle of disease pathogenesis, then, is the propensity of virus to replicate at mucosal surface, be transported to dorsal root ganglia become latent. Although replication can sometimes lead to disease and can frequently in life-threatening central nervous system (CNS) infection, it is usually the host-virus interaction leading to latency which predominates. After latency is established, a proper provocation stimulus will cause reactivation to occur, the virus will become evident at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers. It is conceivable that with primary infection the virus can spread beyond the dorsal root ganglia, thereby becoming systemic; however, this event is unusual. Such circumstances include disseminated neonatal HSV infection with multiorgan involvement disease of pregnancy, and, infrequently, dissemination in patients who require significant immunosuppressive therapy. It is reasonable to presume that widespread organ involvement is the consequence of viremia in a host not capable of the limiting replication to mucosal surface.

HSV-1 and HSV-2 are common human pathogens that cause herpes labialis (cold sores or fever blisters), encephalitis, keratitis, and genitalis. HSV infections most commonly occur on the genitalia (genital herpes) and oral mucosa (gingivostomatitis, pharyngitis, and herpes labialis), while lesions can occur at nearly all visceral and mucocutaneous sites and the outcome can be serious (e.g. blindness and damage to the central nervous system) (197-208). HSV encephalitis is the most common form of sporadic encephalitis in the United States, while herpes keratitis affects approximately 400,000 individuals and it is the leading infections cause of corneal blindness in the United States

(209,210). Herpes genitalis is a common sexually transmitted disease, with HSV-2 accounting for approximately two-thirds and HSV-1 accounting for one-third of new cases in the United States (204).

HSV consists of two known serotypes of types 1 (HSV-1) and 2 (HSV-2), which albeit distinct are closely related (93,94). HSV-1 is common in general populations and is often acquired non-sexually during childhood years. HSV-2 is rarely a cause of oral lesions and serological evidence of HSV-2 is regarded as a good marker of infection acquired genitally. The frequency of oral-labial or genital herpes recurrences during the first year primary infection was reported to be highest in case of genital HSV-2, less frequent with oral-labial HSV-1, still less frequent with genital HSV-1, and least frequent with oral-labial HSV-2 infection (212). Thus, the frequency of recurrences appears to be influenced by the virus serotype and site of infection. Although previous evidence suggested that the majority of genital herpes infections were HSV-2 related, recent reports suggested an increase in HSV-1 genital infection in at least several countries (e.g. U.K.,U.S.A., and Japan), sometimes outnumbering case caused by HSV-2, especially in women (199-201,203-5,207-8,213-21). HSV-2 genital infection is frequently associated with instances of multiple sexual partners, however, a change in sexual practices and increase in oro-genital contact may be contributing to increase in patients with HSV-1 genital infection. While HSV-1 is becoming the major cause of primary genital infection, HSV-1 genital infection is less likely to recur (a long time to recurrence and fewer recurrences) than that caused by HSV-2 (198-201,205,210,222).

The ability of HSV to remain latent in the human host for its lifetime is the unique and intellectually most challenging aspect of its biology. Introduction of virus replication by axonal injury was reported by Walz *et al.* (223). They reactivated latent ganglionic infection by sectioning a peripheral nerve, resulting in the appearance of virus within the ganglia three to five days after the surgical manipulation. Reactivation of latent virus appears to be dependent on an intact anterior nerve route and peripheral nerve pathway (195). Accumulated clinical experiment suggests that after primary infection, replication of virus at the portal of entry, usually oral or genital mucosal tissue, results in infection of sensory nerve ending, the virus is transported to

the dorsal root ganglia. The resulting current lesion contains infectious virus. Recurrences appear in the presence of both cell-mediated and humoral immunity. No virus can be isolated from patients during interim periods (namely, those times between recurrences) at or near the usual site of recurrent lesions. Recurrences are spontaneous, but there is an association with physical or emotional stress, fever, exposure to UV light, tissue damage, and immune suppression (224-227). Reinfection with the same strain of HSV can occur by autoinoculation at a distant site. Thus, HSV-1 could be mechanically transmitted from one site to either an adjacent site or a distal one as occurs in cases of mouth-to-genital transmission (228) or intentional inoculation of vesicle fluid to "bolster immunity" (229-233). Depending on the host immune response, the resulting lesion may vary considerably in severity, from barely visible vesicle to rather severe, debilitating lesions in immunosuppressed individuals.

The natural history of HSV infections is influenced by both specific and nonspecific host defense mechanisms, as recently reviewed (233), which the characteristic ability to establish latency after primary infection, and thus to persist in the human host for life. For virus to do so, it must able to avoid immune eradication both after primary infection and after subsequent reactivation. Although the importance of immunity in HSV infection has been well characterized, it is the degree of adaptive immunity that best correlates with the severity of consequent clinical disease. Persons with primary infection have provided the majority of address the relationship between host defense and disease pathogenesis. Host genetic background, macrophages, natural killer cells, specific T-cells subpopulations, specific antibodies, and lymphokine responses have been implicated as important host defense against HSV infection. Host response to HSV infection may well influence (i) the acquisition of disease, (ii) the severity of infection, and (iii) the host resistance to the development, maintenance, and reactivation of HSV. Clearly, humoral immunity does not prevent either recurrences or exogenous reinfection. Thus, it is not surprising that transplacentally acquired antibodies from the mother are not totally protective against newborn infection but with conflicting results (234-239). Humoral antibodies can usually be detected within one to three weeks. With certain serological method it is also possible to demonstrate

an early rise of IgM antibodies to HSV, followed by IgA and IgG antibodies. In newborns, IgM to HSV can be detected within one to four weeks after birth and are persisted for six or more months. A cell-mediated immune response appears within one to two weeks after the onset of infection in both human and experimental animals. The role of both humoral and cell-mediated immune responses have been investigated. Determination of sera from patients previously infected with HSV-1 and HSV-2 indicates antibodies reacting with a range of HSV-specific proteins and glycoproteins (240-241). Although most viral polypeptides elicit an antibody response, it is generally thought that viral surface glycoproteins represent the most important of the viral antigens (241).

For HSV infection, the most prominent antibody response is directed against gB and gD (240). The initial host response to primary HSV-1 infection, whether genital or oral, is directed against gB whereas in primary HSV-2 genital infection, the initial response is against gG (241). Cellular immunity is considered important in the host response of the newborn. Newborns with HSV infections have a delayed T-lymphocyte proliferative response as compared with that of older individuals (236). Most infants studied had no detectable T-lymphocyte responses to HSV two to four weeks after the onset clinical symptoms (237,243). Cellular immune defects are more closely associated with severe HSV disease than are humoral immune defects (243), which points to the role for T cells in the control of HSV infection (244). Precursors of HSV-specific $CD4^+$ and $CD8^+$ T cells are found in peripheral blood, the exact frequency depending on the detection technique used. HSV evolved multiple immune evasion strategies to subvert the immune response and thus enable virus survival and replication in the human host. These include resistance to complement-mediated attack by gC (245), prevention of antibody binding (246), and down-regulation of the antigen-presenting machinery through ICP47, which prevents peptide loading onto major histocompatibility complex (MHC) class I and thus interferes with presentation of HSV antigens to T cells (247). One possible target site for HSV is at the level of the antigen-presenting cell (APC). The most potent form of APC known is the dendritic cell (DC) (248). Several viral infections of DCs have been analyzed, and two main points have been observed. DC maturation (increased expression of MHC and costimulatory

molecules) occurs, with subsequent enhanced APC activity. Alternatively, HSV infection results in impaired function and death.

Networks of DCs in epithelial surfaces, including epidermal Langerhans cells (LCs), are the first professional APCs to encounter HSV on mucosal/cutaneous infection, and the importance of DCs in the generation of immune responses to HSV has been demonstrated directly in murine models. The severity of HSV infections in mice is inversely to the number of $CD1a^{+}$LCs in the epidermis (249,250), and vaccination with UV-inactivated HSV-pulsed DCs has resulted in significant survival and reduced severity of illness after genital HSV-2 infection (251). Impairment of expression of costimulatory molecules also reduces HSV-specific $CD4^{+}$ T cell immunity and survival in mice challenged with HSV-1 (252). Therefore, the investigation of the consequences of HSV infection of human DCs is of considerable interest.

Coffin RS *et al.*, were the first who demonstrate that immature human DCs could be infected efficiently *in vitro* by HSV at low multiplicities of infection (MOI) (253). HSV infection of both immature (254,255) and mature (256) DCs has also been investigated in other studies. HSV induces partial maturation of DCs, but this process is counterbalanced by a progressive impairment of function that terminates in delayed cell death. Infection populations of DCs have an impaired ability to stimulate both recall and allogeneic responses but retain the ability to stimulate an HSV recall response in some individuals. Therefore DCs may have a dual role, which contributes to both the establishment and the resolution of the herpetic peripheral lesion (256).

Other investigators have observed delayed blastogenic responses to HSV (257). The correlation between these delayed responses may be of significance in evaluating outcome to neonatal HSV infection. Specially, if the response to T-lymphocyte antigens in children who have disease localized to the skin, eye, or mouth at the onset of disease is significantly delayed, disease progression may occur at a much higher frequency than it does in babies with a more appropriate response (237,258). More recently, natural killer cells have also been involved in the immunity of human to HSV infection. Other cell populations such as macrophages and many other T lymphocytes and lymphokines generated by these cells also play a part in the host's defenses against HSV infection (237).

7. Molecular Epidemiology of HSV

Epidemiologic studies of HSV type 1 and type 2 are difficult to perform on clinical grounds alone because of the frequency of asymptomatic infection. Moreover, antigenic cross-reactivity between the two virus types has only further confused the historical seroepidemiologic studies. HSV infections have historically been considered to be of biologic interest but of little clinical significance. The associated diseases these agent cause, with attendant clinical manifestations, range from the usual case of mild illness, undiscernible in the majority of patients, to sporadic, severe, and life-threatening disease in a very few infants, children, and adults. The cause of infections of HSV varies with respect anatomic site and to virulence. Although HSV type 1 and type 2 are usually transmitted by different routes and involve different areas of the body, as noted previously, there is a great deal of overlap between the epidemiology and clinical manifestations of infections caused by these viruses. When Schneweiss (84) discovered major antigenic differences among strains of HSV and thus defined two serotypes, HSV-1 and HSV-2. In 1967, Nahmias and Dowdle (108) identified HSV-1 as the causative agent in most nongenital infections while HSV-2 was shown to cause the majority of genital and neonatal infection. Subsequently, however, Ejercito *et al.*, (40) in 1968, observed intratypic differences in plaque morphology. In 1970, Terni and Roizman (109) discovered that some isolated identified as HSV-1 were nevertheless serologically distinct. Heine *et al.*, (106) established a molecular basis for the observed variation in intratypic seroreactivities by demonstrating nonrandom variation in the composition and size of virion polypeptides separated by polyacrylamide gel electrophoresis from several strains of HSV-1. Hayward *et al.*, (148) found that fragments resulting from cleavage of HSV DNA by restriction endonuclease enzymes define fingerprints for the virus. Analyses of numerous HSV type 1 and type 2 isolates from a variety of clinical situations and widely divergent geographic areas demonstrated that epidemiologically unrelated strains yielded distinct HSV DNA fragment pattern. In contrast, fragments of HSV virus DNA derived from the same individual obtained year apart, such as mothers and their newborns, monogamous sexual partners, or following short and long passages *in vitro* , had identical fragments after restriction endonuclease cleavage. Analyses of structural polypeptides of several

paired isolates also showed that electrophoretic mobility of structural polypeptides could serve as a useful tool for tracing the epidemiological patterns of HSV infections.

More extensive study involved analyses of the fragments generated by cleavage of HSV-1 DNA with restriction endonucleases, enzymes that recognize and cleave at specific sites within the DNA was done by Buchman *et al.*, (38). Analyses of more than 80 HSV-1 isolates of diverse clinical and geographic origin have shown that no two epidemiologically unrelated isolates yield identical DNA fragment pattern. However, isolates from the same individuals obtained as long as 12 years apart, viruses from a mother and her newborn, virus isolated from sexual partners, and samples from the third and more than 80 serial passages in cell culture yielded identical pattern. The variable characteristics are deletions or acquistions of restriction endonuclease cleavage sites, variability in the size of DNA fragments, and lateral displacement of nucleotide sequences.

The analysis of HSV strains by restriction endonuclease (RE) cleavage sites has been used not only to distinguish between HSV type 1 and type 2 but also for strain differentiation within an HSV type (143,259-269). The extent of intratypic polymorphism in HSV genomes led to the establishment of a new field in terms of molecular epidemiology (270,274). For instance, DNA variability among HSV strains has been employed in the field to trace transmission of HSV in hospitals and to determine whether infection resulted from reactivation of latent virus or from reinfection with exogenous virus (275,276). The markers for strain differentiation are mainly the presence or absence of RE cleavage sites, rather than variable fragment lengths resulting from variation in the copy number of short tandemly repeated sequence. These variable RE sites are considered to be generated by substitution of a nucleotide in the RE recognition sequence (277-279).

According to this model, genomic variability can be expressed in terms of nucleotide diversity, which is estimated from the sites common to homologous DNAs. In addition, for more precise analysis of divergence within and between populations, as many RE sites as possible should be used for a relatively large number of strains. The reports of new data 28 to 50 isolates from Korea, China and the U.S.A. were added to compare the nucleotide

diversity of isolates within and between countries (279-291). Extended the quantitative analysis of genomic polymorphism, epidemiologically unrelated HSV isolates from individuals of geographically separate countries, according to the presence or absence of number of sites for a set of RE, were done (279-291).

8. Detection and Identification of HSV

Laboratory diagnosis of viral infections is traditionally accomplished by the isolation and identification of virus from clinical samples, using cell and occasionally laboratory animals or embryonated eggs, which is inherently a slow process. The delays inherent in viral isolation in cell culture, the "gold standard" for the detection of viruses, created an urgent need for rapid, sensitive, specific, and reproducible methods to augment the slow cell culture procedures (292,293). The ability to detect viral antigens directly in clinical specimens improved greatly with the development of immunoassay, using antibodies labeled with fluorescein, radioisotopes, or enzymes. The following issues contributed to the impetus for rapid viral antigen detection: there are many important viral agents which can not be cultivated in cell cultures and some grow very slowly; the availability of effective antiviral drugs for early intervention: the need for early diagnosis of suspected teratogenic or perinatal viral infection during pregnancy; the ability to prevent and control infections in recipients of organ transplants and blood transfusions as well as in patients with sexually transmitted viral infections; the need to control nosocomial viral infections in hospitals and public institutions; the surveillance and control of epidemic diseases with early identification of the infectious agents to initiate appropriate measures to control the epidemics (292-297).

The appropriate use of laboratory tools is essential if a diagnosis of HSV infection is to be achieved, as was recently reviewed (298). Virus isolation remains the definitive diagnostic method. Many HSV infections are obvious and do not require laboratory confirmation. Some lesions are atypical, however, especially in immunocompromised patients, and clinical diagnosis may not be accurate (299). One study suggested that access to viral cultures improved accurate clinical recognition of HSV lesions (300). If skin lesions are present, a scraping of skin vesicles should be made and transferred in

appropriate virus transport media to a diagnostic virology laboratory. Clinical specimens should be shipped on ice for inoculation into cell culture system (e.g., foreskin fibroblasts, Vero cells, etc.), which are susceptible for the demonstration of the cytopathic effect (CPE) characteristic of HSV replication (301). CPE tends to develop within 24 to 48 hours after inoculation of specimens containing infectious virus. Conventional culture uses cells that are permissive for HSV-1 and HSV-2 as well as other viruses that may be of diagnostic importance. CPE typical for HSV or other viruses is sought by frequent examination with light microscopy. Infected cells develop cytoplasmic granulation and then become large, round, and refractile. Cluster of infected cells appear early after inoculation. Cells then lyse and detach from the plate or flask, with eventual destruction of the monolayer. Most laboratories maintain cultures for at least seven days; critical specimens such as CSF, tissue, or specimens from neonates may be held for 14-21 days to allow low titer virus to replicate to detectable levels. HSV is readily cultured in diagnostic laboratories. The virus grows in a wide variety of cell culture types. Primary rabbit kidney, mink lung, guinea pig embryo, and rhabdomyosarcoma cells particularly sensitive to HSV and allow slightly more rapid detection, although they do not greatly affect overall recovery (302-305). HSV grows rapidly in cell culture, with approximately 50% of positive cultures producing detectable CPE within 24 hours of inoculation of sensitive cells, 85% within 48 hours, and more than 99% within four days (305). HSV-1 and HSV-2 are equally well detected. Most laboratories hold herpes culture for five to seven days. Some laboratories perfrom typing of all positive cultures, whereas others perform typing only by specific request. Some laboratories have adopted shell viral culture techniques to increase the speed of detection. The specimen is centrifuged. After 24 to 48 hours incubation, the infected celles were detected by an antigen detection technique such as fluorescent antibody (FA) staining or enzyme immunoassay (EIA). The sensitivity of these techniques is probably comparable to conventional culture.

A variety of methods have been used to detect HSV antigen in specimens, including FA staining, EIA, immunofiltration, radioimmunoassay (RIA), and latex agglutination (LA). Typing of the virus can be accomplished readily by FA staining perfromed on a positive culture, using monoclonal

antibodies that are specific for either HSV-1 and HSV-2. FA staining of cells obtained from swab or scraping has had high sensitivity compared with viral culture in some studies (306-308). Rapid diagnosis and typing of HSV infection can be achieved using FA staining of material prepared from clinical specimens into a slide by scraping or swabbing material from the base of a lesion and placing it on a glass microscope slide, which is then air-dried and fixed with acetone. The performance of FA staining compared with culture depends greatly on the quality of the specimen submitted. The method detects cell-associated viral antigens, and therefore adequate cellular material must be present to achieve high sensitivity, reported sensitivity has ranged from 70% to 100% (309,310). A disadvantage of FA staining for HSV is that 10% to 30% of specimens tend to have an inadequate number of cells for adequate examination may be particulary difficult from mouth lesions in children and from cervical specimens. (311-317). Regardless of the method used, the sensitivity of a culture for HSV depends on several factors that affect the quality of the specimen, including the stage of the lesion, the amount of material obtained, the type of swab used, and the conditions of transport. The amount of infectious virus is higher in primary infections compared with reactivated infections. Also, the viral titer is the highest in fresh vesicular lesions and decreases progressively as lesions evolve (309,310). In one study, culture was estimated to have an overall sensitivity of 80% for patients with genital HSV. Sensitivity was higher with primary versus recurrent infections and with vesicular versus pustular or ulcerative lesions (310). In another study, HSV culture was positive in 77% of women with first episodes of genital HSV and in 47% of women with recurrent infections (300).

Other antigenic detection methods, EIA can be used to detect HSV in clinical specimens. Most EIAs have been less sensitivity than culture. It uses a polyclonal rabbit capture antibody, streptavidin-horseradish peroxidase, and a chromogenic substrate to detect HSV antigen. The assay detects both HSV-1 and HSV-2, for crusted lesions, its sensitivity exceeds that of culture, most likely because viral antigen persists after infectious viral particles have disappeared (318). For asymptomatic individuals, however, EIA is less sensitive than culture, consistent with the finding that it is 10 to 100 fold less sensitive in detecting dilutions of a stock virus (319).

Serology detection has little use in the diagnosis of currently active HSV infection because culture and antigen detection methods provide rapid direct evidence of infection. Serologic tests have been used in research studies of the epidemiolgy of HSV and are occasionally useful in unusual clinical situation. Methods that have been used to measure HSV antibody (anti-HSV) include complement fixation, neutralization, EIA, and Western blot. Individuals with primary infection are negative for anti-HSV at the time that lesion develops, and they subsequently develop HSV-specific IgG and IgM during the next one to two weeks. Individuals with reactivated infection have HSV-specific IgG at the time of onset and may or may not experience an increase in antibody level as the infection evolves. HSV-specific IgM is detectable in some episodes of reactivation (320-322). Thus the presence of HSV-specific IgM is not a reliable indicator of primary infection. Incerasc in HSV antibodies can also occur as a result of infection with other herpesviruses, especially VZV (323). Extensive antigenic cross-reactivity exists between HSV-1 and HSV-2. Until recently, serologic tests did not provide accurate information about whether a seropositive individual had been infected with HSV-1 and HSV-2 or both viruses (324). Western blot assays have been developed that can provide this information (325). In addition, EIAs using the HSV glycoprotein G (gG) are under development. The antibody response to gG is highly specific, and gG-based assay can accurately determine whether individuals have past infection with HSV-1 and HSV-2 (324,326). Typing of HSV may be done by using several techniques: monoclonal antibodies have been generated which appear to differentiate either HSV-1 or HSV-2 (313,327), and a unique Western blot assay can readily differentiate HSV-1 from HSV-2 infection (324). The PCR has been used in rapid and specific detection of HSV DNA from cerobrospinal fluid and applied to the diagnosis of HSV encephalitis (328-333).

The discovery of PCR by Kary Mullis in 1983 is the most important development in diagnostic virology since the development of cell culture. Mullis was awarded the 1993 Nobel Prize in medicine for his accomplishment (334). PCR can detect as few as one to 10 copies of viral nucleic acid, providing a sensitivity comparable to or greater than viral culture and far exceeding that of other diagnostic tests. PCR has also proved to be very

versatile, having the ability to detect DNA or RNA and to provide quantitative as well as qualitative information. Tests based on PCR have therefore assumed an important role for the laboratory detection of these agents. Event virus such as HSV which can be readily isolated, diagnosis by PCR has become the 'gold standard" for some disease such as herpetic encephalitis. Because infection with different or multiple species of herpesviruses can cause similar symptoms, PCR tests have been designed to detect more than one herpesvirus at a time.

The PCR might then be an attractive method for use in most routine microbiological laboratories, because it can be applied to all development stages of herpetic lesion, and to atypical mucocutaneous manifestation in an immunocompromised population. Moreover, results can be obtained within one day, whereas virus culture may take weeks (334-336). So far the only target (DNA polymerase gene) has been exploited for consensus PCR amplification of human herpesviruses DNA. They proposed a single pair of consensus primers to conserved regions of the DNA polymerase gene for detection of HSV-1, HSV-2, CMV, and EBV (331). The detection and species identification of HSV is based on a seminested PCR technique with primers followed by restriction enzyme analysis, the assay has been specially designed for clinical application. Methods used to achieve this have included parallel PCRs, multiplex PCRs with several primer pairs, and tests with a consensus primer pair targeting conserved genomic regions. Methods for subsequent confirmation of the species of the virus detected have included hybridization with a species-specific probe, nested PCR with species-specific primers, and restriction enzyme analysis (331,337-340). Herpervirus species identification has been achieved by digestion of amplicons with endonuclease *Bam*HI and *Sma*I. Recently, in 2000, Johnson *et al.* improved the assay by redesigning the primer and substituting *Bst*UI for *Sma*I. The improved assay provided detection and species identification of herpesviruses (341).

Typing or subtyping of viruses by detection of RFLP is a versatile and widely used method. Its basis is the activity of restriction endonucleases, which are enzymes that cleave DNA at specific four-nucleotide or six-nucleotide recognition sequences. The presence of nucleotide differences (i.e. mutations) at restriction endonuclease clevage sites in different strains of virus results in

different patterns of fragments after digestion of viral DNA with restriction endonuclease. This phenomenon is termed RFLP. The technique requires (i) fairly large amounts of purified or partially purifired viral DNA, (ii) a set of restriction enzymes with which to cut the DNA, (iii) the ability to separate the DNA fragments by electrophoresis, and (iv) a method for documenting the results. The results are usually displayed as patterns of bands in an agarose gel stained with ethidium bromide.

The RFLP typing method is directly applicable to DNA viruses but may be used with an RNA viral genome if it is first converted to complementary DNA (cDNA) in a reverse-transcription reaction. Viruses with large DNA genomes may have 20 to 50 bands, whereas viruses with smaller genomes have only 10 bands. The method is often not applicable to viruses with very small genomes because of an insufficient number of bands. The restriction enzyme used for the digestion is an important factor in the resultant number of bands. No easy way exists, and generally there is no need, to correlate the pattern (e.g. a missing band or an extra-band) with mutations at specific locations in the genome without extensive molecular hybridization studies or even sequencing of the genomes being compared. A significant limitation is that the RFLP method can not detect a mutation unless it falls within the recognition sequence of the restriction endonuclease being used for DNA digestion. If different serotypes of a virus are known, as with HSV, RFLP analysis of the DNA will identify the serotype as well as the subtype.

Timothy *et al.*, (259) analyzed 14 isolates of HSV and showed that among more than 50 total restriction enzyme cleavage sites, variability was present in at least 16. An RFLP analysis concluded that two HSV-1 isolates had been introduced independently into a pediatric intensive care unit. RFLP analysis of the isolates in an outbreak of HSV encephalitis in Boston showed that all isolates from seven patients had different restriction patterns and thus were not epidemiologically linked (241). Several restriction endonuclease enzymes that recognize and cleave at specific nucleotide sequences within DNA, have been found suitable for analyses of the HSV DNA because they cleave the DNA into relatively small number of fragments that can be separated by electrophoresis primarily according to size. The location in the intact DNA of the fragments generated by restriction endonuclease *Eco*RI, *Hsu*I,

*Xba*I, *Hpa*I, and *Bgl*II has been determined (342). Because HSV DNA consists of four populations differing in the orientation of L and S components, the fragments generated by restriction enzymes that do not cleave within the reiterated sequences *ab* and *ac* form three classes that differ in relative concentration. These studies permitted several conclusions. First, most epidemiologically unrelates were readily differentiated from each other by at least one of the five enzymes used in that study. The differential characteristics were loss of restriction enzyme cleavage sites resulting in an apparent fusion of two fragments or a gain or loss of 10% of the molecular weight of the fragment. Second, epidemiologically related strains, for example, repeated isolates from the same individual, viruses isolated from a mother and her newborn, or viruses isolated from sexual partners, were identical. Last, the genotypic differences between the isolates appeared to be stable both *in vivo* and *in vitro*. Thus, isolates obtained more than 12 years apart from recrudescent lip lesions of the same individual were identical, as were the third and greater than 80th serial passages in cell culture of the same virus strain (259).

9. Intratypic Polymorphism of HSV

Although the genetic maps of HSV-1 and HSV-2 are largely colinear, they differ in restriction endonuclease cleavage sites and in the apparent sizes of viral proteins. Thus, the initial locations of viral genomes on the linear map of HSV genomes were based on analyses of HSV-1 and HSV-2 recombinants and took advantage of (i) the intertypic difference in the sizes of the proteins and (ii) the locations of restriction endonuclease cleavage sites (150-1,343-4). The variability in HSV genomes and the use to which this variability can be put to answer specific questions regarding the epidemiology of herpesviruses in human populations.

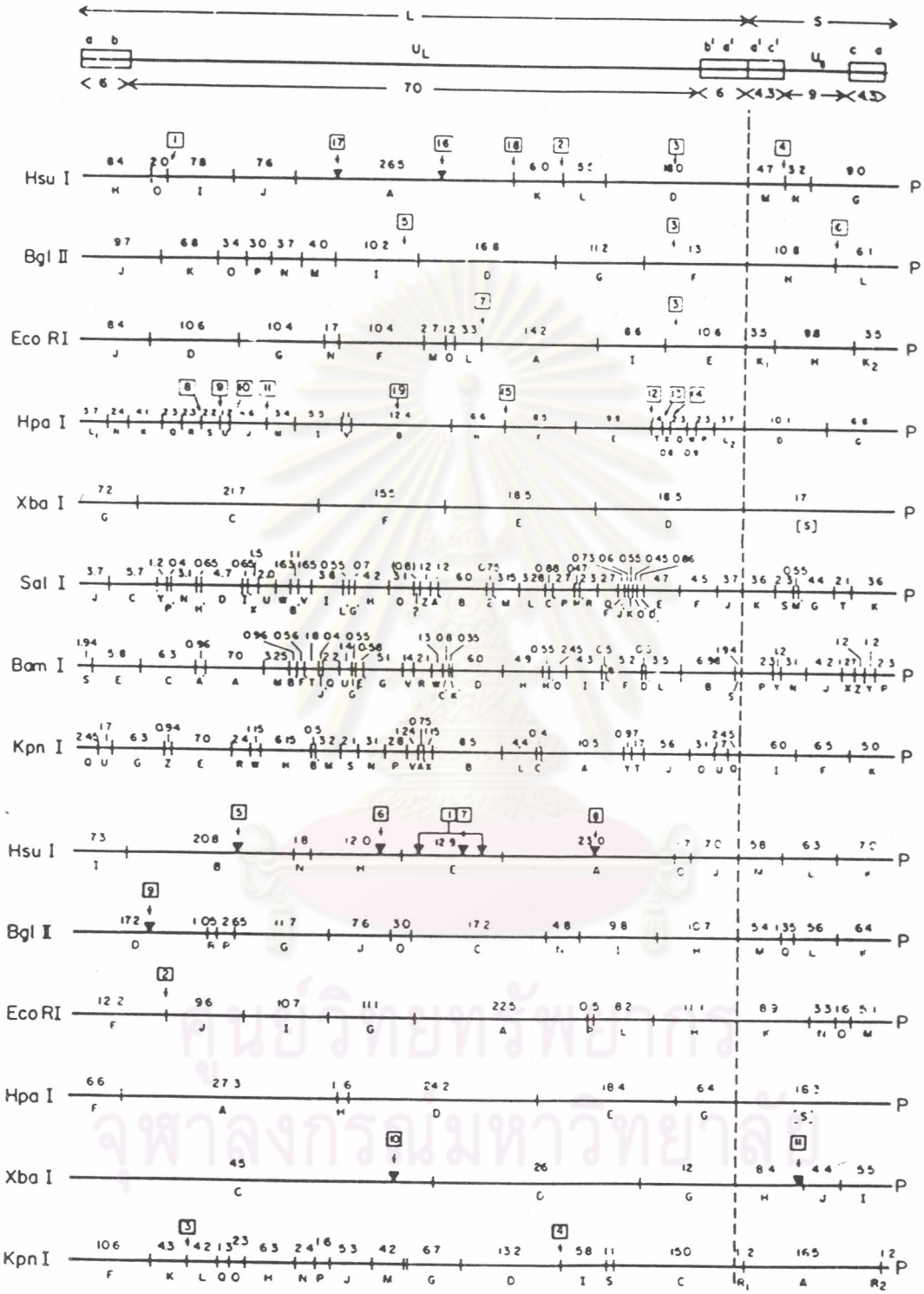
The first evidence of intratypic polymorphism emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers (345). Intratypic variability was also noted by Pereira *et al.*, (110) in their studies on the distribution of epitopes to specific monoclonal antibodies among HSV-1 and HSV-2 isolates. The usefulness of virion proteins as markers for molecular

epidemiologic studies was limited to the effort required to purify virions for such analyses. At the DNA level, differences between HSV-1 strain appear to result from (i) base substitutions which may add or eliminate a restriction endonuclease cleavage site and which may, on occasion, change an amino acid or (ii) variability in the number of repeated sequences present in a number of regions of the genome. The restriction endonuclease patterns of a given strain are relatively stable, whereas the number of repeats are not (145,347-349). Thus, no changes in restriction endonuclease patterns is in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphism was readily noted in isolates from epidemilogically unrelated individuals (272). On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population (272,347-349), and restriction endonuclease analyses of coded virus isolates have been used to trace the spread of infection from patients to hospital personnel (259), from hospital personnel to patient (249).

10. Variability in Restriction Enzyme Clevage Sites of HSV

Restriction endonuclease analysis of the virus gives positive identification of HSV strains and provides a powerful tool in epidemiological studies (352-358). Evidence suggesting that the HSV genome may be altered in the course of transmission and adaptation to a new patient was obtained by this sensitive technique (359). Methylation and sequence rearrangement of intracellular HSV DNA were also related to the mechanism of virus latency, on the basis of clear differences in restriction patterns (360-361). The results presented in this section center on two well-documented conclusions that HSV-1 and HSV-2 DNAs vary with respect to the presence or absence of restriction endonuclease cleavage sites, and that these differences do not obscure the lack of correspondence of cleavage sites in the DNAs of these two subtypes of HSV. The lack of correspondence between the mapped restriction endonuclease cleavage sites in HSV-1 and HSV-2 DNAs emerge from examination of the maps of colinear arrangements of the two DNAs (Figure 5). This figure also shows the variable restriction endonuclease cleavage sites as well as the

differences in the number of cleavage sites and the size of the fragments. It can be readily expected that HSV-1 and HSV-2 DNAs could be differentiated from each other even if all of the variable cleavage sites noted for any one enzyme show were to vary at once. The variable cleavage sites were determined from analyses of the DNAs of more than 120 HSV-1 and 50 HSV-2 isolates with *Xba*I, *Hsu*I, (*Hin*dIII isoschizomer), *Bgl*III, *Hpa*I, and *Eco*RI restriction endonuclease.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Kpn I
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Eco RI
Hpa I
Xba I
Kpn I
P

Fingure 5. Topology of the DNA of herpes simplex virus (HSV). A, sequence arrangement in HSV type 1 (HSV-1) DNA. The size of the unique sequences (U_L and U_S) as well as of the sequences *ab* and *ca* and their inverted repeats *a'b'* and *a'c'* are give as molecular weight (x 10^6). B, *Hsu*I restriction endonuclease maps of the four arrangements of HSV-1 DNA. P = prototype arrangement; I_S, I_L, and I_{SL} correspond to inverted L, inverted S, and inverted SL arrangements. Fragment sizes are given as molecular weights (x10^6). C, *Xba*I, *Hsu*I, *Bgl*II, *Eco*RI, and HpaI restriction endonuclease maps of HSV-1 DNA. Only the P arrangements are shown. The remainder can be generated by invertion endonuclease maps of HSV-1 DNA. Only the P arrangemets are shown. The remainder can be generated by inverting L,S, or both components. Boxed numerals 1-19 identify sites of variability in HSV-1 DNA. D, *Xba*I, *Hsu*I, *Bgl*II, *Eco*RI, *Hpa*I, and *Kpn*I restriction endonuclease maps of HSV type 2 (HSV-2) DNA. Only the P arrangements are shown. The remainder can be generated by inverting L, S, or both components. Boxed numberals 1-4 identfify sites of variability in HSV-2 DNA observed in the isolates used in this study. Note the absence of correspondence in presence and location of restriction endonuclease cleavage sites between the DNAs of the two types of HSV.

The variability observed with *Bam*HI, and *Kpn*I restriction endonucleases. The extent of variability is reflected in the observation that, to date, 19 of the 57 cleavage sites produced by four restriction endonucleases, *Hsu*I, *Bgl*II, *Eco*RI, and *Hpa*I, have been variable, a finding indicating that the smallest number of potentially differentiable strain is 2^{19}, or 524,288. The large number of fragments generated was also very extensive by each enzyme, it was never tabulated (362).

Several comments should be noted in connection with the variability in restriction enzyme cleavage sites observed to date. The restriction enzyme cleavage pattern of the DNA of any given isolate appears to be quiet stable. This conclusion is based chiefly on two studies. First, HSV-1 (MP) propagated serially at least 80 times between 1985 and 1986, when the practice of serially propagation viruses was terminated, could not be differentiated from either the second serial pasage or a concurrently, examined high-passage stock of the parent, HSV-1 (MP) virus. The second study was stimulated by the observation that the HSV-1 (KOS) strain obtained from Dr. P.A. Schaffer did not correspond with respect to restriction enzyme cleavage sites to the KOS strain obtained from Dr. F. Rapp. Subsequent comparisons with a fresh isolate from the same individual established that the virus obtained from Dr. Rapp could not be differentiated from the isolate obtained from the same patient 12 year later (2).

Variation in the presence or absence of restriction endonuclease cleavage sites reflects spontaneous insertions, deletions and substitution of bases at nonlethal sites in HSV DNA. A recent conference on genetic variation of viruses (326) made it apparent that variation of the type observed with HSV DNAs had also been observed with a varity of other DNA and RNA viruses. Although most of the epidemiologic studies carried out to date involved enzymes which recognize nucleotide, a single base-pair deletion, insertion, or substitution would sulfice to preclude cleavage, such mutations probably occur in human infection at a slow rate; they are perpetuated and disseminated when the mutated virus is transmitted from one individual to another. The observed variability in HSV DNA probably association of the virus with the human population. The apparent stability of the viral DNA probably reflects the test conditions rather than a moratorium on mutations. Specifically, it is likely the

people are infected with relatively low dose of virus and hence the progeny of the infecting virus, and hence the pool available for transmission to other people, are clonally related. Because mutant viruses generated after infection would represent a small fraction of the toial pool, the probability of transmission to another person is small and reflects the fraction of the mutants in the pool. In addition, the total number of HSV DNAs cleavage sites tested with *Xba*I, *Kpn*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Bgl*II, and *Hpa*I is of the order of 160 and involves approximately 1,000 base pairs. Since HSV DNAs has approximately 160,000 base pairs, the probability that a mutation would occur in one of 1,000 base pairs being tested and that the particular mutant rather than the parent would be transmitted from one individual to another is rather slim. Nevertheless, transmission of mutated viruses is likely to occur and be documented, especially as the fraction of the genome being sampled with restriction endonucleases increase. Because the distribution of restriction enzyme cleavage sites is obviously non-random, the question arises whether some restriction sites are more likely to accumulate mutations than other by virtue of being located in regions of the genome which tolerate changes in base composition.