CHARACTERIZATION OF WILD-TYPE AND TEMPERATURE SENSETIVE MUTANTS OF HSV-1 DNA POLYMERASE

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F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
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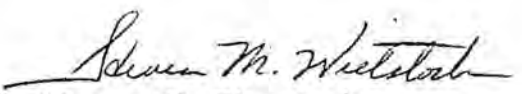

Steven M. Wietstock
Department of Biochemistry
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: Characterization of Wild-type and Temperature Sensitive Mutants of HSV-1 DNA Polymerase

Steven M. Wietstock, Doctor of Philosophy, 1988

Dissertation directed by: William T. Ruyechan, Ph.D., Department of Biochemistry

This study was undertaken in order to gain a better understanding of the activities and functional domains of HSV DNA polymerase. HSV polymerases were partially purified from cells infected with HSV-1 wild-type strains $\overline{m}$P and KOS, and temperature sensitive mutants, tsC4 and tsC7 (derived from KOS) whose mutations map within the HSV polymerase gene. The activities of these enzymes were characterized as to salt and pH optima, divalent cation optima, and DNA synthesis requirements. Differences between parental and mutant induced polymerases were observed in divalent cation use and sulfhydryl reagent requirements. The polymerases from tsC4 and tsC7 also showed decreased affinity for deoxynucleoside triphosphates as compared to the parental strain. The HSV $\overline{m}$P, tsC4, and tsC7 HSV polymerases were more sensitive to phosphonoacetate than KOS polymerase. The rate of thermal inactivation of partially purified polymerases and crude extracts of infected cells (including the polymerase from an additional ts mutant, tsD9) was determined. Little difference was seen between the wild-type and putative temperature sensitive enzymes with the exception of tsD9 polymerase in crude extracts. Velocity sedimentation of native and heat-treated nuclear extracts on neutral glycerol gradients revealed that the residual polymerase activity in the heat-treated extracts for all strains was consistently shifted to a slightly slower sedimentation

value indicating the possible dissociation of a macromolecular complex. These studies did not uncover any obvious differences in the catalytic activity of the mutant HSV DNA polymerases (with the exception of tsD9) that would account for the temperature sensitivity seen *in vivo* with the HSV-1 mutants. The temperature sensitive phenotype of these viruses is therefore likely due to some other interaction of the HSV replication system and not the basic deoxynucleotide polymerization activity.

CHARACTERIZATION OF WILD-TYPE AND TEMPERATURE SENSITIVE MUTANTS OF HSV-1 DNA POLYMERASE

by

Steven M. Wietstock

Dissertation submitted to the Faculty of the Department of Biochemistry Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1988

DEDICATION

To my wife, Anne, and our Parents

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I would like to sincerely thank my advisor, Dr. William T. Ruyechan for his support, guidance, and assistance in completing this research.

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Most of all, I am blessed for all of the love, support, and understanding that I received from my wife, Anne, who was always there with a smile and a willingness to do all that she could to see that this work was completed. Without her encouragement, this dissertation would not have been possible.

Abbreviations:

Ad, adenovirus;

EBV, Epstein-Barr virus;

HBLV, human B-lymphotropic virus;

HCMV, human cytomegalovirus;

HSV-1, herpes simplex virus type 1;

HSV-2, herpes simplex virus type 2;

SV40, simian virus 40;

TMV, tobacco mosiac virus;

VZV, varicella-zoster virus;

MOI, multiplicity of infection

pfu, plaque forming units

α-TIF, α-trans-inducing factor;

BSA, Bovine serum albumin;

Vhs, virion host shut-off

dNTPs, deoxyribonucleotide triphosphates;

dATP, 2'-deoxyadenosine-5'-triphosphate;

dCTP, 2'-deoxycytidine-5'-triphosphate;

dGTP, 2'-deoxyguanosine-5'-triphosphate;

TTP, thymidine-5'-triphosphate;

rNTPs, ribonucleotide triphosphates;

ATP, adenosine-5'-triphosphate;

CTP, cytidine-5'-triphosphate;

GTP, guanosine-5'-triphosphate;

UTP, uridine-5'-triphosphate;

A_{260}, Absorbance at 260 nm;

A_{280}, Absorbance at 280 nm;

Ci, Curie;

cpm, counts per minute;

Da, Daltons;

dpm, disintergrations per minute;

Km, Michaelis constant;

PAGE, polyacrylamide gel electrophoresis;

TLC, thin-layer chromatography;

Ammediol, 2-amino-2-methyl-1,3-propanediol;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

PBS, phosphate buffered saline;

PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid)

Tris, tris(hydroxymethyl)aminomethane;

DNA, deoxyribonucleic acid

RNA, ribonucleic acid;

mRNA, messenger RNA;

ssM13, single stranded M13 DNA strain mp9;

ssDNA, Single-stranded DNA;

hdDNA, heat denatured DNA;

ngDNA, nicked-gapped DNA;

ARS, yeast autonomously replicating sequences;

DBP, DNA binding protein;

ICP, infected cell protein;

Ori, origin of replication;

DMEM, Dulbecco's Modified Eagle Medium;

SMEM, Suspension Modified Eagle Medium;

FCS, fetal calf serum;

PEI, polyethyleneimine;

DEAE, diethylaminomethyl;

DEAE-T-M, DEAE-Trisacryl-M chromatography resin;

HAP, Hydroxyapetite

PC, phosphocellulose;

APS, ammonium persulfate;

BIS, N,N'-methylene-bis-acrylamide;

DMSO, dimethyl sulfoxide;

DTT, Dithiothreitol;

EDTA, ethylenediamine-tetraacetic acid;

β-ME, 2-mercaptoethanol;

NP-40, Nonidet P-40;

PEG, polyethylene glycol;

PMSF, phenylmethylsulfonyl fluoride;

SDS, sodium dodecyl sulfate;

TCA, trichloroacetic acid;

TEMED, N,N,N',N'-tetramethylethylene-diamine;

ACG, Acycloguanosine

BrdUrd, 5-bromodeoxyuridine;

NEM, N-ethylmaleimide;

NTG, nitrosoguanidine;

PAA, phosphonoacetic acid;

UV, ultraviolet;

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I. INTRODUCTION

I. A. History of Viral Infections

In ancient times, the Latin term *virus* was used nonspecifically to denote any poison or noxious agent. Viral infections have been described for centuries, although the causative agents of the infections were unknown until the past several hundred years. Rabies, for example, was known to have been accurately diagnosed by Greek and Roman physicians who based their diagnoses on earlier writings which described the course of the disease. The earliest report describing the course of the rabies infection was by Democritus circa 500 B.C. In 322 B.C., Aristotle described the behavior of rabid animals and reported that the transmission of rabies was accomplished by an infected dog biting an uninfected animal. The first account that related rabies to a "virus" was that of Celsus in the first century A.D., although he used the term "virus" in its classical sense as a "disease producing vapor". The causative agent still was not known. Other writings also described viral infections; for example, medieval writings described shingles, but it was not until the eighteenth century that this disease was attributed to a viral infection [Johnson, 1982]. Finally, a Chinese account from about the tenth century B.C., is believed to be a description of smallpox [Kucera, 1985]. It was not until the development of new technologies that the causitive agents for these diseases were discovered.

Many of the technological advances for the discovery of the agents for viral infections were developed during the nineteenth century. Until this time, it was known that certain diseases were

passed on by contact with "contagions", but what these agents were was not understood. Before the isolation of any virus was accomplished, Jenner introduced the practice of smallpox vaccination in 1798, by using exudate from cowpox lesions. Also without recognition that he was working with a viral agent, Pasteur developed a vaccine for rabies in 1884. During the late 1800s, major advances were made in determining the cause and effect of various infectious agents. It was during this time that Pasteur, Koch, Erlich, and others made significant progress in isolating and culturing pathogenic bacteria and describing their effect in specific diseases. These workers also found infectious agents that were unable to be cultured, although they remained infectious after passage through "bacteriologic filters". It was this particular group of agents that was given the name *virus*. [Kucera, 1985]

The first virus to be isolated for study was the tobacco mosaic virus (TMV). Iwanowski isolated TMV in 1892 by passage of extracts of TMV infected plants through "bacteriologic filters". However, Iwanowski drew the wrong conclusion from his results. He believed that the TMV was either a toxin or a bacterium that had passed through his filters. In 1899, the significance of Iwanoski's work became apparent when Beijerinck proved that TMV could be serially transmitted by bacteria-free filtrates. Soon after the isolation of this virus, other infectious agents were found that passed through these "bacteria-proof filters".

It was with the advent of the electron microscope that virologists were able to visualize and distinguish these agents from ultra-small bacteria or other molecules that may act as infectious particles. Much of the physiochemical characterization of viruses has been done

since the crystallization of the TMV was accomplished in the 1930's. The molecular-biological study of viruses was initiated with the development of cell culture methods in the 1950's. As is clearly evident, much of what we currently know about viruses has been discovered in the last 30-40 years [Johnson, 1982; Kucera, 1985].

I. B. The Herpesviruses

I. B. 1. History of the Human Herpesviruses

There are six human herpesviruses that have been identified to date. The term *herpes* originates with the Greek word ερεπιυ which means "to creep", and has been used since the era of Greek medicine to describe spreading cutaneous lesions of varied etiology. An observation made by a Roman physician, Herodotus, circa 100 A.D., described "herpetic eruptions which appear around the mouth at the crisis of simple fevers" [Nahmias & Josey, 1984]. What was first described by Herodotus is now known as herpes simplex virus (specifically described was herpes febrilis or herpes simplex virus type 1 (HSV-1)). In the 1500s, a French physician, Astruc, first reported cases of herpes infection of the genital tract (the causative agent is now called herpes genitalis or herpes simplex virus type 2 (HSV-2)). It was not until the 1900s that the term *herpes* was restricted to describe diseases that were associated with vesicular eruptions. In the early 20th century herpes zoster was differentiated from herpes febrilis and herpes genitalis based on cytopathological differences of the infections. This was shown by Grüter and others who demonstrated that the herpes febrilis and genitalis could be transmitted to rabbit corneas, whereas,

herpes zoster could not. The causative agent of the herpes zoster infection is now known to be varicella zoster virus (VZV) [Gelb, 1985; Straus et al., 1984].

Though it was postulated in 1920 by Lipschütz that the herpes febrilis and herpes genitalis were related but etiologically different, it has not been until recently that this hypothesis has been confirmed [Nahmias & Josey, 1984]. It was shown by Schneweis [1962] and again by Plummer [1964] that there are antigenic differences between the HSV serotypes. Nahmias and Dowdle [1968] showed that the majority of newborn infections and genital infections are due to HSV-2, while most of the non-genital infections are caused by HSV-1 [Nahmias & Dowdle, 1968]. Within the last ten years, it has been possible to show via the SDS PAGE patterns if infected cell polypeptides and restriction endonuclease analysis of HSV DNAs that there are strain differences within each serotype of HSV [Pereira et al., 1976; Buchman et al., 1978, 1979, 1980].

In addition to HSV-1, HSV-2 and VZV, there are three other human herpesviruses whose existence has been more recently recognized. The clinical presentations associated with human cytomegalovirus (HCMV), like HSV, were described years before the causative agent of these infections was known [reviewed by Alford & Britt, 1985]. Early observations of HCMV infections showed that this virus produced intranuclear inclusions and cellular enlargement in cells of infected individuals. This observation gave the disease the name "cytomegalic inclusion disease" (CID). The epidemiology and etiology of this disease led Lipschütz to postulate that the causative agent of the disease was a virus. CMV infections of other species such as mouse and guinea pig,

were being studied along with HCMV infections, and the virus had even been isolated from some of those species [Goodpasture & Talbot, 1921; Cole & Kuttner, 1926; Smith, 1954]. The isolation of CMV from other species led Smith [1956], Rowe et al. [1956], and Weller et al. [1957] to independently isolate HCMV from various tissues in infected individuals.

Several years after the isolation of HCMV, Burkitt reported on a particular lymphoma that was isolated from the jaws of African children [Burkitt, 1961, 1962; Burkitt and Davies, 1961]. The etiology of Burkitt's lymphoma, as this disease is now known, led Epstein & Barr to show that a herpes-type virus was associated with this lymphoma [Epstein & Barr, 1964; Epstein et al., 1964, 1965], and more recently that the Epstein-Barr Virus (EBV) is probably the causative agent of this disease [reviewed by Roizman & Kieff, 1974]. It has also been shown that EBV is the causative agent of infectious mononucleosis [Evans et al., 1968; Niederman et al., 1968, 1970; University Health Physicians, 1971; Sawyer et al., 1971, reviewed by Roizman & Kieff, 1974], and is associated with nasopharyngeal carcinoma [zur Hausen, 1980; Miller, 1985]. The last and most recent human herpesvirus to be isolated was the human herpesvirus 6. This virus was isolated in 1986 by workers at the National Institutes of Health, and is the focus of ongoing studies [Salahuddin et al., 1986; Josephs et al., 1986].

I. B. 2. Classification of the Human Herpesviruses

The human herpes viruses are six of over eighty known herpesviruses with a wide host range [Roizman, 1982]. The six known human

herpesviruses can be divided into three subfamilies based on their biological properties such as host range, length of their reproductive cycle, cytopathology and characteristics of latent infections. The six human herpesviruses and their properties are listed in Table I with their appropriate subfamily designations and International Committee for the Taxonomy of Viruses (ICTV) name. The most recent addition to this list occurred in 1986 with the isolation of the human herpesvirus 6 [Salahuddin et al., 1986; Josephs et al., 1986] which recently has been classified as belonging to the betaherpesvirinae subfamily [Honess, et al., 1988].

I. B. 3. Characteristics of the Herpesviruses

All of the herpesviruses studied to date share some common structural, replicative, and biological characteristics. The common structural characteristics of herpes virions include: 1) a fibrillar core around which the linear, double-stranded DNA is wrapped, 2) a 100-110 nm diameter icosahedral capsid made up of 12 pentameric capsomeres and 150 hexameric capsomeres, 3) a variable amount of globular material asymmetrically surrounding the capsid which is known as the tegument, and 4) a membrane surrounding the entire structure which is usually derived from the inner lamella of the nuclear membrane and which contains virus specific glycoproteins [Wildy et al., 1960; Roizman & Furlong, 1974]. The infectious virus particles are 120 to 200 nm in diameter and exclude negative stains used in electron microscopy [Roizman, 1982]. The virions of the herpesviruses cannot be distinguished from each other by electron microscopic observation. The

TABLE I

THE HUMAN HERPESVIRUSES

Alphaherpesvirinae

Host Range[1]: In Vivo: Very Limited to very wide
In Vitro: Very Limited to very wide
Duration of Reproductive Cycle: Short (18-24 hrs)
Cytopathology: Rapid lytic infection, spreads rapidly in cell culture
Latent Infections: Frequently Ganglia

Viruses[2] and Their Diseases:

- Human Herpesvirus 1 (Herpes Simplex Virus Type 1)
  "Cold Sores" (herpes febrilis & labialis), herpetic gingivostomatitis, herpetic paronychia, herpetic encephalitis, herpes gladiatorium...
- Human Herpesvirus 2 (Herpes Simplex Virus Type 2)
  genital herpes (herpes genitalis), herpetic meningitis
- Human Herpesvirus 3 (Varicella Zoster Virus)
  chickenpox (varicella), shingles (herpes zoster)

Betaherpesvirinae

Host Range[1]: In Vivo: Narrow
In Vitro: Fibroblasts
Duration of Reproductive Cycle: Relatively long (70+ hrs.)
Cytopathology: Lytic foci, cytomegelia, nuclear/cytoplasmic inclusions
Latent Infections: Secretory Glands, lymphoreticular cells, tissues

Viruses[2] and Their Diseases:

- Human Herpesvirus 5 (Human Cytomegalovirus (HCMV))
  "cytomegalic inclusion disease", numerous birth defects
- Human Herpesvirus 6
  no known diseases associated with this virus at this time

TABLE I (Cont'd)

Gammaherpesvirinae

Host Range[1]: In Vivo: Limited, B or T Lymphocyte specific
In Vitro: Lymphoblastoid cells
Duration of Reproductive Cycle: Variable
Cytopathology: Variable
Latent Infections: Lymphoid Tissues

Viruses[2] and Their Diseases:
Human Herpesvirus 4 (Epstein-Barr Virus (EBV))
infectious mononucleosis, Burkitt lymphoma,
nasopharyngeal carcinoma

[1]Host Range means ability of virus to propagate in species other than its natural host.

[2]ICTV Name, common name in parentheses.

Sources:

1. Melnick, 1984
2. Roizman, 1982
3. Salahuddin et al., 1986
4. Miller, 1985
5. Gelb, 1985
6. Roizman & Batterson, 1985
7. Alford & Britt, 1985
8. Rawls, 1985
9. Honess et al., 1988

viruses can, however, be distinguished by other features including serology and the size, base composition, and physical organization of their genomes.

The herpesvirus genomes range in size from 80-150 x 10^6 Daltons with guanosine plus cytosine (G+C) contents varying from 32 to 75 mole percent. The herpesviruses could be classified based on these characteristics; however, the most useful characteristic for the classification of the genomes of these viruses is the arrangement of reiterated sequences in the genome that are over 100 nucleotides in length. Using this criterion, the herpesviruses can be divided into five classifications as described in Table II and shown in Figure 1. Five of the six human herpesviruses fall into two of these five classifications. The HBLV genome has not been classified since the genomic structure has yet to be determined. The HSV-1 and HSV-2 genomes consist of two unique, covalently linked segments flanked by inverted repeats [Sheldrick & Berthelot, 1975]. These genomes belong to genome classification group E which gives rise to the possibility of four distinct isomeric arrangements of the genome. HSV-1 and HSV-2 demonstrate equimolar distribution of these four isomeric forms [Hayward et al., 1975b; Sheldrick & Berthelot, 1975; Delius & Clements, 1976]. Although VZV has the DNA sequences that would allow for all four isomers to exist, VZV is predominantly found in two genomic isomers with the other two found much less frequently [Straus et al., 1981, 1982; Ecker & Hyman, 1982; Davison & Scott, 1983; Davison, 1984; Kinchington et al., 1985]. The genomic structure of HCMV is similar to that of HSV-1 and HSV-2 and gives rise to four isomeric forms as well [Westrate et al., 1980]. Figure 2 shows the four possible genomic isomers for HSV-1,

TABLE II

CHARACTERISTICS FOR GENOMIC CLASSIFICATION

Group A: Characterized by a single reiteration of a single set of sequences in the same orientation at the termini giving rise to one genomic isomeric arrangement [Chousterman, 1979].

Virus: Channel Catfish Virus (Ictalurid herpesvirus 1)

Group B: Characterized by multiple reiterations of a single set of sequences in the same orientation at both termini giving rise to one genomic isomeric arrangement [Bornkamm, 1976].

Virus: Herpesvirus Saimiri (Saimiriine Herpesvirus 2)

Group C: Characterized by multiple reiterations of a single set of sequences in the same orientation at both termini as well as by internal tandem reiterations of other sequences giving rise to one genomic isomeric arrangement [Raab-Traub, 1980].

Virus: Epstein-Barr Virus (Human herpesvirus 4)

Group D: Characterized by inverted reiteration of a set of sequences from one terminus internally and by reiteration of a subset of those sequences in the same orientation at the other terminus giving rise to two genomic isomeric arrangements [Ben-Porat, 1979; Plummer, 1973].

Virus: Pseudorabies Virus (Suid herpesvirus 1)
Equine Herpesvirus (Equid herpesvirus 1)

Group E: Characterized by inverted reiteration of sets of sequences for both termini internally, as well as by reiteration of a subset of these sequences at both termini in the same orientation giving rise to a possible four genomic isomeric arrangements [Sheldrick, 1975; Wadsworth, 1975; Kinchington, 1985; Westrate, 1980; Lee, 1971].

Virus: Herpes Simplex Virus type 1 (Human herpesvirus 1)
Herpes Simplex Virus type 2 (Human herpesvirus 2)
Human Cytomegalovirus (Human herpesvirus 5)
Marek's disease herpesvirus (Gallid herpesvirus 2)
Varicella Zoster Virus (Human herpesvirus 3)

Figure 1. Organization of the Herpesvirus Genomes.

The herpesvirus genomes may be classified on the basis of the arrangement of tandem and/or inverted repeat sequences within the molecules into five classes as described in the text and in Table II. The arrows indicate the orientations in which the unique sequences may be found.

Classification Group

A

B

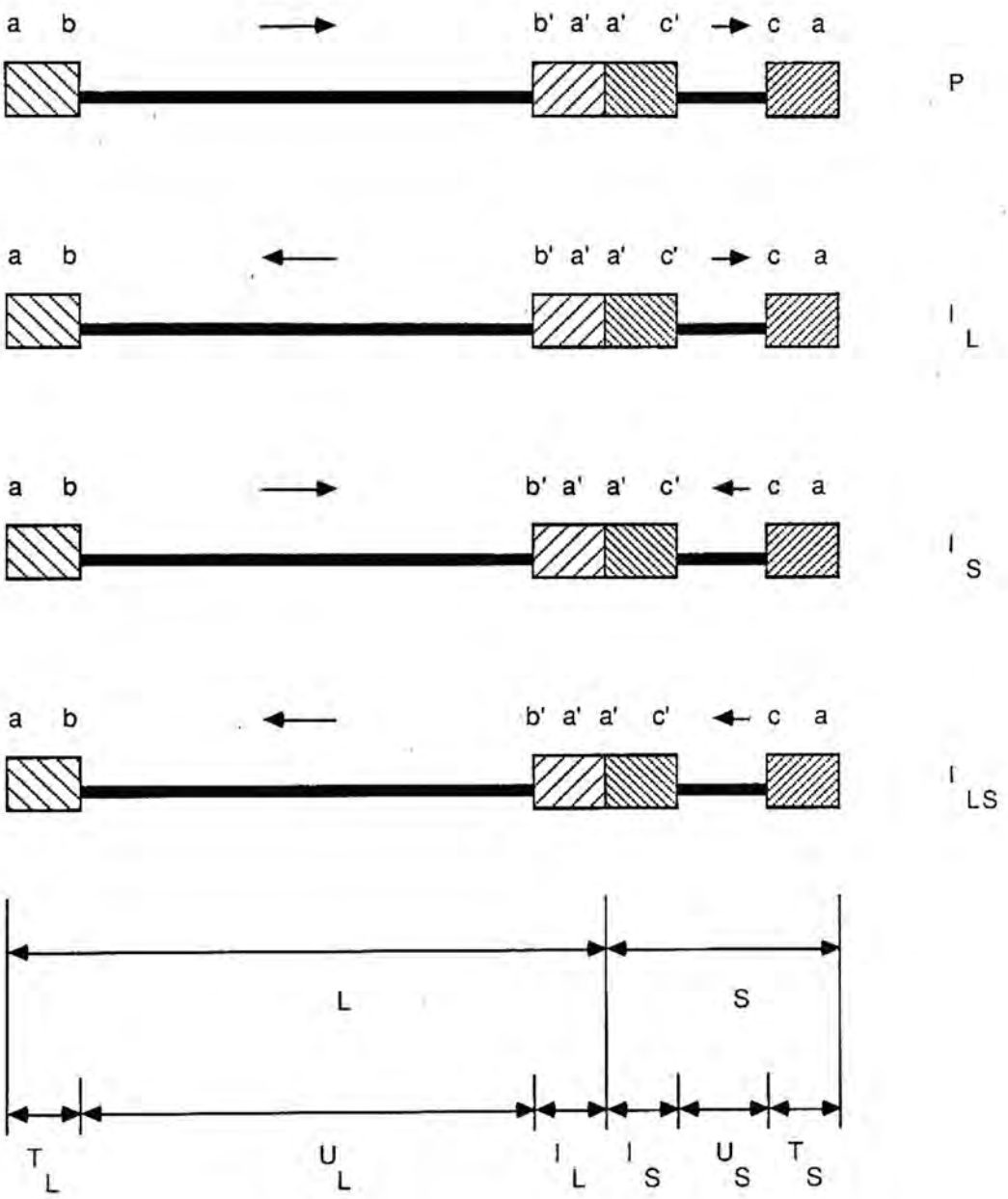
C

D

E

Figure 2. Genomic Isomers of Classification Group E.

The genomes of the viruses in Classification Group E, including HSV, VZV and HCMV, can be found in four isomeric forms as shown. P - prototype isomer; I_S - inverted unique short isomer; I_L - inverted unique long isomer; I_{SL} - inverted unique short and long isomer. The origins of replication are indicated by arrows above the prototype isomer.


a b
b' a' a' c'
c a
P
I L
I S
I LS
L
S
T L
U L
I L
I S
U S
T S

HSV-2, and HCMV with the reiterated inverted repeats (ab & a'b'(comprising 12% of the HSV genomes), ac & a'c' (comprising 8.6% of the HSV genomes) [Wadsworth et al., 1975]), and the unique long (U_L) and unique short (U_s) regions (comprising 70% and 9.4% of the HSV genomes, respectively) as indicated. Figure 1 also shows the genomic arrangement of EBV which belongs to classification group C [Shulte-Holthausen & Zur Hausen, 1970; Given et al., 1979; Given & Kieff, 1979; Kitner & Sugden, 1979; S. Hayward et al., 1980]. As was described in Table II, classification group C is characterized by a genome containing direct terminal repeats and another series of direct internal repeats which gives rise to only one unique genomic isomer. As of this date, three of the six human herpesvirus genomes have been completely sequenced: HSV-1 [McGeoch et al., 1988a], VZV [Davison, 1985], and EBV [Baer et al., 1984]

Another common feature of the HSV-1 and HSV-2 genomes is the molecular weight and base composition. Both of the genomes share a molecular weight of about 100 x 10^6 Daltons [Becker et al., 1968; Keiff et al., 1971; Wilkie, 1973; Sheldrick & Berthelot, 1975; Grafstrom et al., 1975; Wadsworth et al., 1975; Buchman et al., 1978] and nearly identical guanosine plus cytosine (G+C) contents; HSV-1 is 67 mole percent G+C, whereas HSV-2 is 69 mole percent G+C [Roizman, 1980a]. Table III compares these HSV genomic properties to the other human herpesviruses. As well as sharing size and G+C composition characteristics, the HSV-1 and HSV-2 genomes also share about 50% base sequence homology [Keiff et al., 1972]. Although the viruses share this high sequence homology, the two serotypes can readily be distinguished from each other based on their characteristic restriction endonuclease frag-

TABLE III

GENOMIC PROPERTIES OF THE HUMAN HERPESVIRUSES

ICTV Name (Common Name)	% G+C	Size (MW x 10^6)	Genomic Type
Human Herpesvirus 1 (HSV-1)	67	96	E
Human Herpesvirus 2 (HSV-2)	69	96	E
Human Herpesvirus 3 (VZV)	46	80	E
Human Herpesvirus 4 (EBV)	59	114	C
Human Herpesvirus 5 (HCMV)	57	145	E
Human Herpesvirus 6 (HBLV)	N.A.	~73	N.A.

N.A. = Information Not Available

Sources:

1. Straus et al., 1981
2. Dumas et al., 1980
3. Davison, 1985
4. Kinchington et al., 1985
5. Joesphs et al., 1986

ment banding pattern [Hayward et al., 1975a; Cortini & Wilkie, 1978]. In addition, individual strains within each serotype can be distinguished from others by differences in the restriction endonuclease digestion patterns of their DNAs, representing the presence or absence of a restriction enzyme site or deletions of sequences between restriction sites [Hayward et al., 1975a; Skare et al., 1975; Skare & Summers, 1977; Wilkie, 1976]. It is predicted that there may possibly be 2^{19} differentiable strains based on 19 variable cleavage sites out of the 60 that were studied in over 80 clinical isolates [Roizman, 1980b]. This may suggest that the viruses are mutating at a measureable rate. However, it is probable that the rate of spontaneous acquisition or loss of a restriction enzyme site is not ocurring at a rate that is readily measurable for the following reasons:

a. Identical restriction endonuclease cleavage patterns were obtained from viruses isolated from epidemiologically related persons [Buchman et al., 1978].

b. A HSV mutant virus, HSV-1(MP), was shown after 80 passages since its isolation from the parental strain, HSV-1(NT), to yield an identical restriction endonuclease pattern [Hoggan & Roizman, 1959; Roizman, 1980b].

c. The virus HSV-1(KOS) reisolated from the same individual 12 years after the initial isolation yielded identical restriction endonuclease digestion patterns as the laboratory passaged isolate [Roizman, 1980b].

A final property that all the herpesvirus genomes share is randomly placed nicks and gaps in their genomic DNAs. These nicks and

gaps were shown to exist by several methods, including alkaline denaturation [Keiff et al., 1971; Frenkel & Roizman, 1972; Wilkie, 1973; Hyman et al., 1977], and λ-exonuclease digestion (which requires gaps for nucleolytic activity) [Wadsworth et al., 1976]. Ribonucleotides have been shown to exist in HSV DNA [Hirsch & Vonka, 1974; Biswal et al., 1974; Muller et al., 1979b], but it is not likely that they account for the amount of fragmentation that is seen since there is no detectable difference in the velocity sedimentation profile of DNA that was denatured with formamide compared to DNA that was denatured with alkali [Roizman, 1980b]. The role that these nicks and gaps play in the HSV lifecycle have yet to be determined.

In addition to sharing similar genomic properties, the herpesviruses share other characteristics as well. As was mentioned previously, the herpesviruses can be classified based on their host range, cytopathology, and properties of latency [Table I]. The alpaherpesviruses have variable host ranges and relatively short reproductive cycles. These viruses tend to proliferate rapidly in cell culture causing lysis of the host cells. These viruses also do not lend themselves to the establishment of carrier cultures because of the destructive nature of their infections. The betaherpesviruses differ from the alphaherpesviruses in that most of the betaherpesviruses have a very narrow host range in vivo, and replicate best in fibroblasts in vitro. Since the reproductive cycle of the betaherpesviruses is relatively long, and the infection produced does not cause extensive cell death, it is possible to produce carrier cell cultures. In comparison, the gammaherpesviruses have a very narrow host range, usually limited to the family or order of their natural host. These viruses are usually

non-lytic, and specific for B or T lymphocytes; although, some viruses may cause lytic infections in fibroblast or epithelial cells lines in cell culture. The duration of the reproductive cycles of these viruses are variable, as well as the cytopathology of the infection [Roizman, 1982].

Another common feature of the herpesviruses is the ability of some viruses to produce latent infections in their natural hosts. It is known that most of the alpaherpesviruses can become latent in the ganglia of the infected host (this is especially true in the human alphaherpesviruses [reviewed by Roizman & Sears, 1987]), whereas the gammaherpesviruses are usually found to be latent in lymphoid tissues. The betaherpesviruses tend to show the widest variability in the tissues where the latent virus may be found. These tissues include the secretory glands, the kidneys, lymphoreticular cells, and others. [Roizman, 1982]. The mechanism of latency is currently the object of much interest and speculation, although it is beyond the scope of this dissertation (a recent review of this topic has been made [Roizman & Sears, 1987]).

A final characteristic of the human herpesviruses is their potential for being able to transform cells and to cause tumors. All of the human herpesviruses, except for human herpesvirus 6, have been shown to be associated with different neoplasias [Roizman, 1980b]. HSV-1 and HSV-2 (predominantly HSV-2) have been implicated in the formation of, and found to be associated with cervical carcinomas [Naib et al., 1969; Kessler, 1973; reviewed by Roizman & Kieff, 1974; and Nahmias & Norrild, 1980]. Strong circumstantial evidence has linked EBV with two human malignancies, Burkitt's lymphoma and nasopharyngeal

carcinoma [reviewed by Roizman & Keiff, 1974; zur Hausen, 1980]. HCMV has not been directly related to any one specific neoplasia, but has been detected in leukocytes; lymphoblasts; normal and cancerous prostate cells; cervical carcinoma cells; and in human kidney tissue. In addition, HCMV has been shown to have the ability to transform fibroblasts in vitro [reviewed by Geder, 1980]. HBLV was isolated from individuals presenting with associated lymphoproliferative disorders; however, the role that HBLV may play in any neoplasia is not known [Salahuddin et al., 1986].

I. B. 4. Epidemiology of the Herpes Simplex Viruses

At this point in the survey of the herpesviruses, the focus will be turned to the herpes simplex viruses. Infection by HSV is common and widespread. As was mentioned previously, The two serotypes, HSV-1 and HSV-2, usually have different modes of transmission. HSV-1 is usually transmitted by a nongenital route; whereas, HSV-2 is primarily transmitted venereally [Nahmias & Josey, 1984]. It is estimated that 20-40% of the world population is affected by recurrent orofacial HSV-1 infections which is the predominant clinical presentation of HSV-1 [Embil et al., 1975; Young et al., 1976]. HSV infections are found worldwide with equal distribution between the sexes [Reichman, 1984] with a somewhat higher frequency of HSV-1 and HSV-2 antibodies found in black populations [Reichman, 1984; Rawls & Campione-Piccardo, 1981; Smith et al., 1967] This probably reflects differences in socioeconomic positions and sexual activities rather than differences in host response or susceptability to HSV infection. There also appears to be no

seasonal variation in the incidence of HSV infections [Nahmais & Josey, 1984; Reichman, 1984].

The course and serotype of HSV infection appears to be related to the age at which the primary HSV infection occurs. Disseminated herpetic infections, most often HSV-2, occur in newborns as a result of an active maternal genital infection and occur in 1 in 10,000 births [Nahmias & Visintine, 1979]. If untreated, the mortality rate for these infants is about 75% [Reichman, 1984]. During childhood, the most common herpetic infections are due to HSV-1 and are reflected clinically as herpetic gingivostomatitis, and less often as infections of the eyes, skin, and central nervous system. Some investigators believe that many of the primary infections during childhood are asymptomatic. Primary infections of HSV-1 can also occur in adults primarily as oral or genital infections [Glezen et al., 1975; Corey et al., 1981]. HSV-2 infections and their clinical manifestations occur mainly after the adolescent years when venereal exposure becomes the main mode of transmission. Two seroepidemiological studies [Nahmias et al., 1970; Rawls et al., 1974] have shown that between the ages of 1 and 5 years there was a significant rise in the occurrence of HSV-1 antibodies until approximately 50% of individuals studied demonstrated antibodies to HSV-1. This rise continued through adulthood until about 90% of the population studied demonstrated antibodies to HSV-1. This finding is in contrast to HSV-2 where antibodies to HSV-2 were not detected until around the age of 14 years and peaked when the subjects reached the age of 35 with 30-35% of the population showing antibodies to HSV-2.

There are several groups of individuals who are at a higher risk

of HSV infection than the general population. These include nursing, dental and hospital personnel who are at risk of acquiring herpetic paronychia (herpes infections in the folds of skin around the fingernails) [Rosato et al., 1970; Buchman et al., 1980]; prostitutes who are at an above average risk of developing genital herpes infections (often along with other venereal diseases) [Duenas et al., 1972]; and wrestlers or other individuals involved in close contact sports who are at risk for developing "herpes gladiatorium" (herpes infections of the shoulders and back due to close body contact where the skin may become damaged and come in contact with saliva of a person who is shedding virus) [Selling & Kibrick, 1964; Porter & Baughman, 1965; Wheeler & Cabaniss, 1965]. HSV-1 infections are also prevalent in situations where there is prolonged exposure or personal contact with individuals who are carriers of the virus. These situations include nurseries, and families [Nahmias & Josey, 1984].

In addition to the types of infections already discussed, HSV can also cause herpetic keratitis (in 5% of opthalmology patients) [Howard & Kaufman, 1962], herpetic skin infections (in 2-3% of dermatology patients) [Eilard & Hellgren; 1965], respiratory infections [Douglas & Couch, 1970; Sheridan & Herrmann, 1971; Glezen et al., 1975], HSV encephalitis (1 case in 1 million of population with a fatality rate of 70%) [Whitley et al., 1977; Longson, 1979] and meningitis [Nahmias & Josey, 1984]. Some of the more severe complications of HSV occur in patients that are immunocompromised either by other diseases or by the use of immunosuppressant drugs [Merigan, 1981]. Although HSV-2 has been shown to be associated with cervical carcinoma, there is some disagreement among investigators as to the hypothesis

that HSV-2 infections in women may result in a greater probability of developing cervical carcinoma [Naib et al., 1969; Kessler, 1973]. Many of the other vertebrate herpesviruses also share similar epidemiological traits, such as venereal transmission, and the ability to cause dermatological lesions, keratitis, encephalitis, and other neurological problems [Nahmias, 1972].

There are no known animal vectors for the transmission of HSV, although animals can be infected with HSV. The major route of transmission is contact with infectious secretions usually during close personal contact with an infected individual who has a subclinical or clinically inapparent primary or recurrent infection [Roizman & Sears, 1987; Nahmias & Josey, 1984]. A primary infection is defined as an "infection which occurs in the absence of detectable antibody directed against HSV antigens in acute phase sera" [Reichman, 1984]. The incubation period for HSV infections is from 2 to 12 days with the average being 6 days, but the primary infection may be asymptomatic and it may be years before a physical outbreak of the disease occurs [Nahmias & Josey, 1984; Reichman 1984]. In the initial phases of infection, the virus infects parabasal and intermediate epithelial cells where the virus multiplies, eventually leading to cell lysis and an inflammatory response. The virus eventually enters the sensory nerves where it travels intraaxonally to the sensory ganglia where the virus becomes latent [Reichman, 1984; Roizman & Sears, 1987]. The lifecycle and mode of replication of the virus will be described in the next several sections.

I. B. 5. Lifecycle of the Herpes Simplex Viruses

The lifecycles of most of the human herpesviruses are poorly understood. This in part is due to the lack of cell lines that lend themselves to synchronized, productive infections from which useful information about the nature and temporal regulation of infection can be obtained. For this reason, most of the information about the lifecycles of the human herpesviruses have been based by analogy with the lifecycles of HSV-1 and HSV-2, which can support synchronized, productive infections in cell culture and about which a great deal of information has been obtained.

The first step in the lifecycle of the HSV is the attachment of an intact virus particle to specific receptors on the host cell surface [Roizman & Batterson, 1985]. Once the virus adsorbs to the cell wall, it enters the cell by fusion of the viral envelope with the cellular membrane [Morgan et al., 1968; Para et al., 1980; Roizman & Batterson, 1985]. Therefore, in order for the virus particle to be infectious, the outer envelope of the virus must remain intact [Smith, 1964]. Lipid solvents, such as ether, chloroform and alcohol, inactivate the virus, as do proteolytic enzymes and radiant energies (UV light, for example) [Reichman, 1984]. After the outer lipid membrane fuses with the cell wall, the tegument proteins and the viral DNA containing capsid are released into the cytoplasm, where the capsid is transported to the nuclear membrane. The viral DNA is injected into the nucleus through the nuclear pores [Knipe et al., 1981; Batterson et al., 1983], where the DNA circularizes [Roizman and Sears, 1987]. At this point, another virion protein, α-trans-inducing factor (α-TIF), induces the

coordinated and sequentially regulated transcription of the herpesvirus DNA by RNA polymerase II [Constanzo et al., 1977; Roizman & Batterson, 1985]. The resulting viral mRNAs vary in abundance and in stability, and are capped, methylated, and polyadenylated [Bachenheimer & Roizman, 1972; Frenkel & Roizman, 1972; Frenkel et al., 1973; Silverstein et al., 1973, 1976; Jacquemont & Roizman, 1975; Bartkoski & Roizman, 1976, 1978; Stringer et al., 1977]. Some of the viral mRNAs have been detected after the cessation of translation [Johnson & Spear, 1984; Kozak & Roizman, 1974, 1975]. Other characteristics of some of the viral mRNAs include: mRNAs with 5' or 3' coterminal ends [Hall et al., 1982; McLaughlan & Clements, 1982; Wagner, 1985], multiple transcription initiation sites [Frink et al., 1981; Watson et al., 1981; Zipser et al., 1981; Murchie & McGeoch, 1982; Sharp et al., 1983], and derivation of some mRNAs by splicing of larger RNA precursors [Frink et al., 1981]. Some of these properties are shared by host mRNAs.

The herpes simplex genome codes for approximately 72 proteins [Roizman & Sears, 1987; McGeoch et al., 1988a] which are synthesized in three major groups: the immediate early or α gene products, the early or β gene products, and the late or γ gene products [Honess & Roizman, 1974; Roizman et al., 1975]. There is additional evidence that the β gene products can be further divided into two classes: β_1 and β_2 [Honess & Roizman, 1974, 1975; Pereira et al., 1977]. The synthesis of each class of proteins begins, reaches maximum synthesis and declines at various times after infection.

The α gene products are known as infected cell proteins (ICP) α0, α4, α22, α27 and α47 (the system of nomenclature used here is the one used by Roizman & Batterson, 1985; Table IV compares three of five

TABLE IV

NOMENCLATURES FOR SELECTED HSV-1 ENCODED PROTEINS

Protein Function	System of Nomenclature: Infected Cell[1] Protein	Temporal[2] Class	Open Reading[3] Frame
Required Replication Proteins			
HSV DNA polymerase	N.I.	N.I.	UL30
Major DPB	ICP8	$\beta_1 8$	UL29
	N.C.	N.C.	UL5
	N.C.	N.C.	UL8
	N.C.	N.C.	UL9
65 KDa DPB	N.C.	N.C.	UL42
	N.C.	N.C.	UL52
Major Capsid Protein	ICP5	$\gamma_1 5$	UL19
Ribonucleotide Reductase			
Large Subunit	ICP6	$\beta_1 6$	UL39
Small Subunit			UL40
Alkaline Exonuclease	ICP18	$\beta_2 18$	UL12
Thymidine Kinase	ICP36	$\beta_2 36$	UL23
α-TIF			UL48
Immediate Early Genes	ICP0	$\alpha 0$	IE110
	ICP4	$\alpha 4$	IE175
	ICP22	$\alpha 22$	US1
	ICP27	$\alpha 27$	UL54
	ICP47	$\alpha 47$	US12
Glycoproteins			
B			UL27
C			UL44
D			US6
E			US8
G			US4
H			UL22
I			US7

N.I. = not identified
N.C. = not cross referenced

[1]Roizman, 1980
[2]Roizman & Batterson, 1985
[3]McGeoch et al., 1988a

systems of nomenclature used for the HSV proteins). These gene products require no prior infected cell protein synthesis for their expression, and are maximally synthesized 2 to 4 hours post infection in untreated cells [Roizman, 1980b; Honess & Roizman, 1974]. α protein synthesis is mediated via the virion factor, α-TIF, through cis-acting sites on the promoter-regulatory domains that have host transcriptional factors bound to them. These factors are responsible for controlling basal levels of gene expression and whether or not the genes are being expressed [Kristie & Roizman, 1984, 1987]. The functions that most of these gene products play are still in question. However, α4, and possibly one or more of the other α genes, is necessary for the expression of the other herpes genes [Roizman & Batterson, 1985].

Evidence suggests that α4 binds to the 5' transcribed noncoding regions of the herpes simplex genes which results in the positive regulation of the β and γ genes, and the negative regulation of the α genes [Roizman & Batterson, 1985; Kristie & Roizman, 1986a, 1986b, 1987]. The role of the other α genes is not as clear. It appears that α47 is nonessential for viral replication in some cell culture lines [Mavromara-Nazos & Roizman, 1986]; whereas, α22 and α27 appear to be required for late gene expression [Sacks et al., 1985; Sears et al., 1985]. Little is known about the role of α0; although, it appears to act as a general inducer of protein synthesis [Roizman & Sears, 1987].

The β genes primarily code for proteins needed to replicate the viral DNA. This class of genes can be further divided into two groups of genes that are expressed maximally at 5 to 7 hours after infection. The β_1 genes, represented by the major DNA binding protein ($\beta_1$8), and the ribonucleotide reductase ($\beta_1$6), are expressed slightly earlier than

the majority of the β genes which are expressed in the later class of β_2 genes. These include the viral DNA polymerase, thymidine kinase ($\beta_2$36) and the alkaline exonuclease ($\beta_2$18) [Roizman & Batterson, 1985; Wagner, 1985]. During the time that the β proteins are produced, the replication of the viral DNA is initiated. The mechanism of DNA replication will be discussed in section I.C.2 of this work.

The γ proteins can also be divided into two classes based on their requirement for viral DNA replication. The γ_1 proteins (such as α-TIF, glycoproteins B and D, and the major capsid protein ($\gamma_1$5)) are expressed, although at somewhat reduced levels, when viral DNA synthesis is inhibited. The γ_2 genes (such as glycoproteins C and E) require viral DNA synthesis for their expression [Roizman & Batterson, 1985]. It is during this late phase of the infection that capsid formation begins to take place in the nucleus.

In addition to the synthesis of viral gene products, there are significant cellular changes that occur during productive viral infections. These changes include the shut-off of host macromolecular synthesis, and alterations in the structure and integrity of the cell [reviewed by Roizman and Sears, 1987]. The shut-off of host macromolecular synthesis is incremental and takes place in two stages. The first stage of host shut-off occurs immediately after the initial infection of the cell, is a non-essential function, and appears to be accomplished by one or more structural virion proteins which are known as virion host shut-off proteins (Vhs) [Read & Frenkel, 1983]. The second round of host shut-off occurs during the synthesis of the β and γ proteins, and is probably mediated through a second mechanism, since mutants in Vhs are able to complete host shut-off at this time [Read &

Frenkel, 1983].

The infected cell nucleus also undergoes structural changes. An early sign of herpesvirus infection is the condensation and displacement of the chromatin to the sides of the nucleus [Smith and de Harven, 1973; Roizman & Sears, 1987] with the inhibition of host DNA synthesis [Roizman & Roane, 1964; Aurelian & Roizman, 1965; Roizman, 1969; Gergely et al., 1971a, 1971b; Nonoyama & Pagano, 1972]. At the same time, the nucleolus becomes enlarged and is displaced toward the nuclear membrane where late in infection it begins to fragment and disaggregate [Schwartz & Roizman, 1969; Nii, 1971a, 1971b; Roizman & Sears, 1987]. A characteristic of late infection is the distortion of the nucleus at points where long stretches of the nuclear membrane are folded upon themselves [Nii et al., 1968; Schwartz & Roizman, 1969]. In addition to the changes that take place in the nucleus during active infection, there are increases in the size of the polyribosomes such that they contain 15-23 ribosomes [Roizman & Furlong, 1974]. This increased number of ribosomes accounts for the observation that the sedimentation rate of infected cell polyribosomes increases [Sydiskis & Roizman, 1966, 1968]. A final observation that has been made concerning the infected cell is that viral glycoproteins are inserted into cellular membranes, which provide targets for the host immune response [Spear et al., 1970; Keller et al., 1970; Roizman & Spear, 1971; Heine et al., 1972; Heine and Roizman, 1973].

During the synthesis of the γ_2 proteins late in infection, empty capsids begin to accumulate in the nucleus. The newly synthesized viral DNA is then processed and packaged into the empty capsids. The DNA containing capsids then attach to patches of modified inner lamella

of the nuclear membrane where initial envelopment takes place [Roizman & Furlong, 1974; Vlazny et al., 1982]. There is some question as to whether the final envelopment takes place at nuclear membranes or at cytoplasmic membranes [Roizman & Batterson, 1985]. Regardless of where final envelopment occurs, the mechanism by which the mature capsids are secreted from the cell appears to involve the endoplasmic reticulum and Golgi apparatus of the cell, much like the pathway followed by soluble secretory proteins of the cell [Johnson & Spear, 1982, 1983].

It appears that all of the human herpesviruses undergo replication in a fashion similar to that described here for HSV, although the length, and other specific aspects of the reproductive cycle may be different [Roizman & Batterson, 1985]. For example, the length of the reproductive cycle varies with each of the herpesviruses, it may be as short as 18 hours (for HSV-1 and HSV-2), or as long as 70 hours (for HCMV) [Roizman & Batterson, 1985]. The final result for all productive herpesvirus infections, no matter what differences there may be in the reproductive cycle of the virus, is host cell death.

I. C. DNA Replication

I. C. 1. Prokaryotic Replication Systems

The replication of deoxyribonucleic acid has been a source of much investigation and speculation. Early studies of DNA replication began in 1953, when Watson and Crick proposed their double helical model for the structure of DNA. In 1958, the first DNA polymerase was isolated from Escherichia Coli by Kornberg and his co-workers [Lehman et al., 1958]. Since that time, a number of DNA polymerases have been

isolated from a variety of sources, together with the accessory proteins which are necessary for complete replication of double stranded DNA.

Prokaryotic replication systems were chosen for study for several reasons. These reasons included the availability of genetic information on these systems, and the ability to obtain large quantities of material relatively quickly for study. One of the simplest prokaryotic replication systems is that of the T4 bacteriophage.

The T4 replication system is comprised of seven T4 encoded proteins: the products of gene 43 (g43P, DNA polymerase); gene 32 (g32P, single stranded DNA (ssDNA) binding protein); genes 44, 45, and 62 (g44P, g45P, g62P; polymerase accesory proteins); and genes 41, and 61 (g41P, g61P; RNA priming proteins) [Liu et al., 1979; Silver & Nossal, 1979; Nossal & Alberts, 1983]. Each of these proteins has been purified to homogeneity and the genes have been cloned and overexpressed in E. coli [Hinton & Nossal, 1985a, 1985b; reviewed by Nossal & Alberts, 1983]. In vitro, these seven proteins catalyze the priming and elongation reactions necessary to replicate single stranded templates and to carry out strand displacement synthesis from nicked double stranded DNA, but cannot carry out the initiation of new strand synthesis on intact duplex DNA templates [Nossal & Alberts, 1983]. The role(s) that each of these proteins play in the replication reaction is outlined in Table V. The current model for how these proteins initiate lagging strand synthesis is as follows: 1) a g41P/g61P complex has an ATPase activity and unwinds duplex T4 DNA at the replication fork; 2) g32P binds to the single stranded regions of the DNA; 3) periodically the g41P/g61P complex forms the pentaribonucleotide primers

TABLE V

PROTEINS INVOLVED WITH T4 BACTERIOPHAGE DNA REPLICATION

Protein (Function)	Characteristics
43 (DNA polymerase)	DNA polymerase with 3′ to 5′ exonuclease activity
32 (ssDBP)	ssDBP which binds cooperatively to ssDNA, and is required for maximal DNA synthesis
44 (polymerase accessory protein)	Forms a complex with g62P which has ATPase activity in the presence of ssDNA or primer-template junction. This complex stabilizes the interaction of g43P and the template, increases the reaction rate and length of the product, is required for strand displacement synthesis and increases the activity of the 3′ to 5′ exonuclease activity of g43P
62 (polymerase accessory protein)	See g44P above.
45 (polymerase accessory protein)	Stimulates the activities of the g44P/g62P complex.
61 (primase)	Produces primers for lagging stand synthesis. Requires g41P for appropriate primer formation *in vivo*.
41 (helicase)	5′ to 3′ helicase activity that is stimulated by complexing with g61P. Required for formation of physiological primers *in vivo*.

Source: Nossal, 1983

($pppApC(pN)_3$) and stabilizes the interaction of the primer with the template; and 4) the g43P-g44P/g62P-g45P (DNA polymerase/accessory protein) complex then elongates the primers. Leading stand synthesis is completed by the polymerase/accessory protein complex and g32P [Nossal & Alberts, 1983]. The function and biochemical characteristics of each of these T4 replications enzymes are currently being studied alone and in combination with each other to obtain a better understanding of the T4 DNA replication complex.

I. C. 2. Eukaryotic Replication Systems

The study of prokaryotic replication systems has yielded useful information about DNA replication, but the question about comparability to eukaryotic replication systems, especially of the higher eukaryotes, must be answered. The study of eukaryotic replication systems, especially in the higher eukaryotes, is much more difficult for several reasons. The first being that it is more difficult to obtain the quantities of starting material needed for the purification of replication proteins in sufficient yields to carry out meaningful biochemical characterizations. Another reason is that the genetics of the higher eukaryotes are more difficult to manipulate. Therefore, model DNA replication systems studied for eukaryotes have included eukaryotic viruses, Drosophila melanogaster, and yeast.

The first eukaryotic replication system to be studied and fairly well understood is the adenovirus system. The replication system required to initiate and synthesize full length adenovirus DNA (Ad DNA) is comprised of five proteins [Lichy et al., 1983; Nagata et al.,

1983]. Three of these proteins are encoded for by the virus and they are the 59-kd DNA binding protein (Ad DBP); an 80-kd preterminal protein (pTP) which serves as the primer before being processed to a 55-kd terminal protein (TP); and the 140-kd adenovirus DNA polymerase. The other two proteins are cellular factors; one of which is nuclear factor I, a 47-kd initiation protein that binds to a 32 nucleotide terminal DNA sequence, and the other nuclear protein which has topoisomerase I activity. This last protein can be replaced by other eukaryotic type I topoisomerases, but not prokaryotic type I topoisomerases, and is required for elongation. All of the proteins described above, with the exception of the topoisomerase I, are necessary for proper initiation of replication. The first step in proper initiation is the formation of a pTP-dCMP complex by Ad DNA polymerase in the presence of single stranded Ad DNA that has TP bound to the termini [Ikeda et al., 1982; Nagata et al., 1982]. Without the addition of the type I topoisomerase, elongation will occur until approximately 25% of the genome is replicated. Topoisomerase I is the only additional protein that is necessary for complete replication to take place [Nagata et al., 1983]. The model that has been developed for the replication of the adenovirus genome is as follows: 1) pTP and Ad DNA polymerase, facilitated by TP, bind to the terminus of duplex Ad DNA; 2) nuclear factor I then binds and aids in unwinding the end of the duplex Ad DNA; 3) Ad DBP then binds to the single stranded Ad DNA to stabilize it; 4) Initiation occurs as described above; and finally, 5) elongation takes place with the aid of topoisomerase I [reviewed by Campbell, 1986]. The most interesting aspect of Ad DNA replication is in the use of the host initiation factor, nuclear factor I. This factor seems to be

highly conserved among the eukaryotes, as its recognition sequence can replace the adenovirus initiation sequence [Gronostajski et al., 1984a, 1985]. This replication system is a good model for replication systems that are initiated via protein priming.

Another virus system that has yielded information about initiation of replication at internal sites is the simian virus 40 (SV40) system. However, little useful information about elongation and other facets of DNA replication can be gained from this system, as SV40 uses host enzymes for elongation once initiation has taken place [Ariga & Sugano, 1983; Li & Kelly, 1984]. The circular duplex 5243 base pair genome of SV40 is packaged in a chromatin structure similar to the chromosome [Campbell, 1986]. Initiation in the SV40 system is accomplished by the SV40 encoded T antigen. The T antigen, a 708 amino acid protein, is a multifunctional enzyme. It binds specifically through a direct interaction to SV40 DNA or chromatin at the origin of replication [Wilson et al., 1982]. The T antigen also has DNA-dependent ATPase activity [Tjian & Robbins, 1979; Giacherio & Hager, 1979], a nucleotide binding activity [Clertante et al., 1984], nuclear localization sequences [Kalderon et al., 1984; Lanford & Butel, 1984], and helicase activity [Stahl et al., 1986]. In addition, the T antigen can be post-translationally modified [reviewed in Campbell, 1986]. The only functions that are necessary for DNA replication to occur, however, are the origin of replication specific binding, and the ATPase activity [Manos & Gluzman, 1985]. The exact mechanism of initiation is still not understood, but it is believed that the T antigen somehow "activates" the origin of replication which allows a host polymerase-primase complex to bind at the origin of replication and initiate DNA

synthesis. It has been shown that RNA polymerases do not play a role in the initiation of synthesis [Li & Kelly, 1984; Ariga & Sugano, 1983], and the initiation reaction requires ATP and Mg^{2+}. Once initiation occurs, replication of the circular SV40 genome proceeds bidirectionally from the unique initiation site (the θ replication model). Replication of SV40 DNA yields relaxed circular DNA, and addition of nuclear extracts or factors are necessary for the introduction of negative supercoils and formation of chromatin like structures, that are characteristic of the SV40 DNA obtained in vivo [Stillman & Gluzman, 1985]. Although the SV40 system does not yield any additional information about the reactions that are taking place at the replication fork, this system may prove to be a model for the initiation of chromosomal replication in the cell.

A third eukaryotic replication system that has been studied in some detail is the yeast replication system. Yeast has several advantages that make it useful for study. First, it can be grown in large quantities. Second, the classical genetics of the yeast system have been studied and are fairly well understood, and can be manipulated through genetic engineering. Finally, and perhaps most importantly, the yeast's chromosomal organization and replication cycle are typical of that of the higher eukaryotes. The yeast genome is divided into 17 linear chromosomes, mitochondrial DNA (comprising 15% of the total cellular DNA), and a 2 μm circular plasmid DNA (4% of the total cellular DNA). Although the chromosomes are much smaller than most of those found in other eukaryotes, they are packaged in typical chromatin structures in the nucleus. Additionally, a feature of the yeast chromosomes are that they are composed of multiple replicons of which there

are an estimated 400 in the haploid genome. There are features of yeast DNA (telomere, and centromere) replication that are beyond the scope of this work, but have been recently reviewed [Campbell, 1986].

Initiation of replication in these replicons takes place only once in the S phase of the cell cycle, and reinitiation of these replicons is rarely seen. The sequences that appear to be responsible for the initiation of replication in yeast have been identified, cloned and are termed "autonomously replicating sequences" (ARS) [Struhl et al., 1979]. It has been demonstrated that ARSs are most likely to be the origin of replication for the following reasons: 1) there are approximately 400 ARS sequences per cell, which is the same as the number of estimated replicons; 2) there are sequence similarities with the origin of replication of E. coli, and SV40 T antigen binding sites; and 3) DNA synthesis in in vitro replication systems appears to initiate specifically at ARS sites [Campbell, 1986].

Plasmids containing the ARS sequence or the 2 μm plasmid (which has a similar ARS sequence) can be replicated by extracts of yeast proteins. Replication of duplex DNA in these systems requires ATP, Mg^{2+}, and the dNTPs. It has also been shown via the use of antibodies that DNA polymerase I and yeast single-stranded DNA binding protein 1 (SSB-1) are required for replication [Campbell, 1986]. The replication of single stranded DNA by the same extracts requires the addition of the rNTPs to the reaction. It appears that the initiation of replication is catalyzed by a DNA polymerase-primase complex and not through RNA polymerase, since addition of the RNA polymerase inhibitor α-amanitin does not inhibit the reaction [reviewed by Newlon, 1984].

Although it has been shown that yeast extracts contain the

appropriate enzymes for DNA replication, it has been far more difficult to isolate these enzymes and reconstruct the replication fork in vitro. Several methods have been used to attempt to demonstrate the proteins that are necessary for replication of duplex DNA. One goal was to generate replication mutants. However, classical genetics have not been very successful in this pursuit. Instead, mutants were made and protein extracts of these mutants were assayed in hopes of finding a specific lesion in a replication protein. This method has resulted in finding mutants in topoisomerases I and II [Thrash et al., 1984, 1985; DiNardo et al., 1984]. Another method that is currently employed for the isolation of replication proteins involves using the known genetic sequences from other replication systems to prepare reagents. These reagents include oligonucleotide probes which can then be used to isolate the gene for that replication protein, which can then be used in mutagenesis studies. In addition, the oligonucleotide sequences could be used to prepare synthetic peptides for use as antigens for antibody preparation. The antibodies can then be employed to isolate large amounts of purified proteins for biochemical analysis. So far, topoisomerase II, DNA polymerase I and SSB-1 have been isolated in this way [Campbell, 1986]. Although much has been learned about the replication of yeast DNA, it appears that there is much more work to be done to reproduce the replication fork in vitro and understand the reactions on a biochemical level.

I. C. 3. Herpes Simplex DNA Replication

There are several characteristics of the HSV system that make it an ideal candidate for use as a model replication system. The first is that a relatively large amount of infected cells can be obtained for the purification of the replication proteins. Second, the virus is simple in that an active infection produces approximately 70 viral proteins [Roizman & Sears, 1987; McGeoch et al., 1988a], but is complex enough that it may code for most of its own replication machinery. The third characteristic is that the genetics of HSV are very well understood and can be manipulated rather easily. This has been demonstrated in that a number of temperature sensitive mutants have been isolated in most stages of HSV replication, some of which are DNA$^-$ (no viral DNA is produced) and may prove to be useful in studying specific DNA replication proteins and processes [Schaffer et al., 1973].

Based on the enzyme activities that are involved in DNA replication in other systems, it would be hypothesized that HSV would encode the following activities: 1) DNA polymerase, 2) single stranded DNA binding protein, 3) origin (of replication) binding protein, 4) DNA primase, 5) deoxyribonuclease, 6) topoisomerase, 7) ribonucleotide reductase, 8) helicase and 9) thymidine kinase. At this time, it has been shown that the HSV does encode for its own DNA polymerase (HSV DNA polymerase) [Keir & Gold, 1963; Keir et al., 1966a], single standed DNA binding protein ($\beta_1$8) [Honess & Roizman, 1974; Powell & Purifoy, 1976], exonuclease [Morrison & Keir, 1968; Hay et al., 1971], thymidine kinase [Kit & Dubbs, 1963], ribonucleotide reductase [Cohen, 1972], and an origin of replication binding protein [Elias, et al., 1986; Elias &

Lehman, 1988]. A unique primase activity has been detected in HSV-1 infected cells, but has not yet been shown to be encoded for by the virus [Holmes et al., 1988]. Of the proteins listed above, only two have been shown to be absolutely essential for DNA replication based on use of temperature sensitive mutants. These two proteins are the HSV DNA polymerase [Aron et al., 1975] and $\beta_1 8$ [Weller et al., 1983].

Recently, Challberg [1986] developed a complementation assay for identifying HSV gene products that are essential for origin of replication specific DNA synthesis. The procedure involved transfecting cells with fragments of HSV KOS DNA and a plasmid that contained the HSV S region origin of replication (ori_s) to find genes that are necessary for the replication of the plasmid. From this study, seven gene products were shown to be required. These gene products included the HSV DNA polymerase ($U_L 30$), and $\beta_1 8$ ($U_L 29$) that were previously shown to be required; as well as five other previously unidentified gene products - $U_L 5$, $U_L 8$, $U_L 9$, $U_L 42$, and $U_L 52$ [Wu et al., 1988]. The $U_L 42$ gene product has recently been identified as the 65 kDa DNA binding protein [Gallo et al., 1988]. By the use of a temperature sensitive mutation in the $U_L 42$ gene, the protein produced by this gene has been shown to be essential for DNA replication [Marchetti et al., 1988], thus confirming the results of Wu et al. [1988]. The nomenclature used for these genes was derived by numbering the open reading frames left to right in the prototype genomic isomeric arrangement as determined from the complete HSV genomic sequence [McGeoch et al., 1988a]. The sequences of all seven genes are known and have been compared to DNA sequence data of other human herpesviruses to determine if there may be any homology between other predicted gene products in the other viruses

[Quinn & McGeoch, 1985; McGeoch et al. 1988b]. From these studies, it was found that there are varying amounts of homology on the amino acid level between some of the VZV and EBV proteins and the seven essential replication genes of HSV. Significant homology was seen between HSV and VZV proteins, where all seven genes had at least 21.6% homology with a predicted VZV gene product. The homology with EBV was not as striking, as only four of the essential HSV genes (U_L5, U_L52, $\beta_1 8$ and HSV DNA polymerase) had significant homology (>20%) with an EBV predicted gene product [McGeoch et al., 1988b]. One other noteworthy observation based on this homology comes from the comparison of the viral DNA polymerases. The HSV-1 DNA polymerase and the VZV DNA polymerase shared 52.5% homology on the amino acid level whereas the EBV DNA polymerase shared 33.3% homology with the HSV-1 DNA polymerase [McGeoch et al., 1988b; Quinn & McGeoch, 1985]. There is currently much interest in assigning enzymatic activities to the five unidentified gene products, and in purifying these products to determine the roles that they play in the replication of the HSV genome.

In addition to studying the proteins involved in the replication process, the structure of replicating DNA has also been investigated. First of all, it has been shown that a small fraction (<5%) of the input HSV DNA enters the replicative pool [Jacob & Roizman, 1977]. The properties of replicating HSV DNA have been studied by a number of laboratories, and have been reviewed in the literature [Ben-Porat, 1982]. Briefly, replicating DNA has been studied by gradient centrifugation which has demonstrated that viral DNA synthesis can be divided into two phases. During the early rounds of replication, the newly synthesized DNA sediments heterogeneously with S values up to two

times that of mature viral DNA. However, at later times in DNA synthesis, the newly synthesized DNA can sediment at S values up to 100 times (>230 S) that of mature viral DNA [Ben-Porat & Tokazewski, 1977]. The newly replicated DNA also contains more nicks and gaps than mature viral DNA. Sedimentation analysis has failed to show continuous replication of one DNA strand, however, it is not clear if this is due to a DNA excision-repair function at work on the newly synthesized DNA. It has also been demonstrated that there are oligoribonucleotides associated with the replicating and mature DNAs [Biswal et al., 1974; Hirsch & Vonka, 1974], which would lend support to the theory that initiation of some HSV DNA is done via a DNA primase [Muller et al., 1979, Holmes et al., 1988].

Electron microscopic analysis of the DNA present in the nucleus prior to the initiation of DNA synthesis reveals several forms of DNA. These forms of DNA include full length linear molecules with single stranded ends, full length circular molecules, and some DNA molecules that are longer than unit length [Jacob & Roizman, 1977]. Restriction endonuclease analyses of these molecules indicate that most of the DNA is circularized. Therefore, it is believed that some of the structures observed with the electron microscope are artifacts due to the fragility of the viral DNA.

Initiation of replication is believed to take place at one of three origins of replication (ori) in HSV; one of the two ori_S located in the short unique repeat regions, or at ori_L in the unique long region of the HSV genome. This hypothesis may be supported by electron microscopic studies [Hirsch et al., 1977; Friedman et al., 1977], and by pulse chase experiments [Ben-Porat & Veach, 1980]. Restriction

analysis of DNA during the early stages of replication indicates that the DNA molecules appear to be "endless" [Ben-Porat, 1982]. Again, the same multiple forms of viral DNA are observed at this stage of replication when they are studied under the electron microscope.

Late in infection, other forms of DNA are observed. These include linear, unit and larger than unit length molecules, circular unit and smaller than unit size molecules, lariat structures of various sizes, and large concatemeric tangles [Jacob & Roizman, 1977; Ben-Porat, 1982]. Supporting the finding of large concatemeric structures is the restriction endonuclease data which shows underrepresentation of the terminal fragments with the appearance of fragments representing "joint" fragments (comprised of the joining of the two free end fragments) [Jacob et al., 1979]. It has been shown that cleavage of the concatemeric DNA into unit sized molecules is accomplished by a virus specific protein(s). This has been shown by temperature mutants that have a DNA^+ phenotype, but are unable to cleave the concatemers into mature viral DNA molecules [Ben-Porat, 1982]. Cleavage of the concatemers takes place at the a sequence (Figure 2) [Locker & Frenkel, 1979; Vlazny et al., 1982; Stow & McMonagle, 1983]. Several complex mechanisms have been described to account for the synthesis of the terminal repetitions that may be lost when the concatemeric DNA is cleaved, and have been reviewed in the literature [Roizman, 1980; Ben-Porat, 1982; Roizman & Batterson, 1985]. Based on this data, Roizman [1980] and Ben-Porat [1982] have proposed models for the replication of unit length viral DNA via a rolling circle mechanism. Although the eukaryotes do not use a rolling circle mechanism for the replication of their DNA, the HSV replication system may provide useful information

about the biochemistry of replication at the replication fork.

I. D. DNA Polymerases and Primases

I. D. 1. Eukaryotic DNA Polymerases and Primases

The first DNA polymerase isolated from an eukaryotic source was the calf thymus DNA polymerase [Bollum 1960]. Since that time, DNA polymerases have been purified from a number of sources [Weissbach et al., 1971; Baril et al., 1971; Holmes et al., 1971; Smith & Gallo, 1972]. Early studies revealed that several forms of DNA polymerases could be isolated. For that reason, the current system of nomenclature was proposed which separates the DNA polymerases into classes based on their size and reaction properties [Weissbach et al., 1975]. The various classes of eukaryotic DNA polymerases and their properties are described in Table VI.

At present it is thought that DNA polymerase α is responsible for the replication of the nuclear DNA, although the involvement of a newly discovered class of eukaryotic DNA polymerases, DNA polymerase δ, can not be ruled out [reviewed by Lee et al., 1981; Fry, 1983] . DNA polymerase α is a high molecular weight (>100 kDa) enzyme that comprises 80-90% of the DNA polymerase found in dividing cells; it is sensitive to sulfhydryl group inhibitors such as NEM; and replicates gapped duplex DNA. DNA polymerase α can also use synthetic RNA primed DNA templates, but not synthetic DNA primed RNA templates. DNA polymerase β is low molecular weight, NEM insensitive enzyme located predominately in the nucleus. This enzyme uses activated DNA and synthetic RNA templates for DNA synthesis, but cannot use RNA initiated

TABLE VI

CHARACTERISTICS OF EUKARYOTIC DNA POLYMERASES

	DNA Polymerase		
Characteristic	α	β	γ
Molecular Weight (kDa)	>100	40-50	180-190
NEM Sensitive	Yes	No	Yes
PAA Sensitive	++	-	+
Template Useage Activated DNA	+	+	+
poly(dT)•oligo(rA)	++	-	+
poly(rA)•oligo(dT)	-	++	+++
Salt Optimum (mM KCl)	0	75-100	100-150

Sources:

1. Bolden et al., 1975
2. Weissbach, 1975
3. Holmes et al., 1983
4. Fry et al., 1983

DNA templates.

The third class of DNA polymerases is DNA polymerase γ. DNA polymerase γ is now known to be the mitochondrial DNA polymerase. This DNA polymerase is a high molecular weight (180-190 kDa) enzyme that comprises approximately 1-5% of the total DNA polymerase found in the cell. This DNA polymerase is NEM sensitive, although it is less sensitive than DNA polymerase α, and like DNA polymerase β, it can use synthetic RNA templates.

In addition to these characteristics, none of these DNA polymerases contain an associated exonuclease activity [all the properties of the DNA polymerases described above are reviewed by Weissbach, 1975; Holmes et al., 1983; Fry, 1983]. The new class of eukaryotic DNA polymerase, DNA polymerase δ, does contain a 3′ to 5′ exonuclease activity [Lee, 1981; Fry, 1983].

As mentioned earlier, DNA polymerase α can use a short piece of RNA as a primer for DNA synthesis. These RNA primers are produced by DNA primase which has been copurified in tight association with DNA polymerase α [Kaguni et al., 1983; Chang et al., 1984; Gronostajski et al., 1984b; Wang et al., 1984; Campbell, 1986].

I. D. 2. Herpesvirus DNA Polymerases

In the 1960s and 1970s, a number of laboratories noted that infection of cells with HSV was accompanied by an increase in several enzymatic activities including those of a thymidine kinase [Kit et al., 1967], a deoxyribonuclease [Morrison & Keir, 1968; Hay et al., 1971], and a DNA polymerase [Keir & Gold, 1963; Keir et al., 1966a]. The DNA

polymerase activity was observed in cells infected with various herpesviruses. These included pseudorabies herpesvirus [Halliburton & Andrew, 1976], Marek's disease virus [Boezi et al., 1974], HCMV [Hirai et al., 1976; Huang, 1975], equine herpesvirus [Cohen et al., 1975; Kemp et al., 1975] and HSV [Keir & Gold, 1963]. Since then, several herpesvirus DNA polymerases have been purified, and their properties analyzed [Leinbach et al., 1976; Allen et al., 1977; Miller et al., 1977; Allaudeen & Bertino, 1978]. Most of these DNA polymerases have characteristics that are similar to the HSV DNA polymerase in that they are activated by high ionic strength buffers, and are inhibited by the pyrophosphate analog, phosphonoacetic acid (PAA). The properties of the HSV DNA polymerase are described in the following section.

I. D. 2. a. Wild Type HSV DNA Polymerases and Primases

Early studies on the induction of the HSV induced DNA polymerase activity suggested that the induced enzyme activity was more heat stable than the host enzyme (DNA polymerase α) and was stimulated by salt concentrations which inhibit the host enzymes [Keir et al., 1966a, 1966b]. Weissbach and his coworkers [1973] were the first to partially purify the HSV-1 DNA polymerase from strain MPdk$^-$. Weissbach found that a majority of the HSV DNA polymerase was located in the nuclei of infected cells, whereas, only 20% of the host DNA polymerase α could be detected in the nucleus. Using the partially purified HSV DNA polymerase, the following characteristics of the HSV DNA polymerase were determined: 1) the HSV DNA polymerase was optimally stimulated by 150 mM potassium sulfate, potassium phosphate, and ammonium sulfate,

whereas the monoanionic salts (potassium chloride, sodium chloride, etc.) effect was maximal at 250 mM; 2) the pH optimum was at pH 8.0; 3) magnesium ions were required at a concentration of 2 mM for maximal DNA synthesis; 4) the HSV DNA polymerase activity was optimal at 0.5 mM dithiothreitol (DTT); 5) the presence of 50 μM N-Methylmaleimide (NEM) in the reaction mixture inhibited the polymerase by 95%; 6) omission of dCTP or TTP from the reaction reduced the activity by 50 to 60%, whereas omission of dGTP resulted in the loss of 70% activity; 7) the HSV DNA polymerase had an apparent molecular weight of 180 to 200 kD based on chromatography on Sephadex G-200; 8) the HSV DNA polymerase required a gapped template (single stranded, and duplex templates were not used); 9) synthetic templates could be used in the replication mixtures with varying efficiencies; oligomer-homopolymer templates that had a polyribonucleotide template strand in place of the polydeoxynucleotide template strand however could not be used by the polymerase; 10) the HSV DNA polymerase did not incorporate the rNTPs into products when assayed under conditions for the HSV DNA polymerase or for DNA-dependent RNA polymerases; and 11) it was shown that the HSV DNA polymerase had no significant nuclease activity under conditions of maximal DNA synthesis, with the omission of the dNTPs, and using either labelled single stranded or duplex DNAs; however, some activity (5% the rate of synthesis) was observed when using synthetic duplex polymers [Weissbach et al., 1973].

Other investigators have also described many of the same properties of HSV DNA polymerase from other strains. Additional properties described include inhibition of HSV DNA polymerase by Zn^{2+} ions [deRoeth, 1963; Gordon et al., 1975; Shlomai et al., 1975; Fridlender

et al., 1978; Knopf, 1979b], and inhibition of HSV DNA polymerase by PAA in vivo and in vitro [Shipkowitz et al., 1973; Overby et al., 1974; Mao et al., 1975; Knopf, 1979a, 1979b]. Knopf [1979b] further purified the HSV DNA polymerase (strain Angelotti). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of this preparation yielded ten polypeptide bands ranging in size from 144 kD to 14 kD, with three major polypeptides with molecular weight of 144 kD, 74 kD, and 62 kD. As with other strains of HSV DNA polymerase, HSV DNA polymerase (Angelotti) was inhibited by PAA, NEM and Zn^{2+} and had similar substrate requirements. HSV DNA polymerase (Angelotti) was also tested for endo- and exonuclease activities. The only detectable nuclease activity was a 3' to 5'exonuclease activity when assayed under conditions of DNA synthesis in the absence of dNTPs [Knopf, 1979]. This is supported by the findings of other investigators, who did not detect nuclease activity under conditions of DNA synthesis, but did observe exonuclease activity in the absence of dNTPs [O'Donnell et al., 1987a]

Similar results as those described above were obtained for the KOS strain [Smith, 1964] HSV DNA polymerase and for the HSV-2 HSV DNA polymerase (strain 333) [Ostrander & Cheng, 1980]. The molecular weight of the HSV KOS DNA polymerase KOS was determined to be 126 kDa and that of HSV-2 DNA polymerase (333) was determined to be 129 kDa, based on sedimentation in high ionic strength sucrose gradients.

In all the cases reviewed so far, except for Weissbach, the associated exonuclease was not detectable under conditions of DNA synthesis and is inhibited by PAA. The HSV DNA polymerases in the above studies were also shown to be stimulated by physiological concentra-

tions of polyamines at physiological ionic strengths [Ostrander & Cheng, 1980]. This study also yielded apparent K_m values for the dNTPs, and those are presented in Table VII together with a later report of K_m values for the HSV KOS DNA polymerase [Frank et al., 1984b]. Derse and Cheng [1981] reported a K_m of 8.6 μg/mL (26 μM in terms of nucleotide) for activated DNA for the HSV KOS DNA polymerase under conditions of optimal DNA synthesis. Finally, a recent report states that the HSV DNA polymerase purified from HSV-1 strain F infected cells is a highly processive enzyme [O'Donnell et al., 1987a]. In addition, a turnover number of 0.25 nucleotides/sec was determined for this enzyme [O'Donnell et al., 1987a], making this a poor DNA polymerase in comparison to eukaryotic DNA polymerase α which has a turnover number on the order of 50 nucleotides/sec [Holmes et al., 1976]. The HSV DNA polymerase can be activated by $\beta_1 8$ using: 1) activated and heat denatured templates [Ruyechan & Weir, 1984; Ruyechan et al., 1986] and 2) using circular duplex DNA templates in the presence of other nuclear factors found in infected cells [O'Donnell et al., 1987b]. This may suggest the activity of HSV DNA polymerase is mediated through additional virus induced proteins in vivo.

Other investigators have shown that the HSV DNA polymerases can be inhibited by a number of drugs and nucleotide analogs, including PAA (K_i=970 μM) [Derse et al., 1982]; phosphonoformic acid (K_i=640 μM) [Ostrander & Cheng, 1980; Derse, et al., 1982]; 9-β-D-arabinofuranosyladenine (araA) (K_i=0.14 μM) [Muller et al., 1977]; 9-(2-hydroxyethoxymethyl)guanine (also known as acycloguanosine, acylovir, or ACG), which must be phosphorylated to the triphosphate form (ACVTP) for antipolymerase activity (K_i=.08 μM (strain H27 [Elion

TABLE VII

COMPARISON OF APPARENT Kms FOR HSV KOS DNA POLYMERASE

Substrate	K_m (μM) Study 1[1]	Study 2[2]
dATP	0.11	0.40
dCTP	0.09	0.50
dGTP	0.05	0.16
TTP	0.14	0.45

[1]Ostrander & Cheng, 1980

[2]Frank et al., 1984b

et al., 1977]), K_i=.18 μM (strain KOS [Derse et al., 1981; Furman et al., 1984])); purine ribonucleoside monophosphates (5'-GMP and 5'-AMP) [Frank & Cheng, 1986]; 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate (K_i=.03 μM) [Frank et al., 1984a]; and aphidicolin [Frank et al., 1984b]. The genes of several strains of the HSV DNA polymerase have been sequenced including strain KOS [Gibbs et al., 1985; Quinn & McGeoch, 1985].

The sequence information, together with information derived from conditional lethal mutants in the HSV DNA polymerase gene (described in section I.D.2.b) may yield a better understanding of the active site of the HSV DNA polymerase and the role that this enzyme plays in the DNA replication process in the infected cell.

Muller et al. [1979] have suggested that RNA primers are involved in HSV DNA synthesis, thus implicating the presence of a DNA primase activity in the replication process. A novel DNA primase activity found in infected but not mock infected cells has recently been described [Holmes et al., 1988]. This primase activity appears to be closely associated with HSV DNA polymerase. The DNA primase has not been directly shown to be encoded for by the HSV genome, although indirect evidence such as salt and pH optimum, and antibody binding studies suggest that it is not a host enzyme. Further studies are being undertaken to determine the nature and the characteristics of this DNA primase activity and its interaction with the HSV polymerase during viral DNA replication.

I. D. 2. b. Mutant HSV DNA Polymerases

Schaffer and her coworkers used a genetic approach to study the biochemical events involved in viral DNA replication. Temperature sensitive mutants were prepared by mutagenizing HSV-1 strain KOS with 5-bromodeoxyuridine (BrdUrd) [Schaffer et al., 1970], nitrosoguanidine (NTG) and ultraviolet (UV) irradiation [Schaffer et al., 1973]. The permissive temperature for mutant viruses was defined as 34°C, while the nonpermissive temperature was defined as 39°C. A total of 61 mutants were isolated by these mutagenesis procedures, of which 22 were classified into 15 different complementation groups. Four complementation groups A, B, C, and D, containing a total of eight mutant viruses all exhibiting a DNA^- phenotype were obtained. The other mutant viruses fall into remaining complementation groups, E-O, and exhibit DNA^+ phenotypes [Schaffer et al., 1973]. Since there were four DNA^- complementation groups, it was believed that there were at least four proteins involved in HSV DNA replication. Upon further characterization of the mutants [Aron et al., 1975], it was found that two of the complementation groups were defective in regulating the ability of the HSV DNA polymerase to function properly *in vivo*. These complementation groups produce functional, thermostable HSV DNA polymerase, but have DNA^- phenotypes. The other two complementation groups, C and D, appear to produce thermolabile HSV DNA polymerases based on *in vivo* studies; i.e. no HSV DNA polymerase activity is detected at the nonpermissive temperature. It would be expected that the mutations in the HSV DNA polymerase would be found in one complementation group, however, this was not the case, suggesting that the HSV DNA polymerase is a multi-

functional enzyme with one or more functions in the C and other(s) in the D complementation groups. The strains that produce the putative thermolabile HSV DNA polymerase are tsC4, tsC7, and tsD9. Crude extracts prepared from cells infected with these mutant viruses and analysis of the extracts for HSV DNA polymerase activity before and after heating suggested some thermolability [Aron et al., 1975].

Additional studies of these mutants have mapped the temperature sensitive lesions within the HSV DNA polymerase gene [Coen et al., 1984]. From these studies, it was shown that the polymerase gene was located between 0.400 and 0.448 map units on the HSV KOS genome, and the three temperature sensitive HSV DNA polymerase mutants, tsC7, tsC4, and tsD9, were mapped to 0.413-0.420, 0.420-0.422, and 0.422-0.427 map units, respectively. The HSV DNA polymerase gene of strain tsD9 has recently been sequenced and the mutation found to be a substitution of glutamic acid for lysine at amino acid residue 597 in the 1235 amino acid sequence of HSV DNA polymerase [Gibbs et al., 1987]. The drug sensitivities of the mutants have also been characterized. HSV DNA polymerase mutant tsC4 was shown to be ACG resistant [Schnipper & Crumpacker, 1980], however, this result has been disputed [Coen et al., 1984]. The tsC4 HSV DNA polymerase was found to be hypersensitive to the inhibitor PAA, based on an in vivo plaque reduction assay, when compared to the levels of PAA needed to inhibit HSV-1 KOS plaque formation to the same extent [Coen et al., 1984]. The same set of assays showed that the mutant tsD9 was not inhibited by PAA or ACG. The other HSV DNA polymerase mutant, tsC7, was also shown to have wild-type levels of sensitivities to PAA and ACG based on the plaque reduction assay [Coen et al., 1984]. Many other HSV DNA polymerase mutants have

been isolated which have altered responses to various drugs [Derse et al., 1982; Larder et al., 1983]. Mutants in the HSV polymerase gene have been shown to confer altered sensitivity to ACG and ACG analogs [Coen et al., 1980], however, changes in drug sensitivity can also be conferred on viral DNA replication by mutations in the viral thymidine kinase gene [Larder et al., 1983]. In addition, mutations in other genes involved in DNA replication (e.g. $\beta_1 8$) may alter sensitivity to certain inhibitors [Chiou et al., 1985].

II. PROPOSED THESIS

The herpes simplex virus has been proposed as a model for eukaryotic DNA replication. A study of HSV DNA polymerases and the enzymes involved in HSV DNA replication may yield useful information about the eukaryotic proteins involved in DNA replication. Several wild-type HSV DNA polymerases have been purified and characterized [Weissbach et al, 1973; Ostrander & Cheng, 1980]. In an attempt to determine what proteins are required for DNA synthesis, Schaffer et al. [1970, 1973] prepared a panel of temperature sensitive HSV-1 mutants derived from strain KOS. Characterization of these mutants yielded three which have been mapped to the HSV DNA polymerase gene [Aron et al., 1975; Coen et al., 1984]: tsC4, tsC7, and tsD9.

In order to gain a better understanding of the catalytic functions and domains of the HSV DNA polymerase, this study of the wild-type and three putative temperature sensitive mutants tsC4, tsC7 and tsD9 was undertaken. The goals of this study were to: 1) purify the HSV DNA polymerase produced by the parental strain KOS virus and the three temperature sensitive mutants, 2) characterize the purified enzymes as to basic requirements for DNA synthesis, 3) determine the apparent K_m values for the HSV DNA polymerases, 4) study the PAA sensitivity of the HSV DNA polymerases, and 5) investigate the thermal inactivation kinetics of the purified enzymes.

III. MATERIALS

Double deionized water (18 MΩcm) used in the preparation of all solutions and buffers was made from by passing deionized tap water through a Millipore Milli-Q filtration system (Prefilter, Super-C Carbon cartridge, 2 Ion-X cartridges, and a .22 μm Millipore Twin-pak filter). Potassium phosphate (mono- and dibasic), Ammediol, potassium chloride, NP-40, β-ME, glycerol, tetra-sodium pyrophosphate, Tris base, Tris hydrochloride, NaCl, LiCl, EDTA, dGTP, dCTP, dATP, TTP, type I highly polymerized calf thymus DNA, DNase I, type XI protease (proteinase K), RIA grade bovine albumin (BSA) and PEI cellulose coated polyester TLC plates (containing fluorescent indicators) were obtained from Sigma Chemical Corporation (St. Louis, Mo.). Silver nitrate, NaOH (10N), formaldehyde, ammonium hydroxide, HCl, glacial acetic acid, formic acid, sodium bisulfite, and TCA were purchased from Fisher Scientific Company (Pittsburgh, PA). HEPES free base was from Calbiochem Biochemicals (San Diego, CA). E. Coli DNA Polymerase I large fragment ("Klenow" fragment, cloned FPLCpure), and mixed oligo-deoxynucleotides $pd(N)_6$ were purchased from Pharmacia Molecular Biology Division (Piscataway, NJ). E. Coli DNA Polymerase I was purchased from New England Biochemicals (Beverly, MA). DTT was purchased from Boehr-inger Mannheim Biochemicals (Indianapolis, IN). Powdered DMEM and SMEM, as well as fetal calf serum, L-glutamine, 0.25% trypsin solution, and 7.5% sodium bicarbonate used for cell culture work were all obtained from GIBCO Laboratories (Grand Island, NY). Serum Plus (serum free supplement) was obtained from Hazelton Research Products (Lenexa, KS). ssDNA agarose was purchased from Bethesda Research Laboratories

(BRL, Bethesda, Md). IBF Biotechnics (Villeneuve-la-Garenne, France [Gaithersburg, MD, USA offices]) was the supplier for the DEAE-Trisacryl-M chromatography resin. Whatman GF-C paper (22"x18" sheets) and Spectra/Por 4 dialysis tubing were purchased from American Scientific Products (McGaw Park, IL). [8-^{3}H] dATP, and [α-^{32}P]dNTPs were all purchased from New England Nuclear (Wilmington, DE). [Methyl-^{3}H]Thymidine (sterile aqueous suspension) (44 Ci/mmol), and [Methyl-^{3}H]TTP (ammonium salt) (~70 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). SDS, Coomassie Blue R-250, acrylamide, glycine, protein assay kits, BIS, bromophenol blue, β-ME, and TEMED were purchased from BioRad Corporation (Richmond, CA). Ultrapure sodium iodide was obtained from Alfa Products (Danvers, MA). Ready-Solv EP scintillation fluid was purchased from Beckman Instruments, Incorporated (Fullerton, CA). HeLa DNA polymerase β, poly(dT) with chain lengths of 2300 and 3500 were generous gifts from Dr. Lucy M.S. Chang. Anti-DNA primase antibodies, and calf thymus DNA polymerase:primase complex were generous gifts from Dr. Andrew Holmes.

IV. PROCEDURES

IV. A. Cells and Viruses

IV. A. 1. Cell Propagation

Vero cells (African Green Monkey Kidney Cells; obtained from Monroe Vincent, Department of Pediatrics, USUHS) were grown to confluent monolayer cultures in DMEM supplemented with either 10% FCS or 10% serum plus, in stationary cultures or in roller bottles using standard cell culture techniques [Knopf et al., 1976]. HeLa S600 cells (obtained from James Reminick, Department of Microbiology, USUHS) were grown in suspension cultures using Suspension modified Eagle's medium (prepared from powdered media via manufacturer's instructions) supplemented with 10% fetal calf serum. HeLa S600 cells were grown to a density of 1.6×10^6 cells/mL ("confluent") before being used to propagate infections.

IV. A. 2. Virus Propagation

Viral stocks of HSV-1 KOS (parental strain), tsC4, tsC7, and tsD9 were gifts from Dr. Priscilla Schaffer (Dana Farber Cancer Institute, Cambridge, Massachusetts) [Schaffer et al., 1973]. Original stocks of microplaque ($\overline{m}$P (wild-type)) HSV-1 were from the laboratory of David Knipe (Harvard Medical School, Cambridge, MA) [Hoggan & Roizman, 1959]. Viral stocks were prepared at the permissive temperature (34°C) as previously described [Courtney et al., 1970]. The viral stocks were aliquoted and stored at -70°.

IV. A. 3. Viral Titer Assays

Viral titers were determined using confluent Vero cell monolayers in 25 cm^2 tissue culture flasks by the method of Schaffer et al. [1973]. After incubation for the appropriate time at either the permissive (34°C) or non-permissive temperature (39°C), the monolayers were stained with 1% crystal violet stain and the number of viral plaques found in each plate were counted under low power light microscopy and the viral titer was determined.

IV. A. 4. Preparation of Infected Cells

Infected cells used for the production of extracts for viral enzymes were prepared by infecting either confluent Vero roller bottles or "confluent" HeLa S600 cells at an MOI of 20 pfu/cell as previously described [Ostrander & Cheng, 1980]. The cells were harvested by centrifugation, the media decanted, and were washed twice with PBS before being stored at -70°C.

IV. B. Polyacrylamide Gel Electrophoresis

SDS PAGE was used in these studies to follow the purification and to determine the purity of the HSV DNA polymerase polypeptide. The procedure was a modification of the procedure of Laemmli [1970]. The 9.25% polyacrylamide gels were 0.75 mm thick and were run in a Hoeffer Scientific Instruments (San Francisco, CA) Mini-protean II vertical gel

apparatus. Proteins were visualized by one of two methods: Coomassie Blue staining or silver staining [Wray et al., 1981]. Gels were dried down for permanent records using either a Bio-Rad gel dryer (Bio-Rad Corporation, Richmond, CA) or BioGelWrap (BioDesign Inc., Carmel, NY).

IV. C. Purification of HSV DNA Polymerase

IV. C. 1. Purification Procedure I

The initial purifications were carried out using a modification of the procedure developed by Ruyechan & Weir [1984]. All procedures were done at 4°C. Infected cell pellets (10 mL packed cells prepared as described in section IV.A.4) were thawed, and suspended in 50 mL sonication buffer (1.7 M KCl, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 0.5 mM DTT, and 20 µg/mL PMSF). The cells were sonicated on ice for three 30 second pulses. The extracts were centrifuged at 10,000 x g for 30 minutes in a Sorvall RC-5 Superspeed Refrigerated Centrifuge. One-half volume of 30% PEG/sonication buffer was added, and the resulting suspension was incubated on ice with occasional mixing for one hour before repeating the centrifugation step. The supernatant was dialyzed overnight versus 2 x 4 L of TEDGPN buffer (50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 0.5 mM DTT, 20% glycerol, 20 µg/mL PMSF, and 0.2% NP-40). The dialyzed sample was recentrifuged and the supernatant was loaded onto a 30 mL ssDNA agarose column (preequilibrated with TEDGPN buffer). The column was washed extensively with 150 mM KCl/TEDGPN buffer before being eluted with a two step gradient composed of 0.5 M KCl/TEDGPN and 1.0 M KCL/TEDGPN. High and low salt polymerase assays [see section IV.E.1.a], and SDS-PAGE were used after each column to determine the

fractions containing HSV DNA polymerase activity.

Those fractions containing HSV DNA polymerase activity were pooled and dialyzed versus 2 x 4 L of 150 mM KCl/TEDGPN overnight. The pooled sample was loaded onto a 10 mL PC-11 column. The column was washed with 150 mM KCl/TEDGPN buffer and eluted with a 100 mL 0.15-0.4 M KCl/TEDGPN linear gradient. Fractions containing HSV DNA polymerase activity were pooled and dialyzed versus 2 x 4 L of 50 mM KCl/TDGPN (no EDTA) buffer overnight. The HSV DNA polymerase sample was loaded onto a 3 mL ssDNA agarose column, which was washed with 50 mM KCl/TDGPN, and eluted with a 50 mL linear 0.05-0.5 M KCl/TDGPN gradient. Fractions from the ssDNA column that contained HSV DNA polymerase activity were pooled and dialyzed versus 1 x 4 L of 50 mM Tris (pH 7.6), 150 mM KCl, 0.5 mM DTT, 50% glycerol. The polymerase samples were stored at -20°C.

IV. C. 2. Purification Procedure II

The extraction procedure and buffer systems used in this purification scheme are modifications on the procedure that Allen, et. al. [1977] used to purify the equine herpesvirus DNA polymerase. All procedures were performed at 4°C. Initially, 10 mL of packed frozen infected cells [see section IV.A.4] were thawed and suspended in 50 mL extraction buffer (0.4 M potassium phosphate (pH 7.6), 0.5 mM DTT, 20% glycerol, 0.2% NP-40). The suspension was sonicated for three 30 second pulses before centrifugation at 20,000 x g at 4°C for 30 minutes. The supernatant was passed through a 150 mL DEAE-T-M column (preequilibrated with extraction buffer) and the column was washed with one column volume extraction buffer. The proteins not adhering to the

column were collected and dialyzed versus 2 x 4 L of KKDGN buffer (20 mM potassium phosphate (pH 7.6), 25 mM KCl, 0.5 mM DTT, 20% glycerol, 0.2% NP-40) overnight. The dialyzed sample was recentrifuged and the supernatant was loaded onto a 50 mL DEAE-T-M column. The column was washed with KKDGN buffer, and eluted with a 150 mL linear gradient consisting of 0.025-0.5 M potassium phosphate in KKDGN buffer. SDS-PAGE and high and low salt polymerase assays (see section IV.E.1.a) were used to determine fractions containing HSV DNA polymerase after all chromatography steps. Fractions containing HSV DNA polymerase activity were pooled and dialyzed versus 2 x 4 L of KKDGN buffer. The dialyzed pool was loaded onto a 12 mL ssDNA agarose column. The column was washed with KKDGN buffer and eluted with 0.4 M potassium phosphate in KKDGN buffer. Fractions containing HSV DNA polymerase activity were pooled and dialyzed versus 2 x 4 L of KKDGN buffer. The resulting sample was loaded onto a 5 mL PC11 column. The column was washed and eluted with a 100 mL linear gradient of 0.02-0.5 M potassium phosphate in KKDGN buffer. The resulting HSV DNA polymerase was dialyzed versus 1 x 4 L of KKDGN buffer before the sample was loaded onto a 1 mL HAP column. The column was washed and the HSV DNA polymerase was eluted with 0.3 M potassium phosphate in KKDGN. The HSV DNA polymerase was dialyzed versus 1 x 4 L of storage buffer (20 mM potassium phosphate (pH 7.6), 0.5 mM DTT, 150 mM KCl, 0.2% NP-40, and 50% glycerol). The HSV DNA polymerase was then stored at -20°C.

IV. C. 3. Purification Procedure III

The extraction procedure and buffer systems used in this purifi-

cation scheme are modifications on the above procedure (section IV.C.2.). One additional reagent, leupeptin, was added to the original high phosphate extraction buffer at a final concentration of 2 µg/mL. The rest of the methodology was identical until the point in the procedure when polymerase was recovered from the second DEAE-T-M column. The pooled polymerase was loaded onto a 12 mL ssDNA agarose column. The column was washed with KKDGN (see IV.C.2) and then eluted with a 150 mL linear gradient consisting of 0.1-1.0 M KCl in KKDGN buffer. Fractions containing HSV DNA polymerase activity were pooled. If SDS PAGE showed significant levels of contaminants, the pooled fractions were rechromatographed on DEAE-T-M, PC11 and/or ssDNA agarose columns. An additional small (2 mL or less) ssDNA column was used to concentrate the enzyme activity when needed. Fractions containing HSV DNA polymerase activity were pooled and dialyzed versus 1 x 4 L of storage buffer (see IV.C.2). The HSV DNA polymerase was then stored at -20°C.

IV. D. Preparation of DNA Substrates

IV. D. 1. Preparation of Activated DNA

The procedure used for the preparation of activated DNA was a modification of the procedure described by Schlabach et al. [1971]. The reaction mixture consisted of 10 mM Tris HCl (pH 7.8), 10 mM NaCl, 1 mg/mL BSA, 5 mM $MgCl_2$, and 0.1 µg/mL DNase I. A pilot reaction was carried out at 35°C where small aliquots of the DNA were removed at various times and the digestion halted with high salt, EDTA, and heat. The resulting activated DNA was tested in DNA polymerase reactions to determine the optimal time to stop the reaction. The reaction was then

scaled up. The resulting activated DNA was dialysed versus 4 x 4 L of 10 mM Tris HCl (pH 7.8), and 10 mM KCl. The A_{260} of the activated DNA was then obtained and the concentration of the DNA is adjusted to 30 A_{260} units/mL. The activated DNA was aliquoted and stored at -20°C.

IV. D. 2. Preparation of Nick Translated DNAs

Calf thymus DNA was suspended in deionized water at a concentration of 2 mg/mL. The reaction mixture (5 mL) consisted of 1 mg/mL DNA; 0.2 mM each of dATP, dGTP; .04 mM each of ^{3}H-dCTP and ^{3}H-TTP (10,000 DPM/pmol each); 50 mM Tris-Cl (pH 7.5); 10 mM $MgCl_2$; 1 mM DTT; 50 µg/mL BSA; 5 µg/mL DNase I; and 200 units/mL E. Coli DNA Polymerase I. The mixture was incubated at 35°C until such time as the incorporation of the nucleotides had nearly ceased. This was checked by spotting a dilution of the reaction mixture on Whatman GF/C filters and processing the filters as described in section IV.E.1.a. The DNA was then precipitated by adding sodium acetate to a final concentration of 0.3 M before adding 2 volumes of cold ethanol. After the DNA was collected by centrifugation and resuspended in TE buffer (10 mM Tris-Cl (pH 7,6), 1 mM EDTA), the A_{260} was determined to calculate a specific activity for the nick translated DNA. The nick translated DNA was stored at -20°C.

IV. E. Assays

IV. E. 1. Polymerase Assays

IV. E. 1. a. Standard Polymerase Assays

The standard polymerase assay used to detect polymerase activity in the purification of the polymerase was a modification of the procedure used by Weissbach et al. [1973]. Reactions were prepared in Eppendorf tubes in ice water baths. Reaction mixtures (50 µL) contained 50 mM HEPES buffer (pH 8.0); 8 mM $MgCl_2$; 300 µg/mL activated calf thymus DNA; 200 µM each of dATP, dCTP, dGTP; 40 µM ^{3}H-TTP (specific activity 125-1000 dpm/pmol); 2 mM β-ME; 100 µg/mL BSA; 200 mM KCl for high salt polymerase assays or 0 mM KCl for low salt polymerase assays (added in addition to salt carried over from polymerase samples); and 10 µL of the appropriate polymerase fraction. The tubes were gently mixed, and incubated in a 35°C water bath for 30 minutes. The reactions were stopped by placing the tubes back in the ice-water bath. Samples were removed from each tube and were spotted on Whatman GF-C paper. The filters were processed by washing with two changes of ice cold 5% TCA/10 mM sodium pyrophosphate for ten minutes before being washed for ten minutes in 1.0 N HCl. The filters were washed with ice-cold ethanol for five minutes before being dryed under an infrared heat lamp. The dried filters were counted using standard scintillation counting techniques. Data analysis was accomplished through the use of the BASIC computer program Assay (see Appendix A).

IV. E. 1. b. Kinetic Polymerase Assays

The kinetic assays were prepared exactly as in section IV.E.1.a. with the following modifications: 1) each tube contained enough reaction mix for the removal of multiple samples over the course of the reaction period, and 2) reaction mixtures were prewarmed to 35°C before the addition of the polymerase. Samples were remove at appropriate times, spotted on Whatman GF/C, and the filters were processed and counted as described in section IV.E.1.a.

IV. E. 1. c. Polymerase Characterization Assays

IV. E. 1. c. 1. Salt Optimum Assay

The polymerase assays used to study the salt optima for the various enzymes was the same as that described in section IV.E.1.a. with the following modifications. Stock KCl solutions were prepared which would yield the desired final KCl concentration when diluted five-fold. Once prepared, the stock KCl solutions were stored at -20°C. Assays were performed in triplicate.

IV. E. 1. c. 2. pH Optimum Assay

The pH optimum curves were prepared in the same manner as the standard DNA polymerase assay (see section IV.E.1.a.), except various buffers were used at a final concentration of 50 mM in place of the HEPES buffer. The buffers were not corrected for ionic strength. Buffers used included PIPES (pH 6.0, 6.5, 7.0, 7.5), Phosphate (pH 5.0,

6.0, 6.5, 7.0, 7.5, 8.0), Tris (pH 7.6, 7.8, 8.4, 8.8), and Ammediol (pH 8.5, 9.0, 10.0, 10.5). Stock buffer solutions were stored at -20°C once they were prepared. After samples had been removed and spotted on Whatman GF/C filters, the pH of the residual reaction mixtures was determined by using a micro-combination electrode (Microelectrodes, Inc., Londonderry, New Hampshire). The assays were performed in triplicate.

IV. E. 1. c. 3. Divalent Cation Studies

These studies were carried out in a manner identical to that described in section IV.E.1.a. except the divalent cation and concentration of the divalent cation were varied. The divalent cations used included $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, and $CoCl_2$. In order to maintain the cobalt solution as Co^{2+}, 1 mM β-ME was added to the stock solutions. The metal solutions were prepared fresh each time they were needed. Triplicate assays were performed, and the results reported as the mean for triplicate samples.

IV. E. 1. c. 4. Replication Requirement Assays

The polymerase assays used to determine the replication requirements of the HSV DNA polymerase were identical to the standard polymerase assay (section IV.E.1.a.), except in each of the triplicate assays one component of the complete reaction mix was omitted.

IV. E. 2. Primase Assays

IV. E. 2. a. Heat Denatured Template

The substrate that was used in these assays was heat denatured calf thymus DNA. Calf thymus DNA was placed in a boiling water bath for 10 minutes before placing the DNA on ice. Reaction mixtures (50 µL) contained 50 mM HEPES (pH 7.6); 8 mM $MgCl_2$; 300 µg/mL hdDNA; 0.2 mM each of dATP, dCTP, and dGTP; 0.02 mM [^{3}H]TTP (1000 DPM/pmol) or 0.02 mM [α-^{32}P]TTP (1000-2000 CPM/pmol); 0.1 mM each of ATP, CTP, GTP, UTP; 200 mM KCl; 100 µg/mL BSA; 2 mM β-ME; and 5 µL of the polymerase:primase fraction to be assayed. The assays were performed like those described in section IV.E.1.a. Since hdDNA can self prime through snap-back at the ends of the DNA molecules, the above reactions were also done in the absence of the rNTPs. The difference of the two assays ((+rNTPs)-(-rNTPs)) reflects the amount of primase in each sample. This procedure involved the extension of the primers made by using the HSV DNA polymerase present in each sample.

IV. E. 2. b. poly(dT) Template

These assays were performed similar to those described previously (see section IV.E.1.a.). Reaction mixtures (50 µL) consisted of 50 mM Tris-Cl (pH 7.6); 8 mM $MgCl_2$; 100 µg/mL BSA; 1 mM DTT; 50 µM poly(dT) (chain length of 3500); 2 mM ATP; 0.1 mM [^{3}H]dATP (86 cpm/pmol) or 0.1 mM [α-^{32}P]dATP (1000-2000 cpm/pmol); 100 µg/mL BSA; 150 mM or 0 mM KCl; and 5 µL of the appropriate polymerase:primase fraction. These assays were done at high and low ionic strengths, and

samples were processed as described for the polymerase reaction. These assays were also be repeated as described by Holmes et al. [1988] with the addition of E. coli DNA polymerase I to the reaction at a concentration of 20 units/mL. This assay is referred to as the indirect total primase assay. The primers produced in this assay may be elongated by either the HSV DNA polymerase or the E. coli DNA polymerase I. Once again, samples were processed as previously described (section IV.E.1.a.).

IV. F. Exonuclease Assays

Calf Thymus DNA templates used in testing for exonuclease activities were originally prepared by using the nick translation method that was previously described (section IV.D.3.). This material was referred to as the nicked/gapped DNA (ngDNA) substrate. Heat denatured DNA (hdDNA) was prepared by taking a sample of the nicked/gapped template and boiling the sample for 10 minutes followed by an ice quench. Exonuclease assay reaction mixtures (50 µL) contained 50 mM Tris-HCl (pH 7.6); 8 mM $MgCl_2$; 10 µg/mL ^{3}H-DNA (either ngDNA or hdDNA); 2 mM β-ME; 100 µg/mL BSA; 200 mM or 0 mM KCl; and 1.3 units of HSV DNA polymerase. Reactions were incubated at 35°C for appropriate times before samples were spotted on Whatman GF/C filters for counting. Filters were prepared as previously described (see section IV.E.1.a.).

IV. G. dNTPase/Exonuclease Assays

These series of assays were done using [α^{32}P]dNTP labels under

conditions of the polymerization reaction, and the limiting case where one of the dNTPs was absent from the reaction mixture. The [α^{32}P]dNTP (in an aqueous Tricine buffer) of interest was mixed with an equal volume of cold 10 mM dNTP. Reaction mixtures were prepared on ice as previously described for the single point polymerase reaction (section IV.E.1.a.) substituting the [α^{32}P]dNTP in place of the ^{3}H-TTP as the label. In addition to preparing the "complete" reaction mixtures, reaction mixtures were made that were lacking one of the other three unlabeled dNTPs. Controls of "complete" reaction mixtures were also prepared except that polymerase storage buffer was used in place of the polymerase solution. Aliquots (5 µL) were removed before the reactions were incubated at 35°C for 30 minutes. At the end of the incubation period, the reactions were stopped by placing the samples in an ice-water bath. EDTA was added to each sample at a final concentration of 1 mM. One microliter samples were spotted 2 cm from the edge of a PEI plate. The spots were allowed to dry at room temperature before being developed with 1 M formic acid/0.5 M lithium chloride. The plates were developed until the solvent front had migrated 15 cm up the PEI plate. The plates were then remove from the tanks and were dryed under an infrared heat lamp. The dryed PEI plates were then autoradiographed to determine the migration of the various deoxynucleotide mono-, di-, and triphosphates. The plates were then cut up into regions corresponding to the appropriate phosphate derivatives and the pieces counted by Cherenkov radiation using the ^{3}H channel of a liquid scintillation counter. The results of these experiments were analyzed using the BASIC computer program dNTPase (Appendix 2). 25 µL samples of the reactions after incubation were then spotted on Whatman GF/C filters and

were processed as previously described in section IV.E.1.a. The results from these filters gave the extent of the polymerization reaction under the various testing conditions.

IV. H. Thermal Inactivation Studies

The thermal inactivations were carried out in the following manner. A buffer consisting of 50 mM HEPES (pH 7.6), 1 mM β-ME, and 1.25 mg/mL BSA was allowed to equilibrate with water heated to specific temperature (34°, 40°, 45°C) in a Lauda RTM6 recirculating water bath. After the buffer had equilibrated, one-fifth volume of the appropriate polymerase sample was added to the buffer; the mixture was quickly, gently vortexed; and a 10 µL sample removed to a tube in an ice-water bath. Additional 10 µL samples were removed at appropriate times. When all the time points had been collected, the other components of the polymerase reaction were added to the polymerase samples (see section IV.E.1.a.) and the polymerization reaction was allowed to proceed at 35°C. Samples of the polymerase reaction were spotted on Whatman GF/C filters, and the filters were processed as previously described.

IV. I. Glycerol Gradient Analyses

Gradient analysis of crude extracts was accomplished through the use of glycerol gradients. Gradients (5 mL) were 12.5 to 30% glycerol in 50 mM HEPES (pH 7.6), 2 mM β-ME, and 200 mM ammonium sulfate, and were prepared as step gradients. These gradients were allowed to equi-

librate for at least six hours prior to their use. Two confluent VERO monolayers grown in 150 cm^2 flasks were infected with each viral strain at an MOI of 20 as described in section IV.A.4. The infected cells were harvested and washed twice with ice cold HEPES buffered saline (50 mM Tris-Cl (pH 7.6), 150 mM KCl, 0.5 mM DTT) before resuspending the infected cells in extraction buffer (50 mM HEPES (pH 7.6), 0.5 mM DTT, 10 mM sodium bisulfite, 0.5 mM PMSF, and 2 µg/mL leupeptin). The cells were homogenized using a Dounce homogenizer, and the nuclei were collected by centrifugation at 1000 x g. The nuclei were washed once with extraction buffer before they were lysed with lysis buffer (400 mM potassium phosphate (pH 7.6), 1 mM DTT, 0.2% NP-40, and 2 µg/mL leupeptin). Each extract was split into two samples. One sample was retained on ice, and the other was incubated at 40°C for 10 minutes before being ice quenched. Samples (200 µL) of each extract were layered on top of the preformed gradients, which were then centrifuged at 250,000 x g for 18 hours at 4°C. The gradients were fractionated (200 µL/fraction) from the bottom of the tube using an ISCO gradient dripper. The resulting fractions were assayed for high and low salt DNA polymerase (section IV.E.1.a.), and DNA primase (section IV.E.2.a-b.).

V. RESULTS

V. A. Preparation of Viral Stocks

Original stocks of HSV-1 strains KOS, tsC4, tsC7, and tsD9 obtained from P. Schaffer were prepared and titered as described in sections IV.A.1-IV.A.3. Additional stocks of HSV-1 strain $\bar{m}$P (originally obtained from the laboratory of D. Knipe) were also prepared. Table VIII shows the results obtained from the viral titer assay at permissive (34°C) and non-permissive (39°C) temperatures. In order to be classified as temperature sensitive mutants, the viruses must have a 1000 fold less titer at the non-permissive temperature than at the permissive temperature of [Schaffer et al., 1973]. All of the mutants have titers of at least 1×10^{10} pfu/mL at 34°C and no more than 1×10^{3} pfu/mL at 39°C. In contrast, the wild type strains KOS and $\bar{m}$P showed little difference in their titers at the permissive and non-permissive temperatures. These characteristics persisted after several passages of the mutant virus indicating little or no reversion of the temperature sensitive phenotype.

V. B. Induction of HSV DNA Polymerase Activity

VERO cell monolayers were grown to confluency in 150 cm^2 flasks and infected with one of the HSV-1 virus strains or mock infected with uninfected cell preparations. The infected cells were incubated at 34°C. At 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, and 36 hours post-infection one flask was scraped down and the infected cells were harvested. The media from an additional flask was decanted and the

TABLE VIII

TITERS OF VIRAL STOCKS

VIRUS	pfu/mL @ 34°C	pfu/mL @ 39°C
$\overline{m}$P	1×10^{10}	1×10^{10}
KOS	5×10^{11}	4×10^{11}
tsC4	1×10^{11}	10^{3}
tsC7	5×10^{10}	10^{3}
tsD9	1×10^{10}	10^{3}

cells were overlayed with DMEM supplemented with 1% FCS and 0.1 μM [^{3}H]thymidine (4 μCi/mL), and cells were further incubated at 37°C for 1 hour, the label was removed, and the cells harvested. Cell pellets from both flasks were washed twice with PBS, flash frozen in an acetone-dry ice bath and stored at -70°C prior to analysis. The unlabelled cells were thawed and crude cellular extracts were prepared by suspending the cells in 1 mL extraction buffer (section IV.C.1) followed by sonication for one minute on ice. Cellular debris was removed by centrifugation at 20,000 x g for 30 minutes and the extracts were then assayed for high salt HSV DNA polymerase activity. Protein concentrations for each extract were determined using the Bio-Rad protein assay kit. The results of the polymerase assays were expressed as units of high salt polymerase activity per milligram of protein.

The [^{3}H]thymidine labelled cells were extracted in 1 mL of DNA extraction buffer (10 mM Tris-Cl (pH 7.6), 1 mM EDTA, 1% sarkosyl, 2% SDS, 100 mM NaCl, and 1 mg/mL proteinase K) and incubated overnight at 39°C. A_{260} and A_{280} readings were taken to determine the amount of DNA present in each sample. Samples of the labelled DNA were precipitated in 10% TCA, the precipitates collected and counted in a liquid scintillation counter. Results of these studies were reported as CPM/μg DNA and are shown in Figures 3 and 4 together with the results of the DNA polymerase assays.

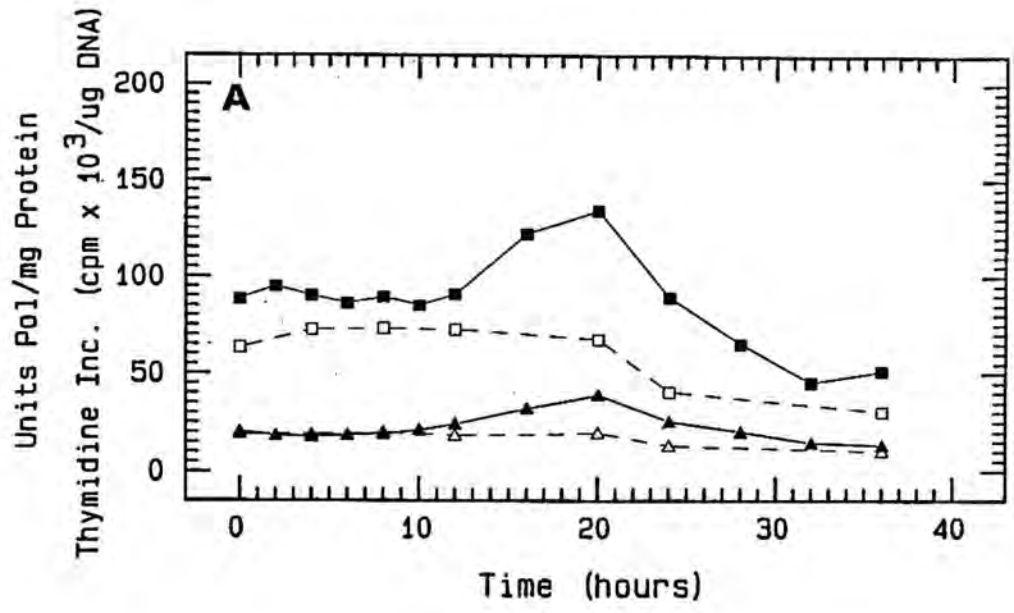
The amount of the parental strain KOS HSV DNA polymerase activity induced upon infection (50 units/mg) was approximately twice that of high salt DNA polymerase activity present in mock infected cells (20 units/mg), with maximum activity occurring between 8 and 12 hours post infection (Figure 3A). In all cases, cells infected with the tempera-

Figure 3. HSV DNA Polymerase Induction Curves - KOS, tsC4.

150 cm^2 flasks of infected Vero cells were scraped down at the times indicated in the figure and were frozen. After the cells were harvested, crude extracts were prepared and assayed for DNA polymerase activity and protein concentration. At the same time, the media was removed from an additional 150 cm^2 flask of infected Vero cells and was overlaid with media labelled with ^{3}H-thymidine (specific activity = 4 µCi/mL). The cells were harvested after an one hour incubation at 37°C and frozen. Crude extracts were prepared as described in the text, and acid precipitable counts determined.

Panel A: results of the HSV KOS (-▲-) and Mock (--Δ--) polymerase assays are plotted as units of DNA polymerase activity per mg of protein. The acid precipitable counts from KOS (-■-) and mock (--□--) infected ^{3}H-thymidine treated cells are expressed as CPM/µg DNA.

Panel B: results of the HSV tsC4 (-▲-) and Mock (--Δ--) polymerase assays are plotted as units of DNA polymerase activity per mg of protein. The acid precipitable counts from tsC4 (-■-) and mock (--□--) infected ^{3}H-thymidine treated cells are expressed as CPM/µg DNA.


A
Units Pol/mg Protein
Thymidine Inc. (cpm x 10^3/ug DNA)
200
150
100
50
0
0
10
20
30
40
Time (hours)

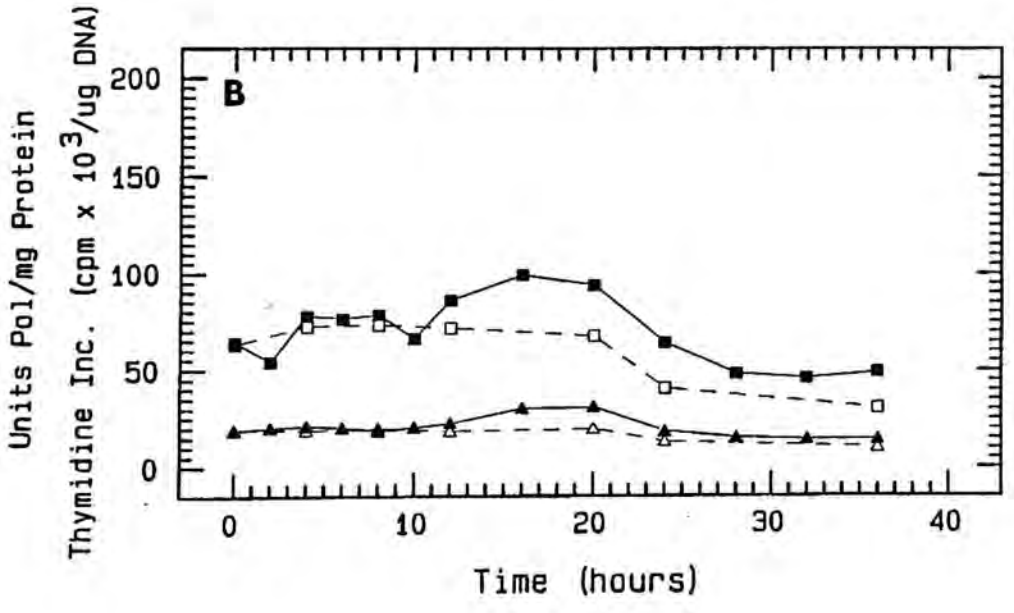
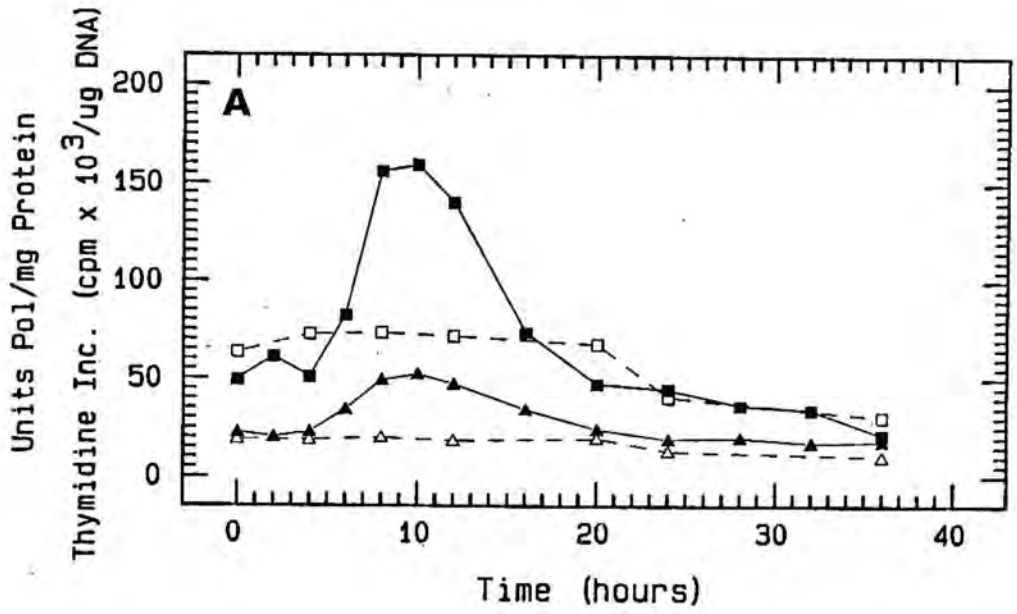

B
Units Pol/mg Protein
Thymidine Inc. (cpm x 10^3/ug DNA)
200
150
100
50
0
0
10
20
30
40
Time (hours)

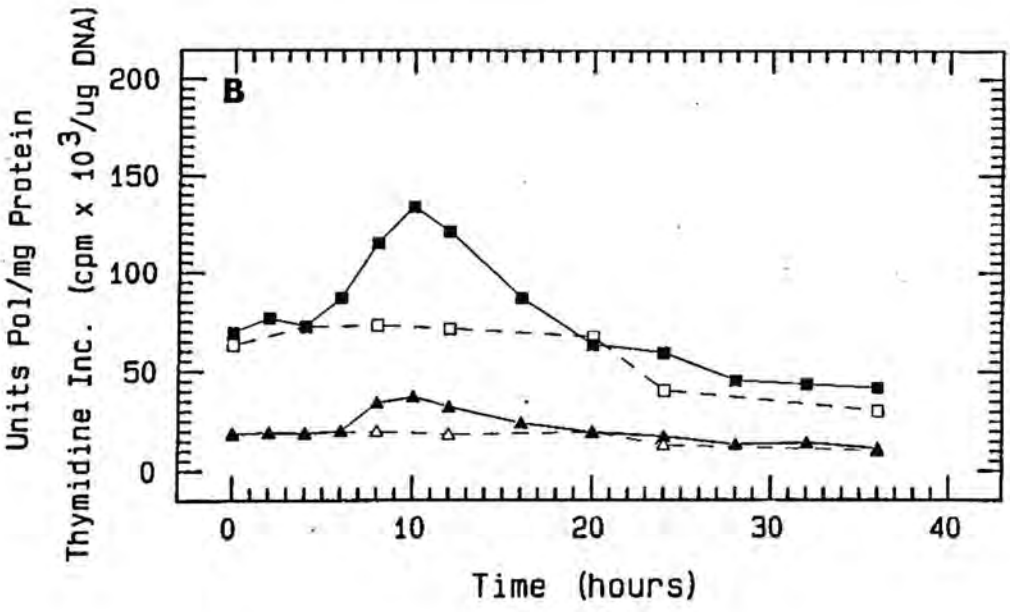
Figure 4. HSV DNA Polymerase Induction Curves - tsC7, tsD9.

150 cm^2 flasks of infected Vero cells were scraped down at the times indicated in the figure and were frozen. After the cells were harvested, crude extracts were prepared and assayed for DNA polymerase activity and protein concentration. At the same time, the media was removed from an additional 150 cm^2 flask of infected Vero cells and was overlaid with media labelled with ^{3}H-thymidine (specific activity = 4 µCi/mL). The cells were harvested after an one hour incubation at 37°C and frozen. Crude extracts were prepared as described in the text, and acid precipitable counts determined.

Panel A: results of the HSV tsC7 (-▲-) and Mock (--Δ--) polymerase assays are plotted as units of DNA polymerase activity per mg of protein. The acid precipitable counts from tsC7 (-■-) and mock (--□--) infected ^{3}H-thymidine treated cells are expressed as CPM/µg DNA.

Panel B: results of the HSV tsD9 (-▲-) and Mock (--Δ--) polymerase assays are plotted as units of DNA polymerase activity per mg of protein. The acid precipitable counts from tsD9 (-■-) and mock (--□--) infected ^{3}H-thymidine treated cells are expressed as CPM/µg DNA.


A
Units Pol/mg Protein
Thymidine Inc. (cpm x 10^3/ug DNA)
200
150
100
50
0
0
10
20
30
40
Time (hours)


B
Units Pol/mg Protein
Thymidine Inc. (cpm x 10^3/ug DNA)
200
150
100
50
0
0
10
20
30
40
Time (hours)

ture sensitive mutants derived from strain KOS exhibited less polymerase activity. Infection using tsC4 yielded slightly less HSV DNA polymerase (37 units/mg), but maximum activity occurred at 8 to 12 hours as with KOS HSV DNA polymerase (Figure 3B). The other two mutants, tsC7 and tsD9, had maximum HSV DNA polymerase activity at 18 to 20 hours post-infection (Figure 4). The tsC7 HSV DNA polymerase activity was present at roughly the same levels as the tsC4 HSV DNA polymerase (38 units/mg) (Figure 4A), whereas, the tsD9 infection showed very little increase in DNA polymerase activity (28 units/mg) above the levels present in mock infected cell extracts (20 units/mg) (Figure 4B). The induction of the viral polymerase was paralleled in all cases by the incorporation of [^{3}H]thymidine into the DNA present of these infected cells.

V. C. Purification of the HSV DNA polymerase

Initial attempts to purify the HSV DNA polymerase used HeLa cells infected with strain $\bar{m}$P HSV-1 and purification procedure I developed by Ruyechan and Weir [1984] described in section IV.C.1. The high salt extract was treated with polyethylene glycol to remove the majority of nucleic acids present, then the extract was adsorbed to the first ssDNA agarose column. The HSV DNA polymerase was eluted from this column with 0.5 M KCl in TEDGPN buffer. The HSV DNA polymerase was then chromatographed on PC11 where the polymerase activity eluted as a single symmetrical peak at a salt concentration near 225 mM KCl. The final purification step of this procedure employed ssDNA agarose eluting with a linear KCl gradient which resulted in the elution of the

HSV DNA polymerase in a single peak at 300 mM KCl. This procedure resulted in the partial purification of the HSV DNA polymerase in a total of seven days. Typical results of this purification procedure are presented in Table IX. HSV DNA polymerase purified using this procedure was subjected to electrophoreis in 9.25% SDS PAGE and typical results obtained are shown in Figure 5. This procedure yielded an impure preparation with the 140 kDa HSV DNA polymerase polypeptide as one of the minor peptides present in this preparation. This procedure yielded partially purified HSV DNA polymerase over a relatively long time course. The final specific activity obtained for the HSV DNA polymerase was 2526 units/mg which was at least five fold lower than that obtained by other investigators [Weissbach et al., 1973; Knopf, 1979; Frank et al., 1984a, 1984b; O'Donnell et al., 1987a].

For reasons stated above, it was felt that the more conventional purification procedure developed by Allen et al. [1977] for the equine herpesvirus DNA polymerases should be employed for the isolation of the HSV DNA polymerase (purification procedure II; see section IV.C.2). The elution salt and buffer system used in this preparation was potassium phosphate. The crude high salt infected cell extract was passed through an initial DEAE-T-M column to remove the nucleic acids. The extract was then adsorbed to and eluted from DEAE-T-M, the HSV DNA polymerase eluting from the column as a relatively sharp peak near 150 mM potassium phosphate in KKDGN buffer. The resulting HSV DNA polymerase was then chromatographed on ssDNA agarose where the polymerase was eluted from the column with 400 mM potassium phosphate. The HSV DNA polymerase was eluted from a PC11 column using a linear potassium phosphate gradient. The HSV DNA polymerase eluted in a symmetrical

TABLE IX

PURIFICATION TABLES FOR PURIFICATION PROCEDURES I, II, AND III

Purification Procedure I
(5 mL packed HSV-1($\bar{m}$P) infected HeLa cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)*	Specific Activity (un/mg)*	Purification	Yield (%)
Crude Extract	51	605	3590	7	1	100
Dialysate	110	340	43081	127	20	1200
ssDNA Agarose	37	26	30577	1181	183	852
PC11	24	5.3	21072	4009	621	587
ssDNA Agarose II	2.4	.67	1738	2526	391	48

Purification Procedure II
(5 mL packed HSV-1($\bar{m}$P) infected HeLa cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)*	Specific Activity (un/mg)*	Purification	Yield (%)
Crude Extract	50	535	17940	335	1	100
DEAE-T-M I	100	28	13859	495	2	77
DEAE-T-M II	150	25	18284	717	2	102
ssDNA Agarose	50	28	5416	1934	6	30
PC11	14.5	1.60	403	2529	8	2
HAP	1.8	.09	323	35937	107	2

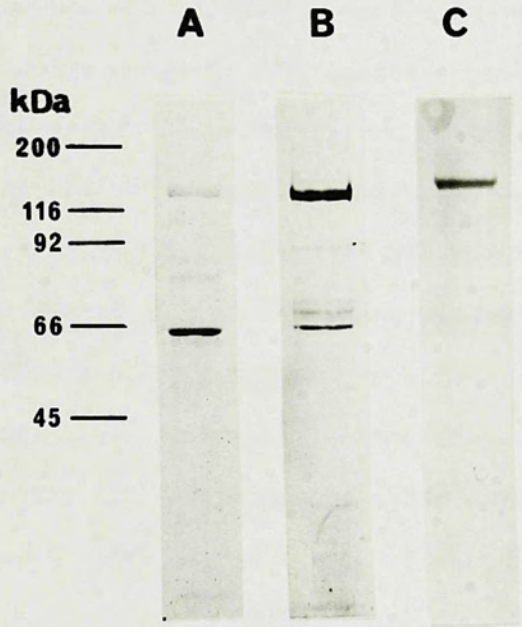
Purification Procedure III
(10 mL packed HSV-1($\bar{m}$P) infected HeLa cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)*	Specific Activity (un/mg)*	Purification	Yield (%)
Crude Extract	69	904	47244	52	1	100
DEAE-T-M I	103	690	60623	88	2	128
DEAE-T-M II	136	185	60399	327	6	128
ssDNA Agarose	49	15	43624	2881	55	92
DEAE-T-M III	23	7.5	32874	4371	84	70
PC11	18	2.68	16276	6069	116	34
ssDNA Agarose II	4	.72	10208	14178	271	22

*1 unit = 1 pmol TMP incorporated per minute at 35°C.

Figure 5. SDS PAGE Analysis of HSV mP Polymerase Purifications I, II, III.

HSV $\overline{m}$P DNA polymerase was purified as described in the text. The resulting HSV DNA polymerase preparations were analyzed by 9.25% SDS PAGE and were silver stained as described in section IV.B.1. Lane A, HSV $\overline{m}$P DNA polymerase purified using Purification Procedure I; Lane B, HSV $\overline{m}$P DNA polymerase purified using Purification Procedure II; Lane C, HSV $\overline{m}$P DNA polymerase purified using Purification Procedure III.


A
B
C
kDa
200
116
92
66
45

peak at 300 mM potassium phosphate. The final purification step in this scheme was a hydroxyapatite column from which the HSV DNA polymerase was eluted with 300 mM potassium phosphate.

Purification procedure II resulted in a much more homogeneous preparation of HSV DNA polymerase as judged by SDS PAGE (see figure 5). However, relatively large loses of polymerase activity occurred during the ssDNA agarose column and the PC11 column steps with small gains in the purification of the enzyme. These losses were reproducible over several preparations, and may have been due to several factors: contamination of the active polymerase by inactive protein, loss of proteins which may enhance enzymatic activity, and/or loss of proteins which initially stabilize the polymerase and which may be removed during purification. Less than 2% of the original HSV DNA polymerase activity was recovered at the end of this purification procedure. However, this preparation yielded a HSV DNA polymerase with a specific activity of 35936 units/mg which was as good or better than the specific activity obtained for the HSV DNA polymerase by other investigators [Weissbach et al., 1973; Knopf, 1979; Frank et al., 1984a, 1984b; O'Donnell et al., 1987a].

Using purification procedure II as a starting point, purification procedure III was developed and gave satisfactory results as judged by the purification table (table IX) and by SDS PAGE of the resulting HSV DNA polymerase (figure 5). The major difference between purification procedure II and purification procedure III is the substitution of potassium chloride for potassium phosphate as the elution salt for all columns and the use of a linear salt gradient on the ssDNA agarose column. If SDS PAGE analysis of the polymerase showed the

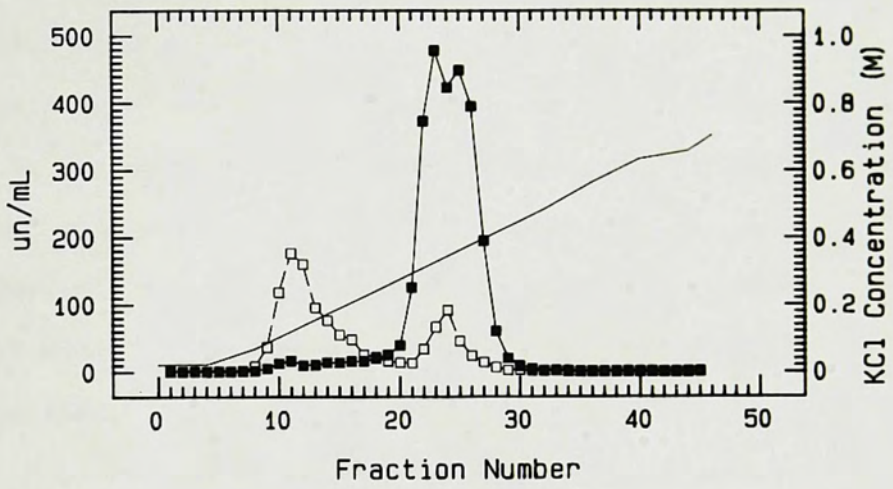
presence of contaminating bands, additional DEAE-T-M, ssDNA agarose, and PC11 columns were used to attempt to purify the HSV DNA polymerase. The elution profile obtained from the final ssDNA column presented in the purification table for purification procedure III is shown in Figure 6. This purification procedure resulted in the purification of the HSV DNA polymerase to 14178 units/mg which is similar to the specific activity obtained by other investigators [Weissbach et al., 1973; Knopf, 1979; Frank et al., 1984a, 1984b; O'Donnell et al., 1987a]. A final yield of 20% of the initial HSV DNA polymerase activity was obtained using this procedure. A second advantage of this procedure is that the purification can be completed within four days.

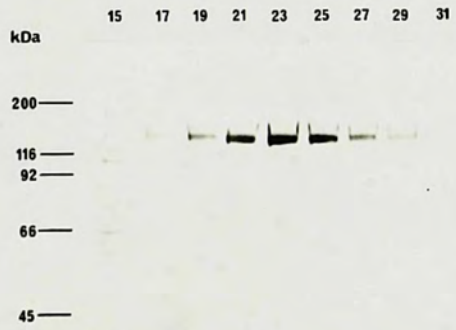
An observation which can be made, by comparing the purification tables presented in Table IX, is that the total HSV DNA polymerase activity obtained from the crude extracts is highly variable. Similar observations have also been made by other investigators [Weissbach et al., 1973; Powell & Purifoy, 1977; Knopf, 1979b]. There are several possible explanations for this observation: 1) a factor present in the crude extract which inhibits the HSV DNA polymerase, 2) variability in the infection characteristics (e.g. the state of the host cells - replicating or growth arrested, the temperature of infection, the incubation temperature), and 3) the presence of variable amounts of nuclease present in the crude extracts which may mask some of the DNA polymerase activity. The differences in total activity are also reflected in the differences seen in the specific activities of the crude extracts and also influence the purification values obtained for the HSV DNA polymerase.

Purification procedure III was then used to purify the HSV DNA

Figure 6. Elution Profile for the ssDNA Agarose Column of Purification Procedure III.

The elution profile for a typical final ssDNA agarose column for the purification of HSV $\overline{m}$P DNA polymerase is shown. High salt (-■-) and low salt (-□-) DNA polymerase activities are plotted. Below, the silver stained 9.25% SDS PAGE results of the indicated fractions are presented.


500
400
300
200
100
0
un/mL
1.0
0.8
0.6
0.4
0.2
0
KCl Concentration (M)
0
10
20
30
40
50
Fraction Number


15
17
19
21
23
25
27
29
31
kDa
200
116
92
66
45

polymerases from cells infected with HSV-1 strains KOS, tsC4, tsC7, and tsD9. The purification tables for KOS, tsC4, tsC7 and the $\overline{m}$P HSV DNA polymerase preparations are given in Table X. The purification of the HSV KOS, tsC4, and tsC7 DNA polymerases yielded preparations with specific activities of 4446, 1634, 3042 units/mg, respectively. Although this procedure yielded KOS HSV DNA polymerase that appeared to be homogeneous based on SDS PAGE analysis (figure 7), tsC4 and tsC7 preparations yielded several polypeptides. The additional polypeptides present in the mutant HSV DNA polymerase preparations may be proteolytic breakdown products of the DNA polymerase peptide. Although the HSV DNA polymerase preparations from the temperature sensitive mutant infected cells were not homogeneous, additional purification steps resulted in large losses of polymerase activity and were, therefore, not used.

An additional observation derived from Figure 7 was that the HSV KOS derived DNA polymerase have an apparent molecular weight of 135 kDa, whereas the HSV $\overline{m}$P DNA polymerase has an apparent molecular weight of 148 kDa. There are a couple of possible explanations for this observation: 1) there are additional peptide sequences present in the HSV $\overline{m}$P DNA polymerase that are not present in the HSV KOS derived DNA polymerases, and 2) the HSV $\overline{m}$P DNA polymerase polypeptide electrophoreses anomalously on SDS PAGE gels.

While it was possible to purify the HSV DNA polymerases from tsC4 and tsC7, it was not possible to purify the HSV DNA polymerase from tsD9 infected cells. Many attempts were made to purify this polymerase, but no demonstrable HSV DNA polymerase activity could be recovered from the second DEAE-T-M column step. There was also a reduced amount of high salt (presumably HSV) DNA polymerase activity in the

TABLE X

PURIFICATION TABLES FOR POLYMERASES USED IN THIS STUDY

HSV-1($\overline{m}$P) (HeLa cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)	Specific Activity (un/mg)	Purification	Yield (%)
Crude Extract	69	904	47244	52	1	100
DEAE-T-M I	103	690	60623	88	2	128
DEAE-T-M II	136	185	60399	327	6	128
ssDNA Agarose	49	15	43624	2881	55	92
DEAE-T-M III	23	7.5	32874	4371	84	70
PC11	18	2.68	16276	6069	116	35
ssDNA Agarose II	4	.72	10208	14178	271	22

HSV-1(KOS) (Vero cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)	Specific Activity (un/mg)	Purification	Yield (%)
Crude Extract	60	3606	53464	15	1	100
DEAE-T-M I	115	2990	45954	15	1	86
DEAE-T-M II	33	229	13997	63	4	26
ssDNA Agarose	10.3	1.78	7923	4446	300	15

HSV-1(tsC4) (Vero cells)

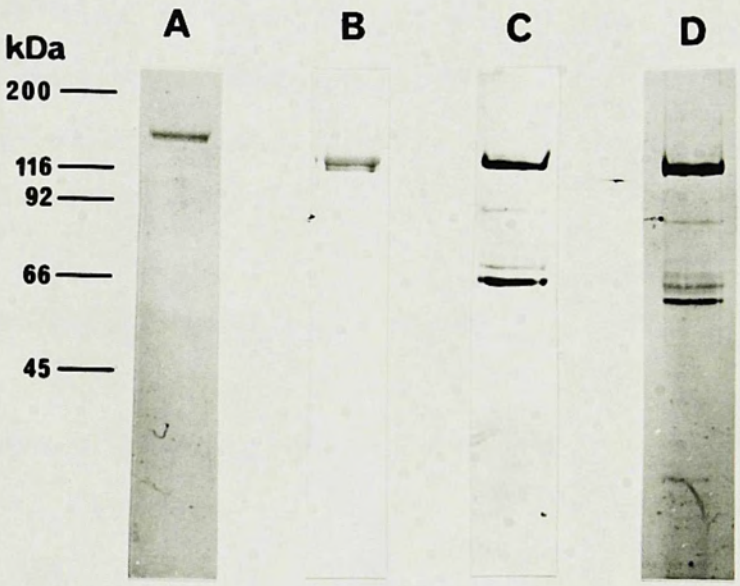
Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)	Specific Activity (un/mg)	Purification	Yield (%)
Crude Extract	60	3522	12586	4	1	100
DEAE-T-M I	115	2300	99912	43	12	794
DEAE-T-M II	35	139	5111	37	10	41
ssDNA Agarose	13.8	1.04	1804	1634	457	14

HSV-1(tsC7) (Vero cells)

Procedure	Volume (mL)	Total Protein (mg)	Total Activity (un)	Specific Activity (un/mg)	Purification	Yield (%)
Crude Extract	62	3224	38750	12	1	100
DEAE-T-M I	110	1815	51398	28	2	133
DEAE-T-M II	60	270	11202	42	4	29
ssDNA Agarose	13.5	3.19	9691	3042	253	25

Figure 7. SDS PAGE Analysis of mP and KOS Derived DNA Polymerases.

The HSV DNA polymerases were purified as described in the text. The resulting HSV DNA polymerase preparations were analyzed by 9.25% SDS PAGE and were silver stained as described in section IV.B.1. Lane A, HSV $\overline{mP}$ DNA polymerase; Lane B, HSV KOS DNA polymerase; Lane C, HSV tsC4 DNA polymerase; Lane D, HSV tsC7 DNA polymerase.


kDa
200
116
92
66
45
A
B
C
D

crude extracts of HSV-1 tsD9 infected cells as compared to extracts prepared from cells infected with the other viruses. This confirmed the result presented in Figure 4B showing a smaller amount of HSV tsD9 DNA polymerase activity during active infections when compared with the other viruses. As stated in the introduction, the HSV tsD9 DNA polymerase gene has been sequenced [Gibbs et al., 1987]. This mutation results in the substitution of glutamic acid for lysine at amino acid residue 597 of the protein and a total charge change of -2. This change may cause a conformational change which may: 1) result in lower polymerase activity, 2) destabilize the protein structure such that on separation from other proteins helping to stabilize the enzyme, the DNA polymerase activity is lost, and/or 3) result in an increased susceptibility to proteolytic degradation.

V. D. Characterization of HSV DNA Polymerases

The partially purified enzymes were used in the characterization of the HSV DNA polymerases.

V. D. 1. Salt Optimum

The first characteristic tested using the partially purified HSV DNA polymerases was salt optimum. The salt optimum has been used to distinguish the wild-type HSV DNA polymerase from the cellular DNA polymerases since the cellular enzymes have salt optima that are lower than the HSV DNA polymerase [Weissbach et al., 1973; Knopf, 1979; Ostrander & Cheng, 1980]. DNA polymerase α has a salt optimum at very

low salt concentrations (0-10 mM KCl) whereas, DNA polymerase β has a salt optimum between 75 and 100 mM KCl [Haines et al., 1971]. The HSV DNA polymerases were assayed as described in section IV.E.1.d.1. using varying amounts of KCl in the assay. Figure 8 shows the results obtained from these reactions. All of the enzymes tested showed nearly identical salt stimulation curves with optima between 200 and 300 mM KCl with almost no detectable activity below 100 mM KCl. These results are similar to those obtained by other investigators [Weissbach et al., 1973; Knopf, 1979; Ostrander & Cheng, 1980]. The salt stimulation curves were almost identical at salt concentrations below 300 mM KCl. However, at 400 mM KCl, HSV $\bar{m}$P and tsC7 DNA polymerases retained a greater percentage of the maximal DNA polymerase activity than did HSV KOS DNA polymerase; HSV tsC4 DNA polymerase showed about 10% of the maximal DNA polymerase activity at this salt concentration.

V. D. 2. pH Optimum

The pH optima of the enzymes was determined using a series of four buffer systems: phosphate, PIPES, Tris, and Ammediol (IV.E.1.d.2.). These assays were carried out at 200 mM KCl without correcting for the ionic strength of the buffers. The wild-type $\bar{m}$P HSV DNA polymerase showed a broad pH optimum between pH 7.5 and 8.7 (Figure 9). The curve also showed smooth transitions between the various buffer systems used. The DNA polymerase produced by HSV-1 KOS, and the mutants, tsC4 and tsC7, all showed broad pH optima between pH 7.5 and 8.5 (Figure 9) similar to that seen with HSV-1 $\bar{m}$P DNA polymerase. These enzymes, however, showed discontinuous curves on going from one buffer

Figure 8. KCl Optimum Curves.

Salt optimum for each of the HSV DNA polymerases was analyzed as described in the text. HSV $\overline{m}$P (-▲-), KOS (-●-), tsC4 (-■-), and tsC7 (-o-) DNA polymerases were analyzed.

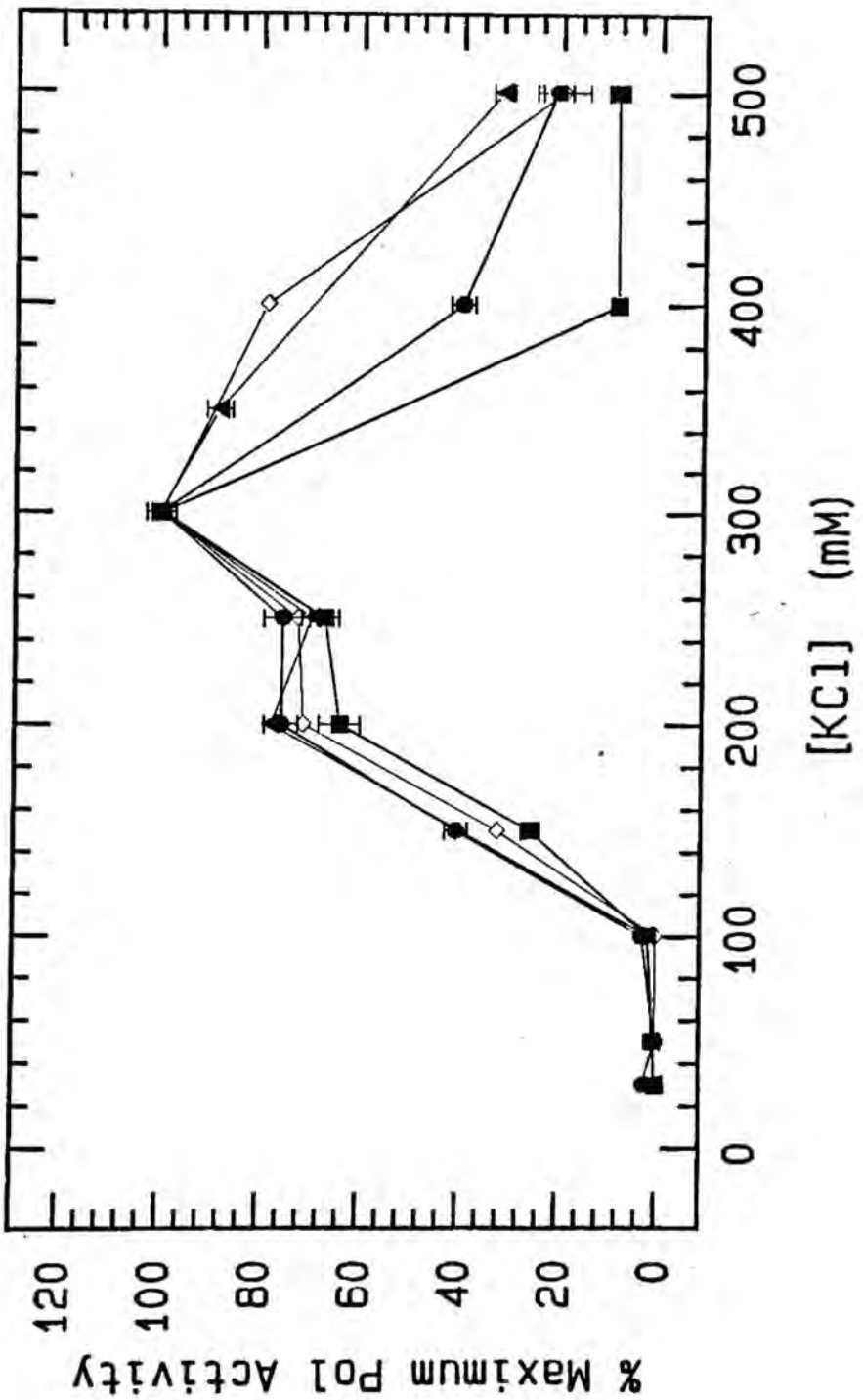
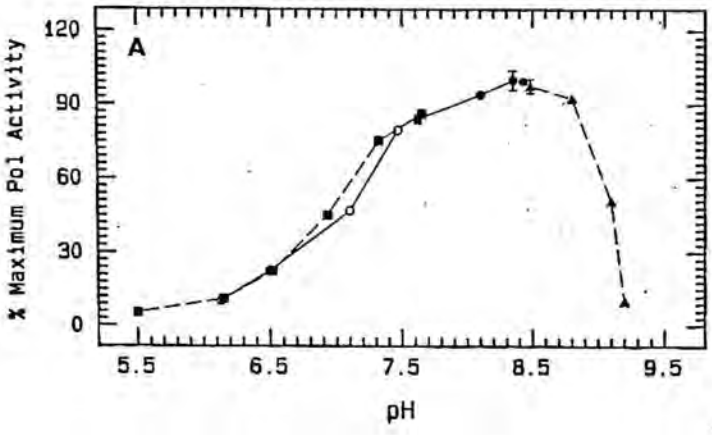
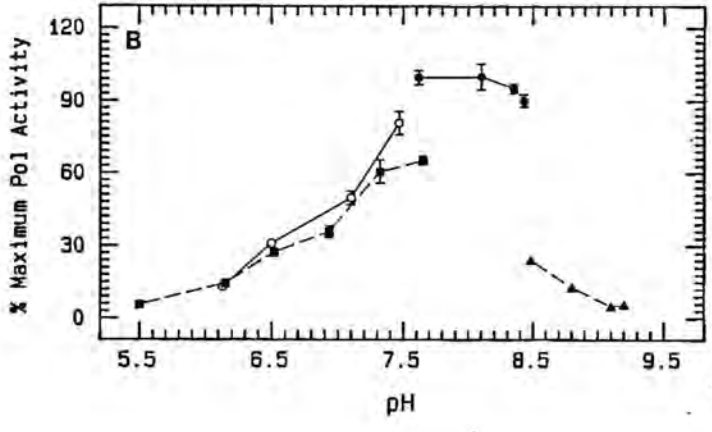
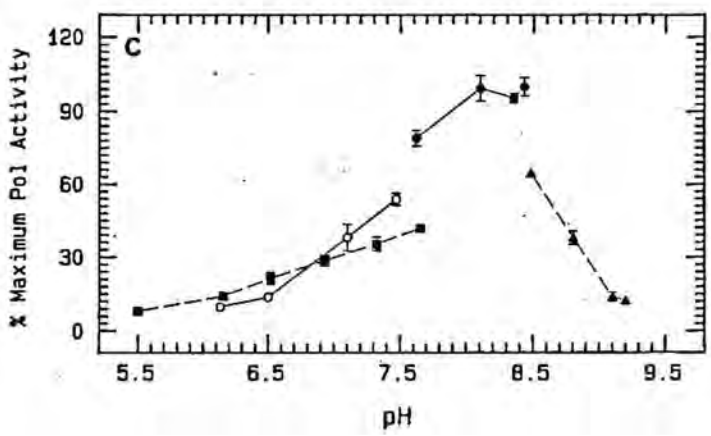

% Maximum Pol Activity
120
100
80
60
40
20
0
0
100
200
300
400
500
[KCl] (mM)

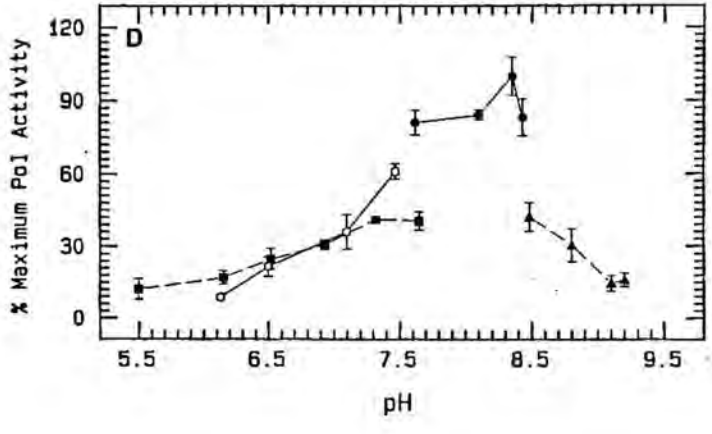
Figure 9. pH Optimum Curves.

Each of the purified HSV DNA polymerases was analyzed for pH optimum as described in the text using phosphate (-■-), PIPES (-o-), Tris (-●-), and Ammediol (-▲-) buffers. Panels: A, HSV $\bar{m}$P DNA polymerase; B, HSV KOS DNA polymerase; C, HSV tsC4 DNA polymerase; D, HSV tsC7 DNA polymerase.


A
% Maximum Pol Activity
120
90
60
30
0
5.5
6.5
7.5
8.5
9.5
pH


B
% Maximum Pol Activity
120
90
60
30
0
5.5
6.5
7.5
8.5
9.5
pH


C
% Maximum Pol Activity
120
90
60
30
0
5.5
6.5
7.5
8.5
9.5
pH


D
% Maximum Pol Activity
120
90
60
30
0
5.5
6.5
7.5
8.5
9.5
pH

system to the next. In addition, HEPES buffers were also used over the same pH ranges as the Tris buffers with identical results (data not shown). Based on this result, either HEPES or Tris could be used with equal effectiveness in the polymerase reaction at a pH between 7.5 and 8.5.

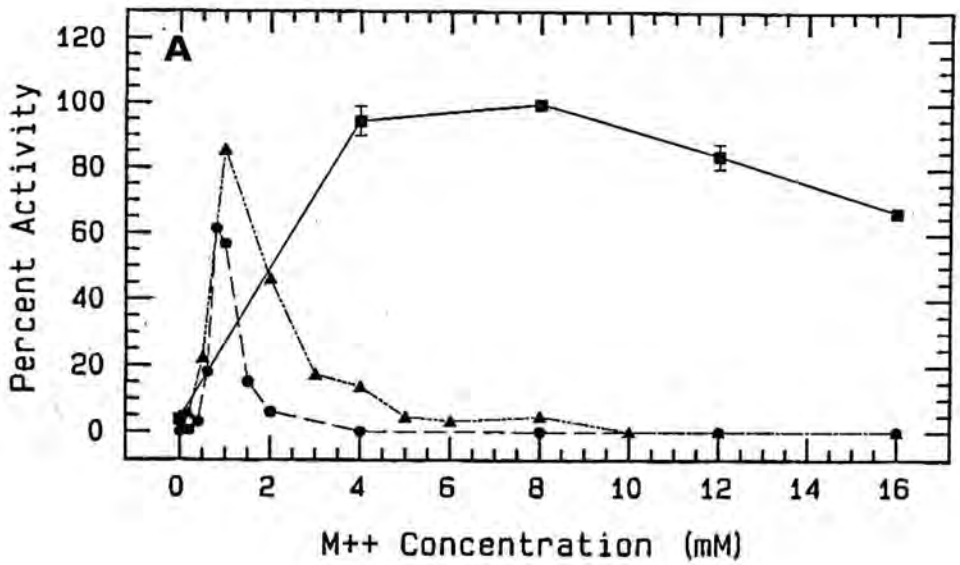
V. D. 3. Divalent Cation Usage

Divalent cations have been shown to be necessary for maximal activity of most enzymes involved in DNA metabolism and essential cofactors for DNA polymerase including the HSV DNA polymerase. Shown in Figures 10 and 11 are the titration results using Mg^{2+}, Mn^{2+}, and Co^{2+}. None of the enzymes showed activity when either Ca^{2+} or Zn^{2+} was the divalent cation.

The HSV $\overline{m}$P DNA polymerase exhibited a Mg^{2+} optimum of between 4 and 8 mM (Figure 10A) and still showed 60% of its maximal activity at 16 mM. In contrast, a sharp Mn^{2+} optimum of about 1 mM was obtained, where about 60% of the optimum Mg^{2+} activity was observed. By 4 mM the Mn^{2+}, the activity was zero. Similarly, Co^{2+} also shared a sharp optimum at 1 mM, where 90% of the optimum Mg^{2+} activity was observed. The HSV KOS DNA polymerase exhibited a Mg^{2+} optimum of between 8 and 16 mM (Figure 10B). A sharp Mn^{2+} optimum of about 1 mM was obtained, where about 70% of the optimum Mg^{2+} activity was observed, and by 2 mM the Mn^{2+}, the activity was zero. In contrast, Co^{2+} showed a broad optimum peaking at 2 mM, where only 25% of the optimum Mg^{2+} activity was observed. The DNA polymerase activity did not return to zero until 10 mM Co^{2+} was present in the DNA polymerase reaction.

Figure 10. Divalent Metal Titration Curves - mP, KOS DNA Polymerases.

Triplicate polymerase assays were performed using purified DNA polymerase with varying concentration of divalent cations. The results of Mg^{2+} (-■-), Mn^{2+} (--●--), and Co^{2+} (-···▲···) are expressed as percent of maximum Mg^{2+} activity. Panel: A, HSV $\bar{m}$P DNA polymerase; B, HSV KOS DNA polymerase.


A
Percent Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
M++ Concentration (mM)

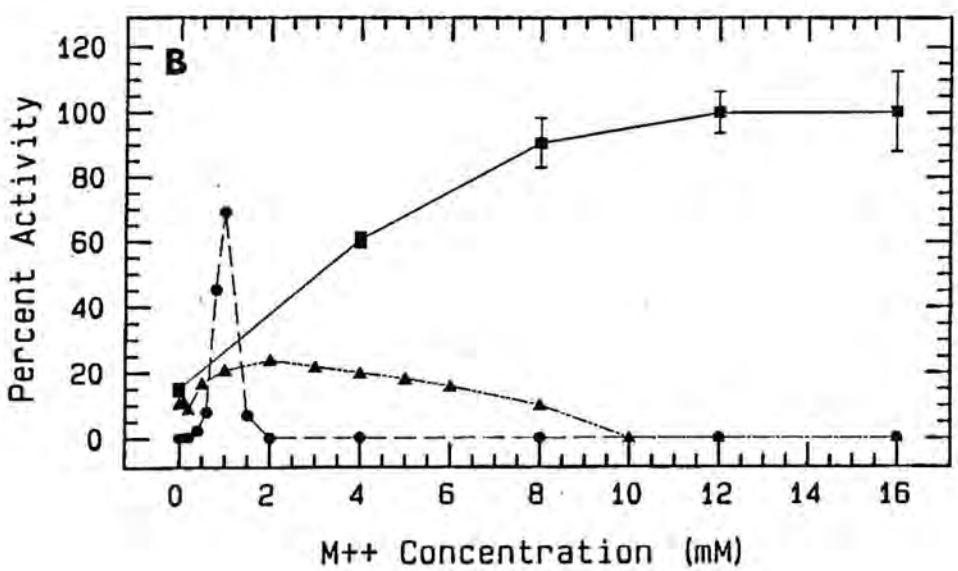
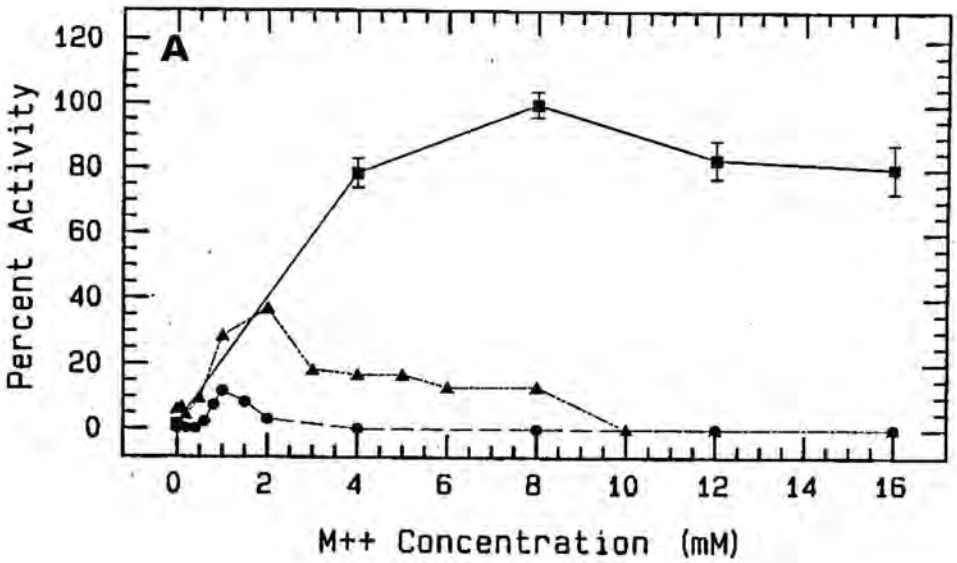
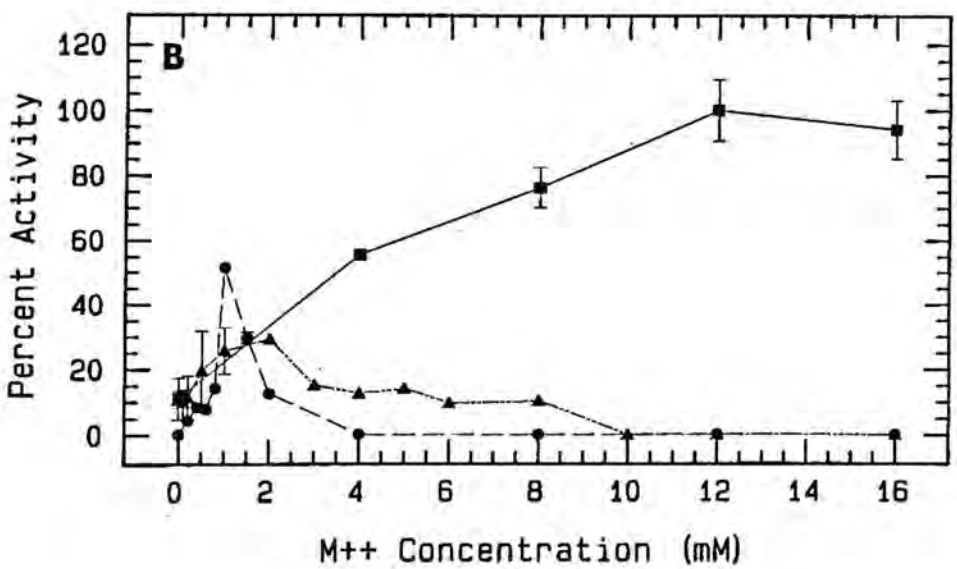

B
Percent Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
M++ Concentration (mM)

Figure 11. Divalent Metal Titration Curves - tsC4, tsC7 DNA Polymerases.

Triplicate polymerase assays were performed using purified DNA polymerase with varying concentration of divalent cations. The results of Mg^{2+} (-■-), Mn^{2+} (--●--), and Co^{2+} (-···▲-···) are expressed as percent of maximum Mg^{2+} activity. Panel: A, HSV tsC4 DNA polymerase; B, HSV tsC7 DNA polymerase.


A
Percent Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
M++ Concentration (mM)


B
Percent Activity
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
M++ Concentration (mM)

The mutant HSV tsC4 DNA polymerase exhibited a Mg^{2+} optimum of approximately 8 mM (Figure 11A) and still showed 80% of its maximal activity at 16 mM. In contrast to the other enzymes above, a Mn^{2+} optimum of about 1 mM was obtained, where only about 12% of the optimum Mg^{2+} activity was observed. By 2 mM the Mn^{2+}, the activity was zero. Like HSV KOS DNA polymerase, Co^{2+} also showed broad optimum at 2 mM, where 40% of the optimum Mg^{2+} activity was observed, and did not return to zero activity until 10 mM Co^{2+}. The HSV tsC7 DNA polymerase exhibited a Mg^{2+} optimum of between 12 and 16 mM (Figure 11B). At 8 mM Mg^{2+}, this enzyme showed 80% of its maximal activity. A sharp Mn^{2+} optimum of about 1 mM was obtained, where about 55% of the optimum Mg^{2+} activity was observed. By 4 mM the Mn^{2+}, the activity was zero. In contrast, Co^{2+} also showed broad optimum at 2 mM, where 30% of the optimum Mg^{2+} activity was observed. The DNA polymerase did not return to zero activity until the Co^{2+} concentration was 10 mM.

The results from these studies indicate that there is a major difference between the HSV $\bar{m}$P DNA polymerase and the HSV KOS derived DNA polymerases in the way that the DNA polymerases utilize Co^{2+}. The HSV $\bar{m}$P DNA polymerase has a relatively sharp Co^{2+} optimum at 1 mM, whereas the HSV KOS DNA polymerases have broad Co^{2+} optima peaking at 2 mM. Another major difference noted was that HSV tsC4 does not use Mn^{2+} efficiently when compared to the other HSV DNA polymerases.

Although there are differences between the parental strain HSV KOS DNA polymerase and the mutant HSV DNA polymerases, the differences observed between the two wild-type HSV DNA polymerases are just as great. This also suggests differences in the enzymes resulting in altered DNA polymerase activities with different divalent cations.

V. D. 4. DNA Polymerase Reaction Requirements

This study was carried out using the procedure outlined in section IV.E.1.d.4. The results of these experiments for all of the polymerases studied are presented in Table XI. There appears to be an absolute requirement for the buffer, Mg^{2+}, and template, with maximum DNA synthesis occurring at high ionic strengths (see section V.D.1.). However, several interesting observations can be made from this table. The first is the relatively high amount of DNA synthesis with $\bar{m}$P HSV DNA polymerase in the absence of one of the dNTPs (60-90%), whereas, the results with the other three HSV DNA polymerases are more in line with what would be expected for this situation (15-40%; this may reflect the base composition and state of the activated DNA substrate). The values obtained for the HSV KOS, tsC4, and tsC7 DNA polymerases are similar to the observations made by other investigators [Weissbach et al., 1973; Powell & Purifoy, 1977]. The result obtained with the HSV $\bar{m}$P DNA polymerase may suggest that this enzyme has a higher rate of misincorporation of the dNTPs in the absence of one dNTP. Another observation derived from Table XI is the apparent need for thiol reducing agents by the KOS HSV DNA polymerase. This may suggest that the HSV $\bar{m}$P, tsC4 and tsC7 DNA polymerases have a cysteine residue participating in the DNA synthesis reaction that is buried or is better protected from the environment and is less prone to oxidation than the cysteine residue in HSV KOS DNA polymerase. Additionally, all of the enzymes studied here are NEM sensitive (data not shown). Other investigators have shown the HSV DNA polymerases to be NEM sensitive [Weissbach et

TABLE XI

HSV DNA POLYMERASE REACTION REQUIREMENTS

	$\bar{m}$P		KOS		tsC4		tsC7	
Reaction Mix	% Act	± S.D.	% Act	± S.D.	% Act	± S.D.	% Act	± S.D.
Complete	100.0	± 5.2	100.0	± 3.5	100.0	± 11.2	100.0	± 5.9
-HEPES	3.0	± 4.3	0.0	± 1.2	2.5	± 2.8	0.9	± 0.5
-$MgCl_2$	.9	± 1.5	0.0	± 1.2	0.9	± 2.4	0.2	± 2.9
-AcDNA	1.5	± 1.5	0.0	± .6	0.9	± 2.6	1.1	± 1.4
-dATP	60.1	± .2	14.2	± 1.4	28.3	± 3.6	20.8	± 3.2
-dCTP	96.1	± 11.6	29.2	± 3.1	44.8	± 6.7	33.1	± 2.3
-dGTP	80.4	± 7.1	28.6	± 2.7	36.1	± 3.6	33.5	± 3.5
-TTP	58.3	± 4.2	27.0	± 2.2	38.8	± 3.3	32.0	± 2.9
-β-ME	83.4	± 10.1	14.2	± 1.4	93.6	± 8.9	81.9	± 7.3

al., 1973; Knopf, 1979].

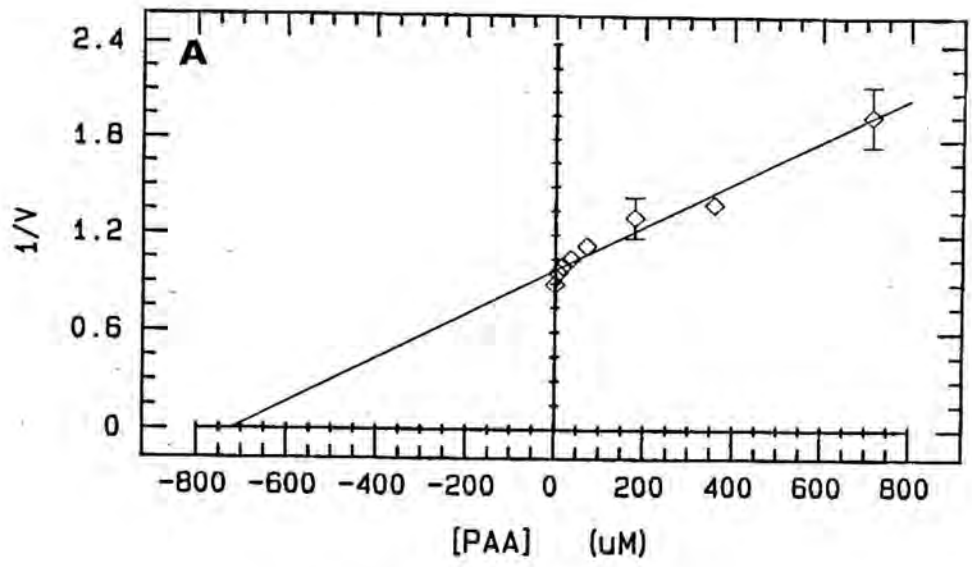
V. D. 5. Phosphonoacetic Acid Sensitivity

Another characteristic of the wild-type HSV DNA polymerases studied to date is their sensitivity to the pyrophosphate analog, phosphonoacetic acid (PAA) [Mao & Robishaw, 1975; Powell & Purifoy, 1977; Ostrander & Cheng, 1980; Derse et al., 1982; Coen et al., 1984]. The inhibition of PAA has been shown to be competitive with respect to pyrophosphate, noncompetitive with respect to dNTPs, and uncompetitive with respect to template [Mao & Robishaw, 1975; Ostrander & Cheng, 1980]. DNA polymerase α is inhibited by PAA at concentrations equal to or higher than those necessary to inhibit the HSV DNA polymerases (10 to 100 µg/mL) [Bolden et al., 1975; Leinbach et al., 1976; Allen et al., 1977; Allandeen & Bertino, 1978].

This study was carried out in order to determine the relative PAA sensitivities of the four DNA polymerases used in these studies. These experiments were carried out by adding various concentrations of PAA to the standard polymerase reaction mix (section IV.E.1.a.). The results from these reactions are shown in Figures 12 and 13 and are summarized in Table XII. The K_i for PAA of 721 µM obtained for HSV KOS DNA polymerase here is similar to the PAA K_i of 970 ± 300 µM obtained by Derse et al. [1982]. Both of the temperature sensitive mutants studied in this experiment appeared to showed increased sensitivity to PAA when compared to the parental strain, KOS. However, the sensitivity to PAA shown by the mutant polymerases was matched or exceeded by the $\overline{m}$P HSV DNA polymerase.

Figure 12. Dixon Plots for PAA Inhibition - KOS, tsC4 DNA Polymerases.

Triplicate DNA polymerase assays were performed with varying amounts of PAA present. Dixon plots for HSV KOS (panel A), and tsC4 (panel B) DNA polymerases are presented.


A
2.4
1.8
1.2
0.6
0
1/V
-800 -600 -400 -200 0 200 400 600 800
[PAA] (uM)

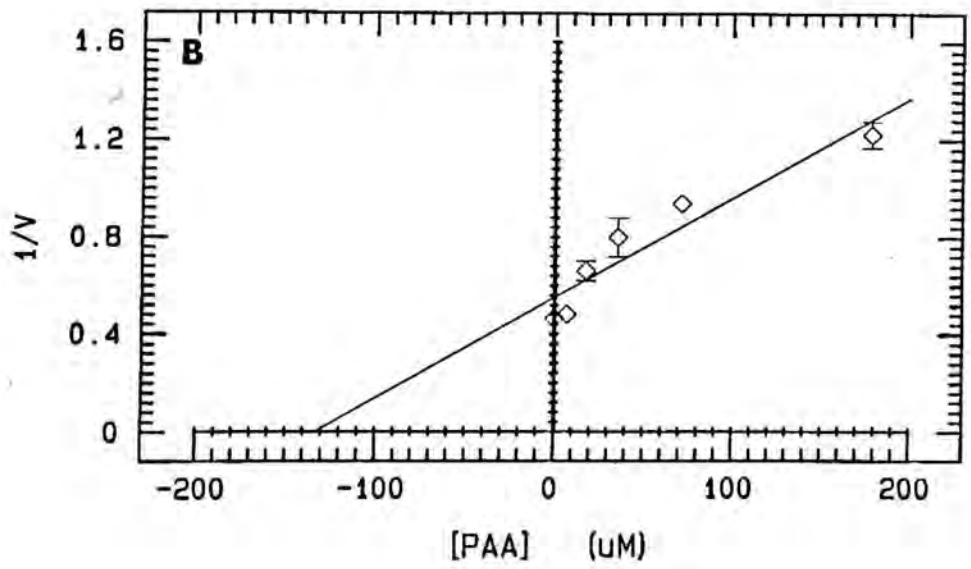
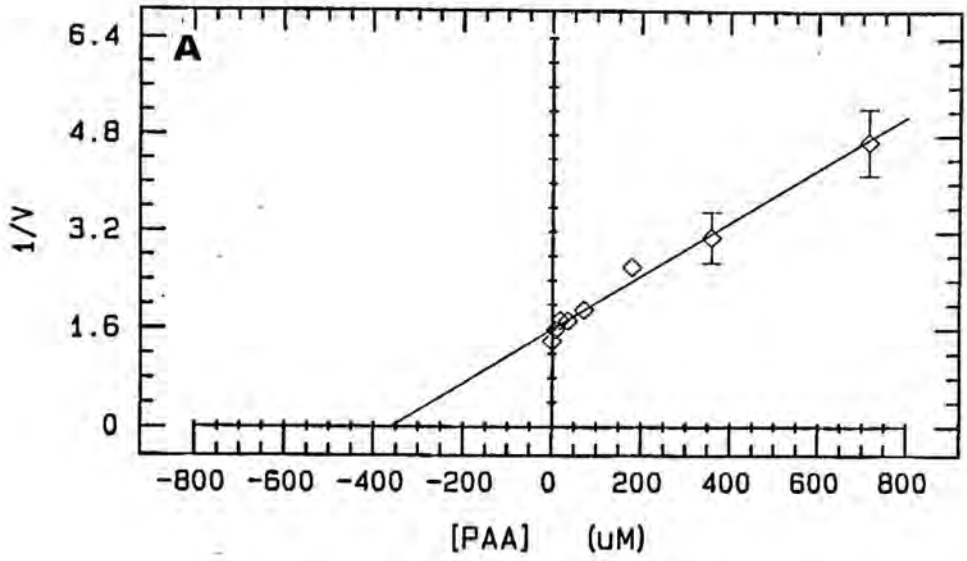

B
1.6
1.2
0.8
0.4
0
1/V
-200 -100 0 100 200
[PAA] (uM)

Figure 13. Dixon Plots for PAA Inhibition - tsC7, mP DNA Polymerases.

Triplicate DNA polymerase assays were performed with varying amounts of PAA present. Dixon plots for HSV tsC7 (panel A), and $\overline{m}$P (panel B) DNA polymerases are presented.


A
6.4
4.8
3.2
1.6
0
1/V
-800 -600 -400 -200 0 200 400 600 800
[PAA] (uM)

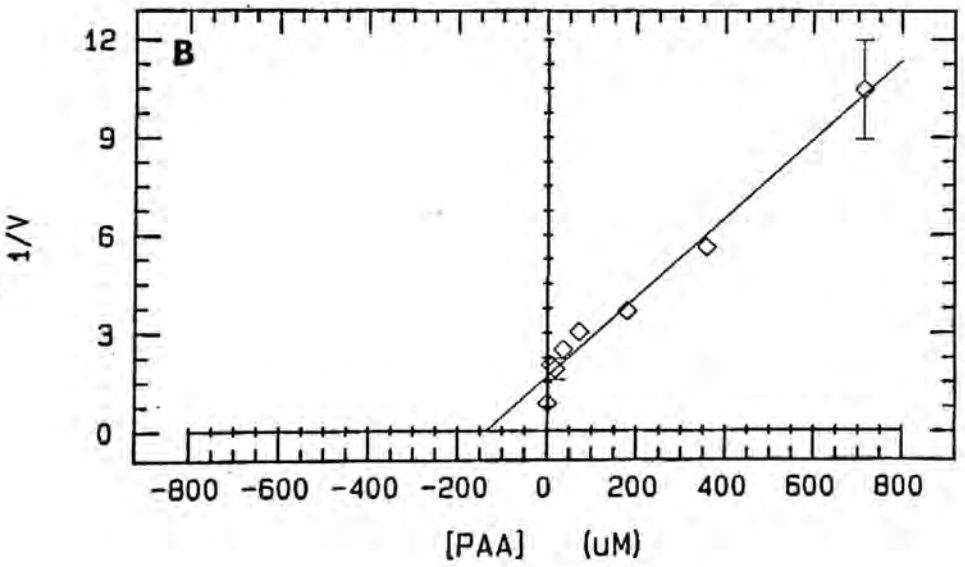

B
12
9
6
3
0
1/V
-800 -600 -400 -200 0 200 400 600 800
[PAA] (uM)

TABLE XII

PAA SENSITIVITY OF HSV DNA POLYMERASES

Strain	Ki (μM)
$\bar{m}$P	137
KOS	721
tsC4	133
tsC7	360

In vivo, the mutant tsC4 has been reported to be "hypersensitive" to PAA when compared to the parental strain and the other ts mutants that were derived from HSV-1 KOS based on a plaque reduction assay [Coen et al., 1984]. Although the HSV tsC4 DNA polymerase is more sensitive to PAA in these studies, the HSV tsC7 DNA polymerase also appears to be more sensitive as compared to HSV KOS DNA polymerase, which is not what is seen in in vivo plaque reduction assays [Coen et al., 1984]. This discrepancy may be due to how hypersensitivity is defined. In the plaque reduction assay, any virus which was at least three-fold more sensitive to PAA was labelled as hypersensitive. The results presented here suggest that the HSV tsC7 DNA polymerase probably fell within the limits of "normal" sensitivity of the HSV DNA polymerase to PAA and was therefore classified as being sensitive but not hypersensitive to PAA. The results of this series of experiments suggest that the mutations found in the temperature sensitive HSV DNA polymerases may alter sites on the enzyme and these changes are reflected in changes in drug sensitivities.

V. D. 6. dNTPase/Nuclease Activities

Each of the HSV DNA polymerase preparations was tested for nuclease activity as outlined in section IV.F. These assays were carried out under conditions of DNA synthesis using nick translated nicked-gapped DNA and heat denatured DNA substrates at high and low ionic strength conditions in the absence of dNTPs. The extent of nucleic acid degradation was assessed, and the results are presented in Table XIII. This particular procedure detects exonuclease activity but also

TABLE XIII

NUCLEASE ACTIVITY PRESENT IN HSV DNA POLYMERASE PREPARATIONS

IN THE ABSENCE OF dNTPs

Substrate	Ionic Strength	Strain of HSV DNA Polymerase[1]			
		$\overline{m}$P	KOS	tsC4	tsC7
ngDNA	200 mM	92	98	98	78
	30 mM	89	85	50	89
hdDNA	200 mM	90	85	87	70
	30 mM	76	74	50	75

[1]Results are expressed as the percentage of DNA remaining after incubation with the enzyme for 30 minutes at 37°C.

is positive for endonuclease activity when there is a lot of activity and short oligonucleotides are produced. The results indicated that nuclease activity was present under conditions where DNA synthesis was not occurring. This confirms the results obtained by other laboratories who show nuclease activity present in HSV DNA polymerase preparations under conditions where DNA synthesis is not occurring [Knopf, 1979; O'Donnell, 1987a].

The amount of DNA dependent dNTPase activity had to be determined for each of the polymerase preparations under the conditions used for DNA synthesis before the Michaelis constants for each of the dNTPs could be determined. In addition, the amount of nuclease activity present in the HSV DNA polymerase preparations under conditions of DNA synthesis had to be determined. Therefore, the dNTPase/exonuclease assay outlined in section IV.G. was developed.

For all of the enzymes tested, no dNTPase or nuclease activity could be detected (data not shown). This result is consistent with the results that other investigators have noted for the HSV DNA polymerase under these conditions [Knopf, 1979; O'Donnell, 1987a].

V. D. 7. Michaelis Constants of HSV DNA Polymerases

DNA polymerase assays were performed at 35°C as described in section IV.E.1.a. by varying the concentration of one of the four dNTPs, while holding the concentrations of the other three dNTPs at saturating levels. The reactions were repeated, holding the concentration of the dNTPs at saturating levels and varying the amount of activated DNA in the polymerase reaction mixtures. The resulting data was

analyzed using Lineweaver-Burke plots (a typical set of results for KOS polymerase are shown in Figures 14, 15, and 16). The results of these experiments are shown in Table XIV, and indicate that there are no significant differences in the apparent K_ms for the dNTPs between the two wild-type HSV DNA polymerases.

The values reported here are three to four fold greater than those reported by Frank et al., [1984b] for KOS HSV DNA polymerase. This may be attributed to differences in HSV DNA polymerase preparations, or may be due to differences in the way the HSV DNA polymerase is assayed in each laboratory. The results obtained from this series of experiments demonstrate a four- to five-fold increase in the K_m values for the dNTPs for the mutant polymerases when compared to the Km values obtained from HSV KOS DNA polymerase. This lends additional support to the hypothesis that there may be differences in the active site of the mutant enzymes when compared to the active site of the parental HSV DNA polymerase.

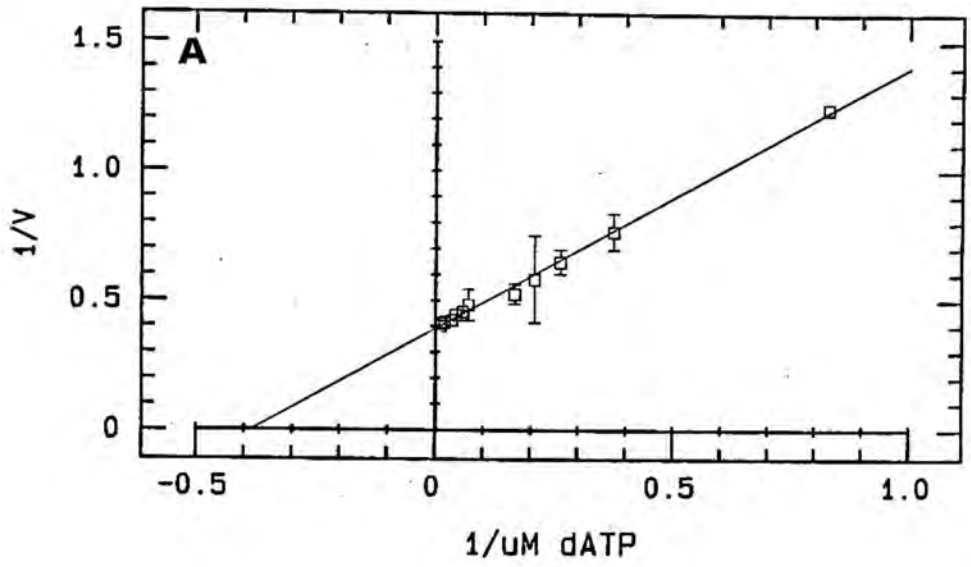
Another result from these experiments was the K_m value for activated DNA. These results indicate that $\bar{m}$P HSV DNA polymerase has five-fold less affinity for activated DNA when compared to the HSV-1 KOS derived enzymes.

V. E. Thermal Inactivation Assays

Although the HSV mutants, tsC4 and tsC7, whose mutations map in the HSV DNA polymerase gene, have been shown to be temperature sensitive <u>in vivo</u>, the temperature sensitivity of the DNA polymerase is in question. For this reason, thermal inactivation studies were carried

Figure 14. Lineweaver-Burke Plots for dATP, dCTP - KOS DNA Polymerase.

Apparent K_m values for the dNTPs were determined by varying the concentration of one of the dNTPs while holding the other three at saturating concentrations in the DNA polymerase reaction. The results were plotted using Lineweaver-Burke plots. The results obtained for HSV KOS DNA polymerase using dATP (panel A), and dCTP (panel B) as the variable dNTP are presented.


A
1.5
1.0
0.5
0
1/V
-0.5
0
0.5
1.0
1/uM dATP

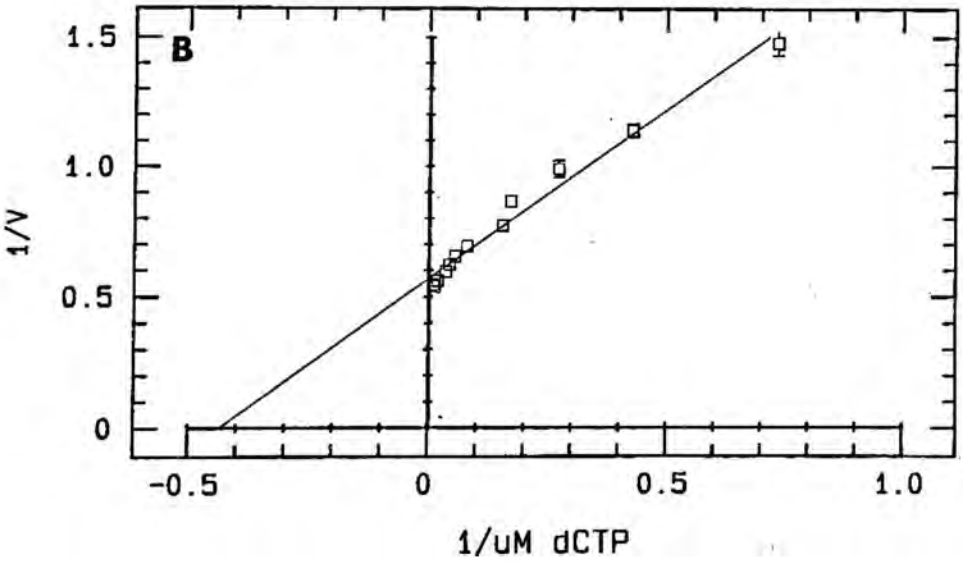
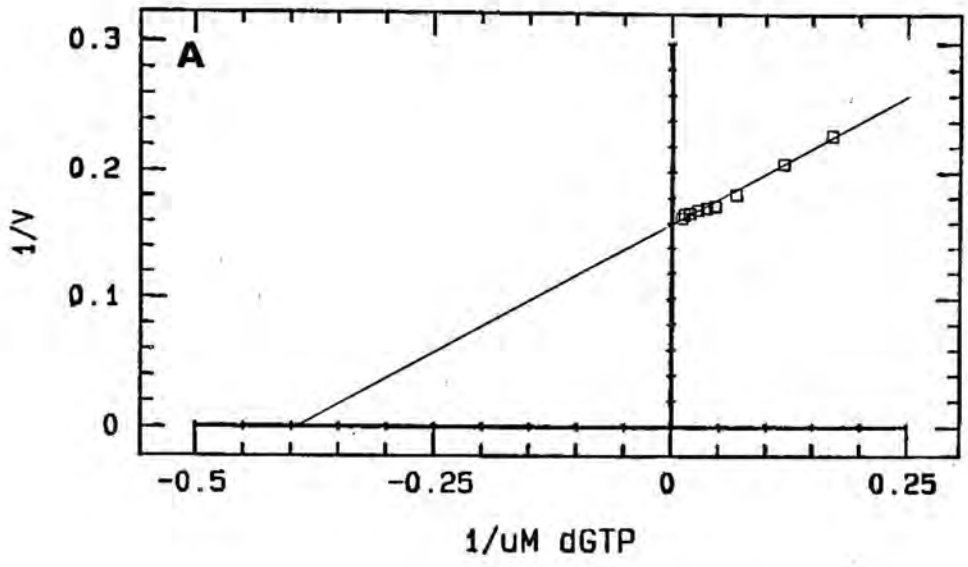

B
1.5
1.0
0.5
0
1/V
-0.5
0
0.5
1.0
1/uM dCTP

Figure 15. Lineweaver-Burke Plots for dGTP, TTP - KOS DNA Polymerase.

Apparent K_m values for the dNTPs were determined by varying the concentration of one of the dNTPs while holding the other three at saturating concentrations in the DNA polymerase reaction. The results were plotted using Lineweaver-Burke plots. The results obtained for HSV KOS DNA polymerase using dGTP (panel A), and TTP (panel B) as the variable dNTP are presented.


A
0.3
0.2
0.1
0
1/V
-0.5
-0.25
0
0.25
1/uM dGTP

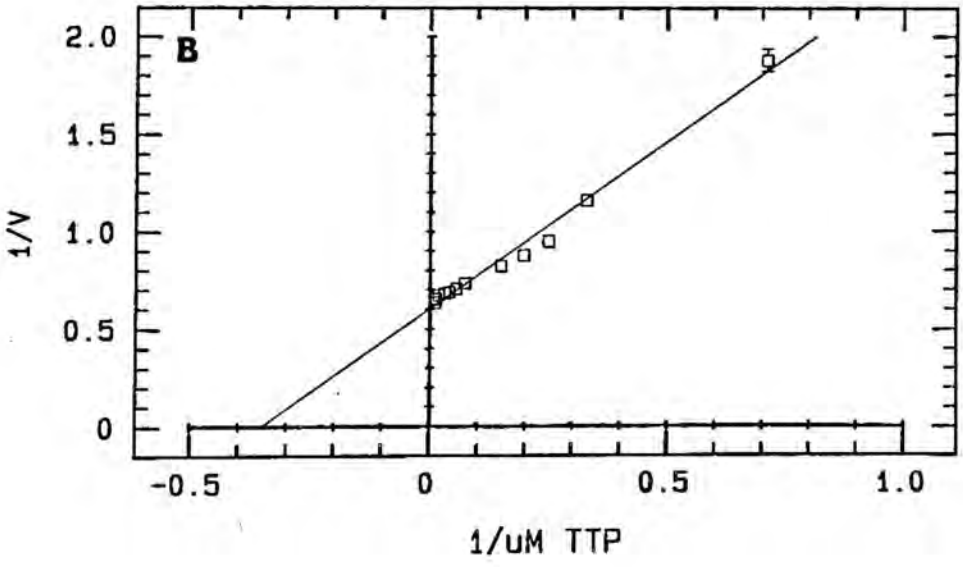

B
2.0
1.5
1.0
0.5
0
1/V
-0.5
0
0.5
1.0
1/uM TTP

Figure 16. Lineweaver-Burke Plot For AcDNA - KOS DNA Polymerase.

The apparent K_m value for activated DNA was determined by varying the concentration of activated DNA present in the DNA polymerase reaction. The results were plotted using the Lineweaver-Burke plot. The results obtained for HSV KOS DNA polymerase is presented.

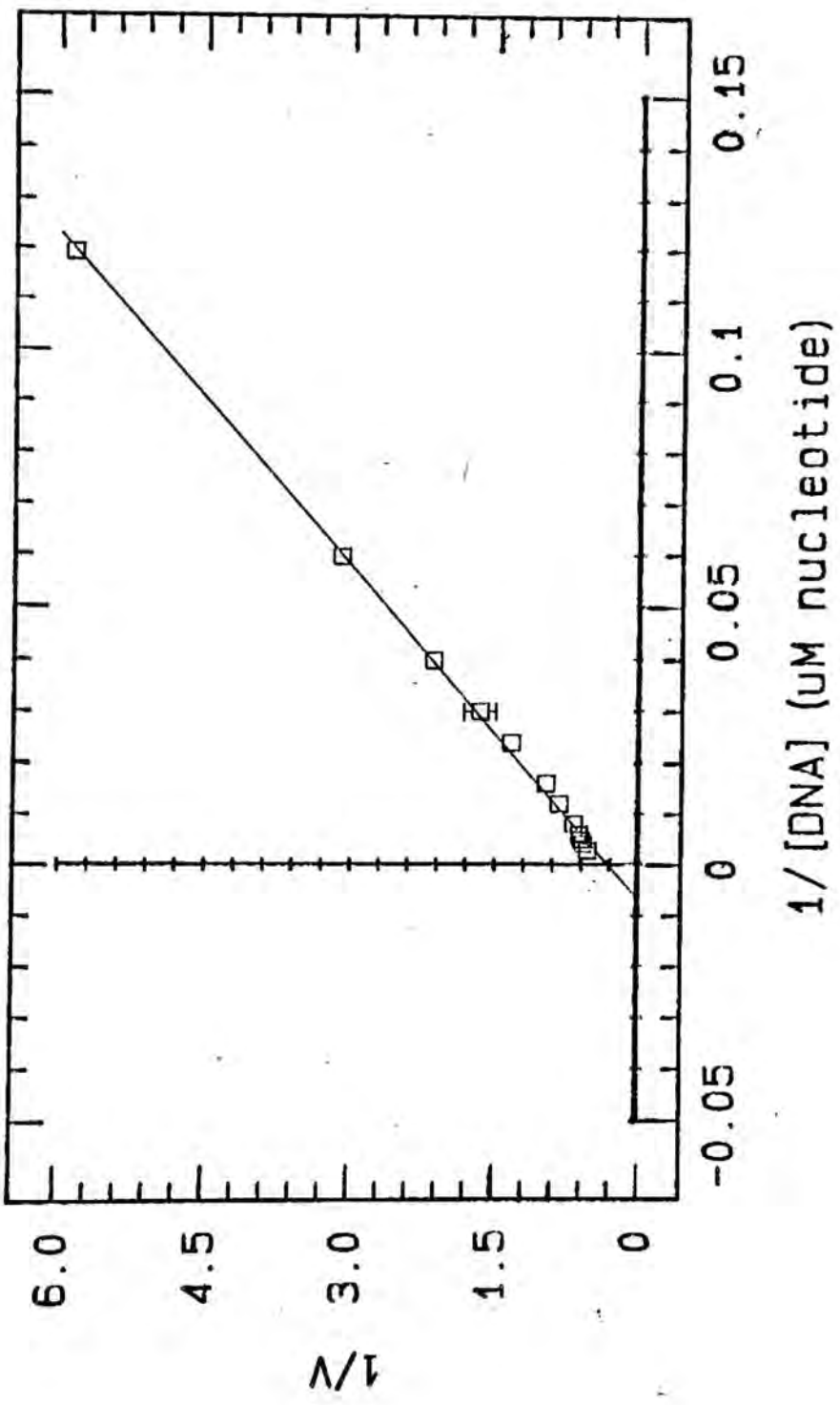

1/V
6.0
4.5
3.0
1.5
0
-0.05
0
0.05
0.1
0.15
1/ [DNA] (uM nucleotide)

TABLE XIV

APPARENT MICHAELIS CONSTANTS FOR THE HSV DNA POLYMERASES

Substrate	Strain of HSV DNA Polymerase			
	$\bar{m}$P	KOS	tsC4	tsC7
Activated DNA[1]	500	105	77	95
dATP[2]	2.4	2.1	14.3	11.1
dCTP[2]	2.4	3.4	11.1	10.0
dGTP[2]	4.3	2.6	12.5	14.3
TTP[2]	2.1	1.9	14.2	11.1

[1]Results expressed as μM nucleotide.

[2]Results expressed as μM.

NOTE: The incubation temperature of the DNA polymerase reactions to determine these K_m values was 35°C.

out on partially purified HSV DNA polymerases as well as on crude extracts of cells infected with these viruses.

V. E. 1. Partially Purified HSV DNA Polymerases

Thermal inactivation studies were performed at 34°, 40°, and 45°C., as described in section IV.H. The results of triplicate assays were plotted as the log of the percent activity remaining versus time, and are shown in Figure 17. In contrast to the expected result, these experiments indicate that the HSV DNA polymerase derived from HSV-1 tsC4 infected cells is actually less susceptible to thermal inactivation than either tsC7 or KOS HSV DNA polymerase at 34°C (Figure 17A), 40°C (Figure 17B), and 45°C (Figure 17C), although all are extremely heat sensitive under the conditions used in this study, for instance losing greater than 60% of their activities by three minutes at 40°C.

The thermolability of even the HSV KOS DNA polymerase would suggest the need for some stabilizing agent <u>in vivo</u>. The most obvious stabilizing agent is DNA. Also, it is possible that the temperature sensitivity seen with the mutant viruses is due to the temperature sensitivity of the interaction of the mutant DNA polymerases with DNA thereby losing any stabilizing effects at the non-permissive temperature. Further, interaction of the mutant DNA polymerases with DNA could make these enzymes even more thermolabile. To examine these possibilities, the thermal inactivation experiments were repeated in the presence of activated DNA. At all three temperatures, DNA stabilized all of the HSV DNA polymerases, with, for instance, less than 10% loss in activity by three minutes at 40°C (Figure 18). The HSV tsC4 and

Figure 17. Thermal Inactivation Plots - 34°, 40°, 45°C - Purified DNA Polymerases.

The thermal inactivation assays were carried out as described in the text. The rate of thermal inactivation of purified HSV KOS (-□-), tsC4 (-o-), and tsC7 (-Δ-) DNA polymerases were determined at 34° (panel A), 40° (panel B), and 45°C (panel C).

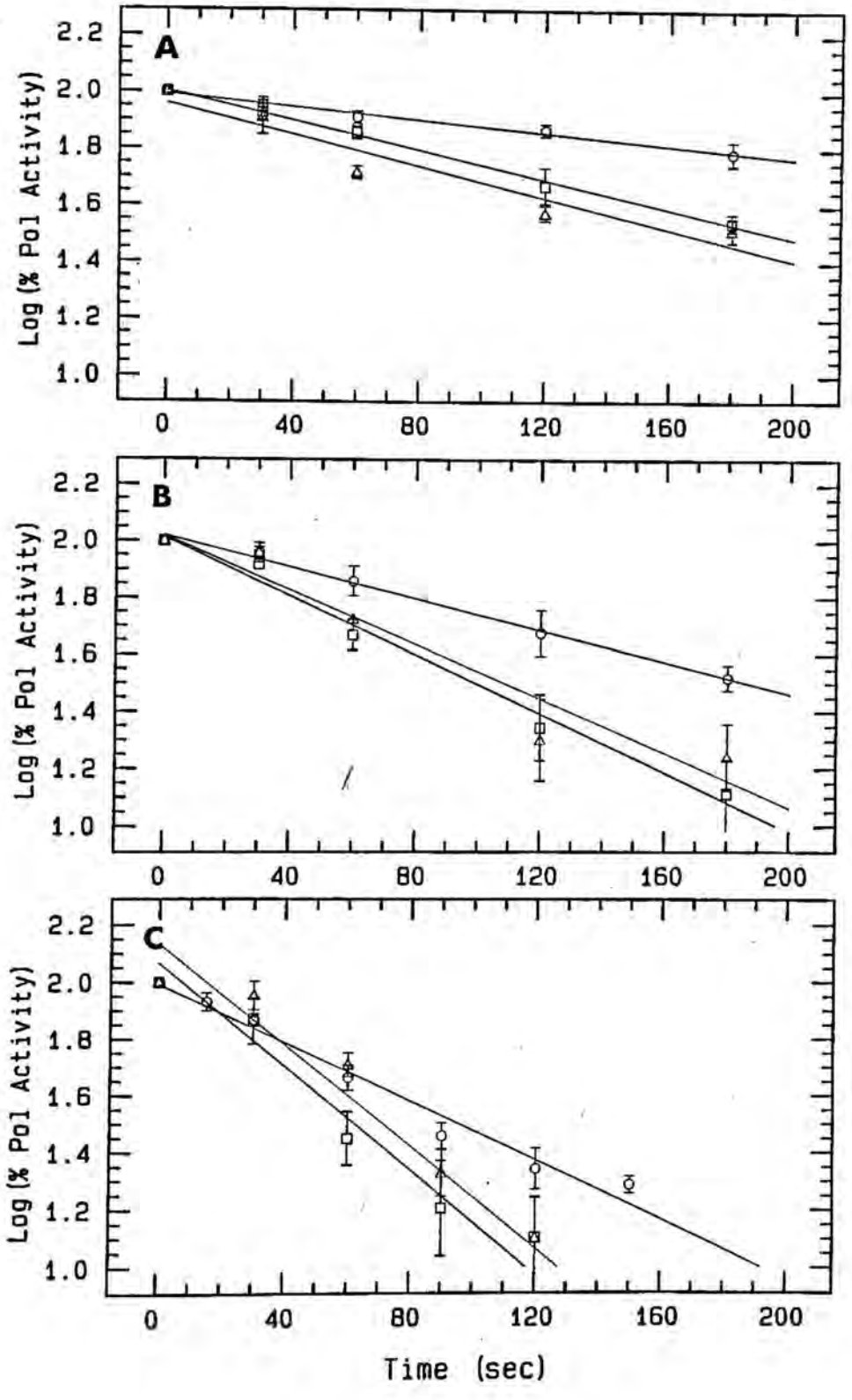
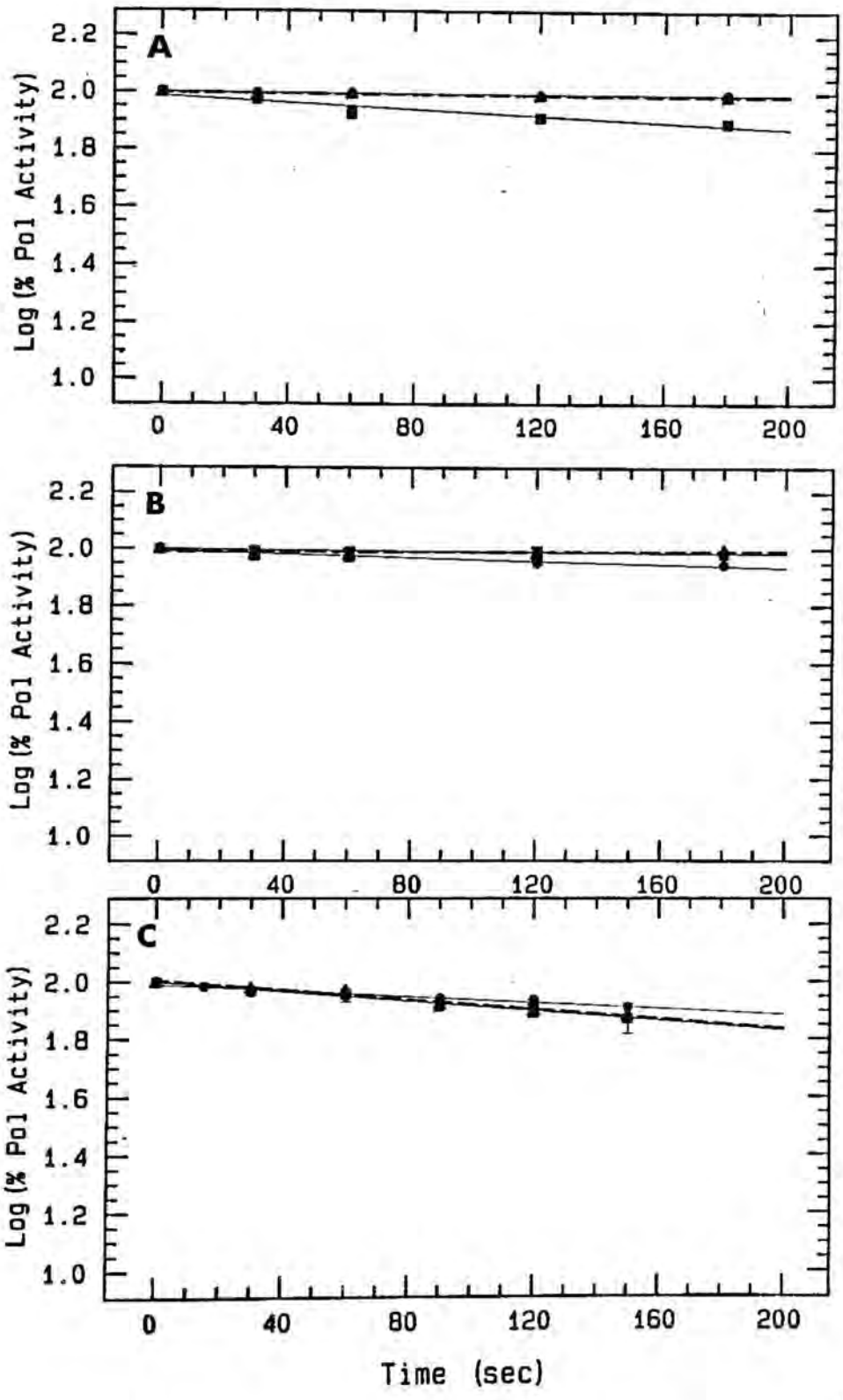

A
B
C
Log (% Pol Activity)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
40
80
120
160
200
Time (sec)

Figure 18. Thermal Inactivation Plots - 34°, 40°, 45°C - Purified DNA Polymerases in the presence of AcDNA.

The thermal inactivation assays were carried out as described in the text. The rate of thermal inactivation of purified HSV KOS (-■-), tsC4 (-●-), and tsC7 (-▲-) DNA polymerases were determined in the presence of activated DNA at 34° (panel A), 40° (panel B), and 45°C (panel C).


A
B
C
Log (% Pol Activity)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
40
80
120
160
200
Time (sec)

tsC7 DNA polymerases are no more temperature sensitive than the HSV KOS DNA polymerase. This suggests that the interaction with DNA is not temperature sensitive, since differential heat inactivation would have been observed at 40° and 45°C. Also, interaction of the DNA did not confer increased temperature sensitivity on the mutant DNA polymerases relative to the wild-type enzyme.

In addition, each of the purified HSV DNA polymerases were assayed at 34° and 40°C, and in each case there was a 5-10% increase in the amount of polymerase activity observed at 40°C when compared to the amount obtained at 34° (data not shown). This also suggests that the HSV DNA polymerases are stabilized by the presence of activated DNA and that the HSV DNA polymerases are not thermolabile.

V. E. 2. Crude Infected Cell Extracts

Since the previous experiments show that the temperature sensitive phenotype observed in the mutant viruses is probably not due to differential thermal stability of the partially purified DNA polymerases, thermal inactivation experiments were repeated using infected cell extracts in place of the partially purified enzymes. These experiments were designed to test the possibility that other proteins could interact with the DNA polymerase and affect the thermal stability of the enzyme. Although it was not possible to purify the HSV DNA polymerase from cells infected with HSV-1 tsD9, it was possible to prepare extracts from cells infected with this virus.

At all temperatures studied, the HSV KOS, tsC4, and tsC7 DNA polymerases were less thermolabile than the partially purified enzymes

(Figures 19-21). At 34°C, there was no difference observed between the KOS and tsC4 HSV DNA polymerases, however, the HSV DNA polymerase activity present in HSV-1 tsC7 and tsD9 infected cell extracts appeared to be slightly more thermolabile than the other two polymerase activites (Figure 19). This was contrasted by the results obtained at 40°C (Figure 20). Although all of the HSV DNA polymerases are more thermolabile at 40°C than at 34°C, there were no differences in the rates of thermal inactivation at this temperature. A difference was noted for the thermal inactivation at 45°C (Figure 21). These results indicate that the tsC4 and tsC7 HSV DNA polymerases are no different (within the error limits presented) than the KOS HSV DNA polymerase. This, however, may not be the case with tsD9 HSV DNA polymerase. This particular enzyme appears to be slightly more thermolabile than the KOS HSV DNA polymerase at 34° and 45°C in crude extracts. Although the HSV DNA polymerase mutants are found in two complementation groups, only the HSV DNA polymerase from complementation group D (tsD9) appears to be temperature sensitive. This may suggest that there is another activity of the HSV DNA polymerase that is temperature sensitive. The results of these studies do not show a clear correlation between the temperature sensitive phenotype of the mutant viruses and the HSV DNA polymerase activity.

V. F. Gradient Analysis of Nuclear Extracts

Based on the results of the preceeding section, it is possible that the temperature sensitive phenotype of HSV-1 tsC4 and tsC7 is due to a temperature sensitive interaction of the HSV DNA polymerase with

Figure 19. Thermal Inactivation Plots - 34°C - Crude Infected Cell Extracts.

The thermal inactivation assays were carried out as described in the text. The rate of thermal inactivation at 34°C of the HSV KOS (-■-) DNA polymerase activity was compared to the rate of thermal inactivation of HSV tsC4 (-●-) (panel A), tsC7 (-▲-) (panel B), and tsD9 (-□-) (panel C) DNA polymerase activities.

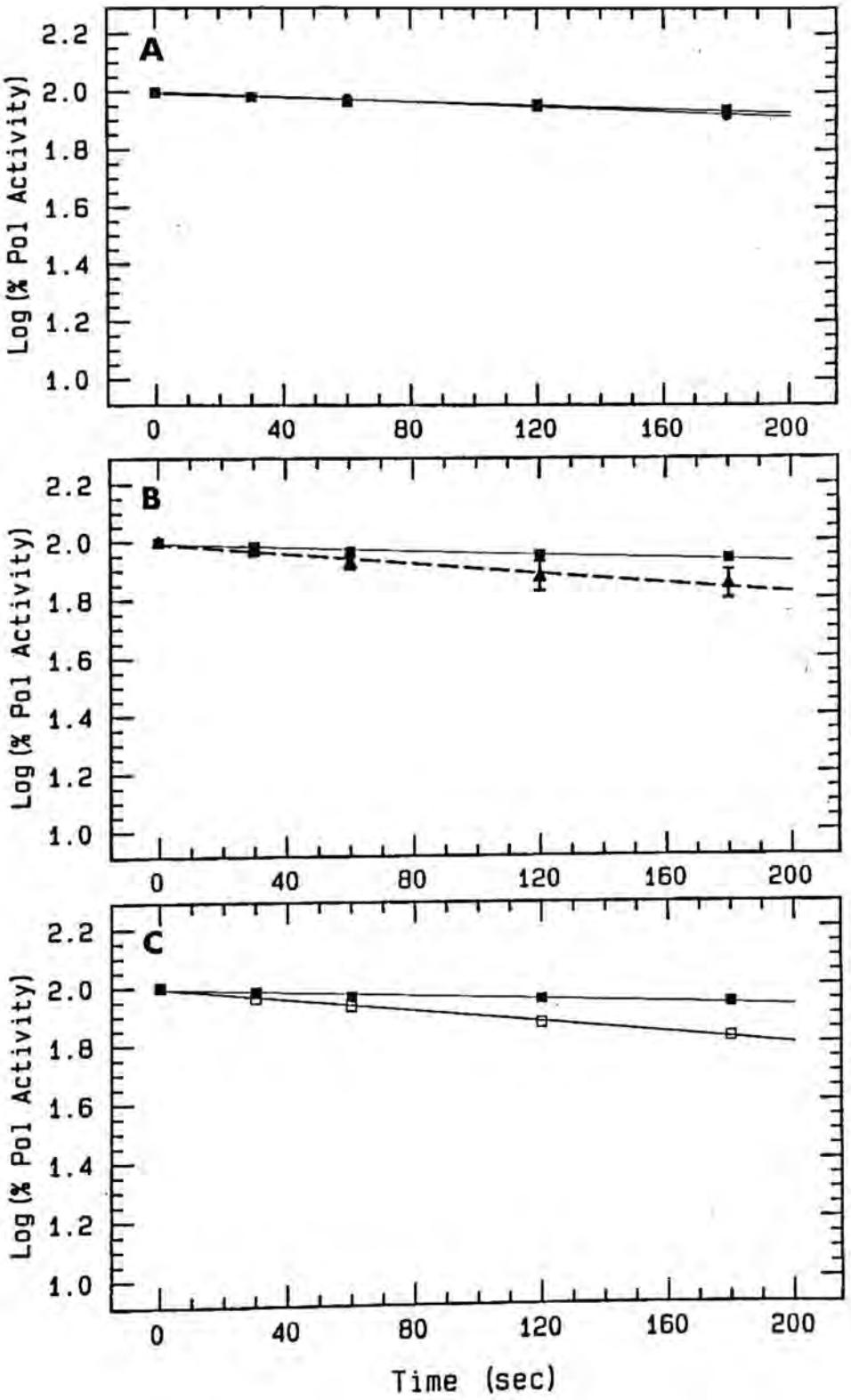

A
B
C
Log (% Pol Activity)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
40
80
120
160
200
Time (sec)

Figure 20. Thermal Inactivation Plots - 40°C - Crude Infected Cell Extracts.

The thermal inactivation assays were carried out as described in the text. The rate of thermal inactivation at 40°C of the HSV KOS (-■-) DNA polymerase activity was compared to the rate of thermal inactivation of HSV tsC4 (-●-) (panel A), tsC7 (-▲-) (panel B), and tsD9 (-□-) (panel C) DNA polymerase activities.

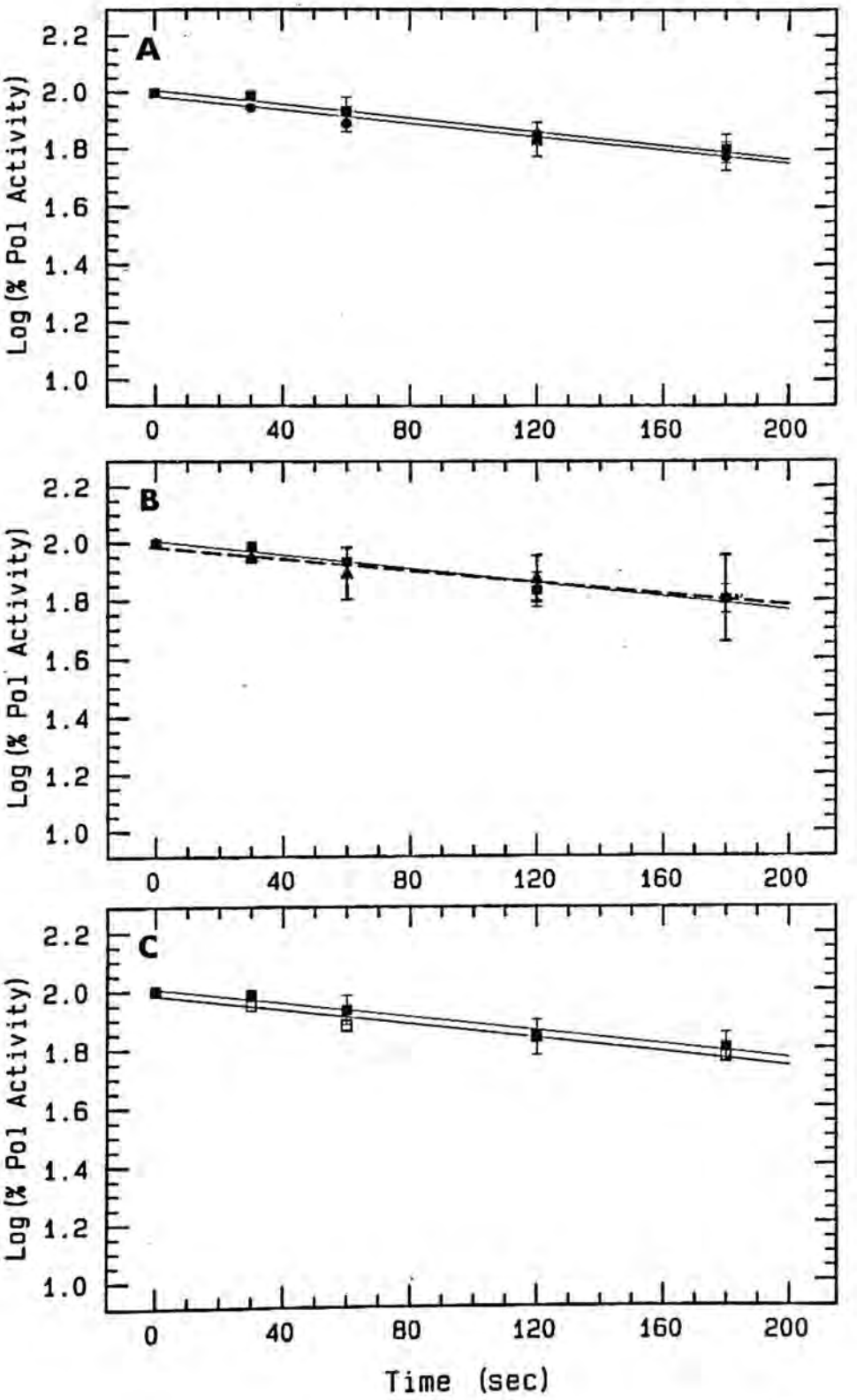
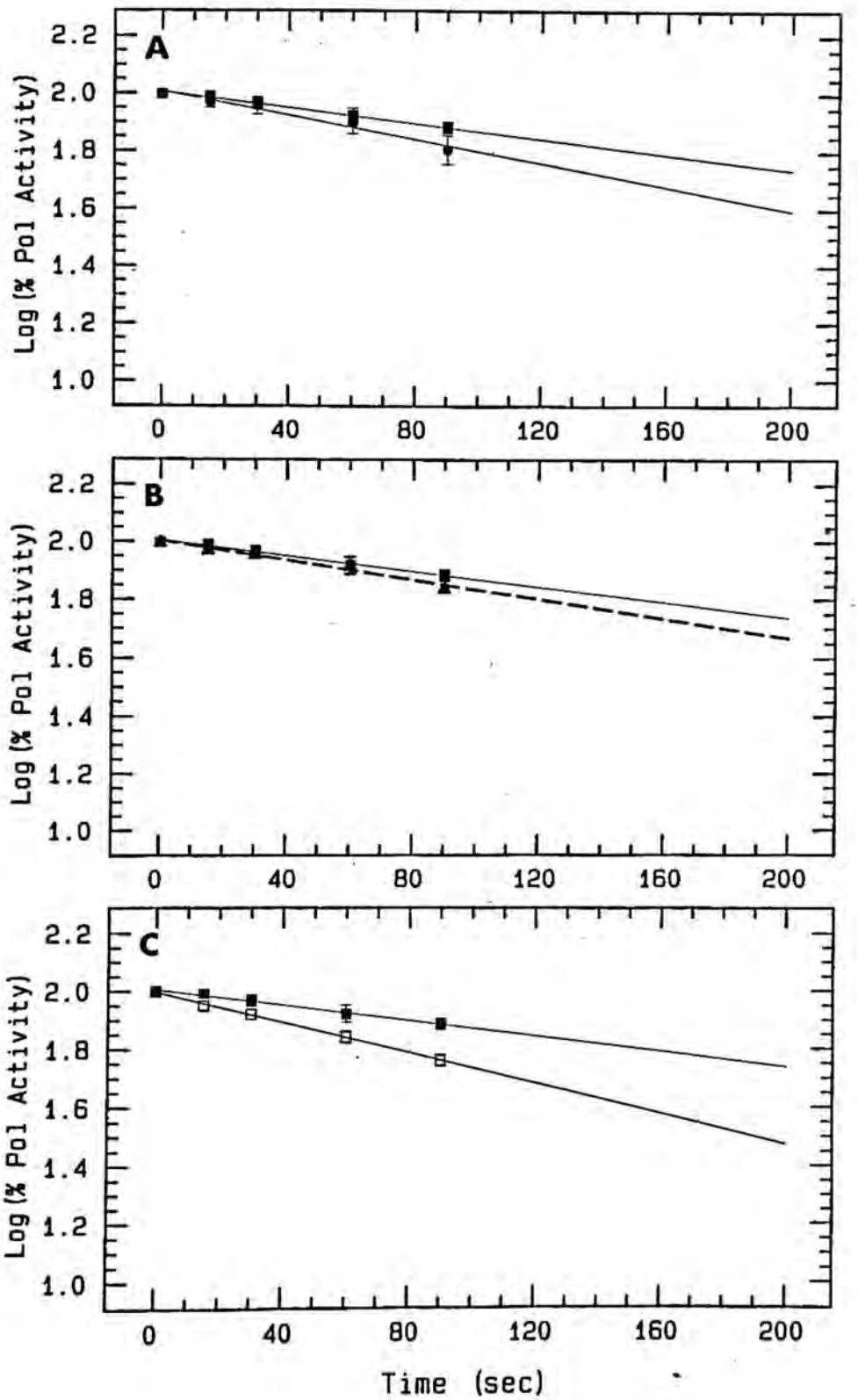

A
B
C
Log (% Pol Activity)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
40
80
120
160
200
Time (sec)

Figure 21. Thermal Inactivation Plots - 45°C - Crude Infected Cells Extracts.

The thermal inactivation assays were carried out as described in the text. The rate of thermal inactivation at 45°C of the HSV KOS (-■-) DNA polymerase activity was compared to the rate of thermal inactivation of HSV tsC4 (-●-) (panel A), tsC7 (-▲-) (panel B), and tsD9 (-□-) (panel C) DNA polymerase activities.


A
B
C
Log (% Pol Activity)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
40
80
120
160
200
Time (sec)

some other component necessary for HSV DNA replication. In some eukaryotic systems, a polymerase: primase complex can be isolated [reviewed by Campbell, 1986], and in prokaryotic systems, such as the T4 bacteriophage, the DNA polymerase can be isolated in a complex with other replication proteins [Nossal & Alberts, 1983]. By analogy with these systems, it may possible that the HSV DNA polymerase participates in a replication complex. For example, recently an unique DNA primase activity has been found in HSV-1 infected cells associated with the HSV DNA polymerase [Holmes et al., 1988]. To examine the possibility that the HSV DNA polymerase does interact with other proteins and that this interaction is thermosensitive in the temperature sensitive mutants, extracts from infected cell nuclei were analyzed by velocity sedimentation on neutral glycerol gradients (see section IV.I.) with and without prior heating of the extracts. In addition, extracts of mock infected cell nuclei were analyzed.

In the initial experiments, the gradients were fractionated and the resulting fractions analyzed for high and low salt DNA polymerase activity, high salt primase activity (section IV.E.2.a.), and indirect primase activity (section IV.E.2.b). Heat denatured templates can snap-back onto themselves, resulting in self priming in DNA polymerase reactions. Therefore, these reactions had to be carried out in the presence and absence of the four rNTPs, and the results presented as the difference of the two assays. This does give some indication of the amount of primase activity in each fraction assayed, however, these assays also present problems in that there may be significant artifacts influencing the results (such as subtraction errors yielding less than accurate results). For this reason, the experiment was repeated, but

the DNA primase assays were altered. DNA primase was assayed in high and low salt, using poly(dT) as template (see section IV.E.2.b.) (data not shown). The indirect DNA primase assay procedure was also carried out.

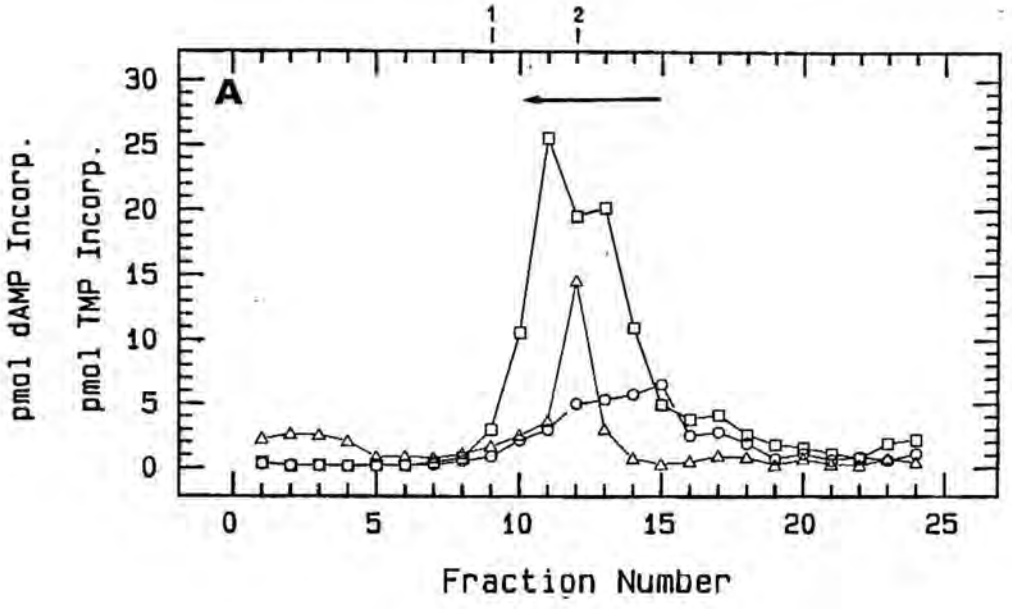
Figure 22 presents the data obtained from the KOS infected nuclear extracts. In the native extract, the major peak of high salt DNA polymerase activity appeared in fraction 11 with a shoulder of high salt activity appearing in fraction 13 (Figure 22A). The shoulder may be due to the presence of DNA polymerase β in these fractions as this was the position of sedimentation of DNA polymerase β activity in the mock infected cells (Figure 23A). This activity was resistant to NEM. The HSV DNA polymerase activity sedimented at 7.5 S. A significant amount of DNA primase is detected under the HSV KOS DNA polymerase peak when analyzed using the indirect primase assay procedure (Figure 22A).

Upon heat treating the extract prior to the gradient centrifugation, several changes are noted in the sedimentation profile: 1) a 50% reduction in the amount of HSV DNA polymerase activity recovered from these gradients, 2) the loss of most of the DNA primase activity, and 3) a shift in the major peak of high salt DNA polymerase activity to a species which sedimented at a slightly slower rate (Figure 22B). Similar results were obtained with nuclear preparations from HSV-1 tsC4 infected cells (Figure 24A & 24B).

Several additional differences were noted in the results obtained from HSV-1 tsC7 infected cells (Figure 25A & 25B): 1) much less tsC7 HSV DNA polymerase activity was detected in gradient fractions, 2) less DNA primase activity was detected, and 3) more of the HSV DNA polymerase activity was lost upon heating the extract. Although these

Figure 22. Glycerol Gradient Analyses - KOS Infected Extracts.

Velocity sedimentation profile of HSV KOS nuclear extracts on 12.5-30% glycerol gradients. High salt DNA polymerase activity (-□-), low salt DNA polymerase activity (-o-), and DNA primase determined via the indirect primase assay (-Δ-) are shown. Panel: A, untreated infected nuclear extract (216 high salt DNA polymerase units loaded, 90% recovered from the gradient); B, heat treated infected nuclear extracts (99 high salt DNA polymerase units loaded, 86% recovered from the gradient). Marker 1 indicates the sedimentation position of rabbit IgG and marker 2 indicates the sedimemtation position of E. coli DNA polymerase I.


1
2
A
30
25
20
15
10
5
0
0
5
10
15
20
25
pmol dAMP Incorp.
pmol TMP Incorp.
Fraction Number

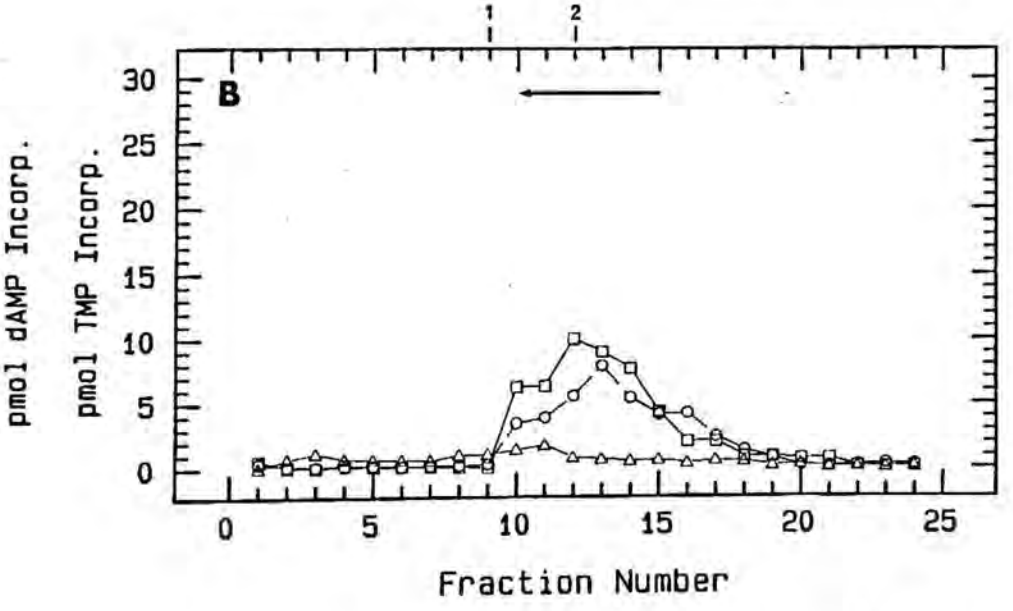
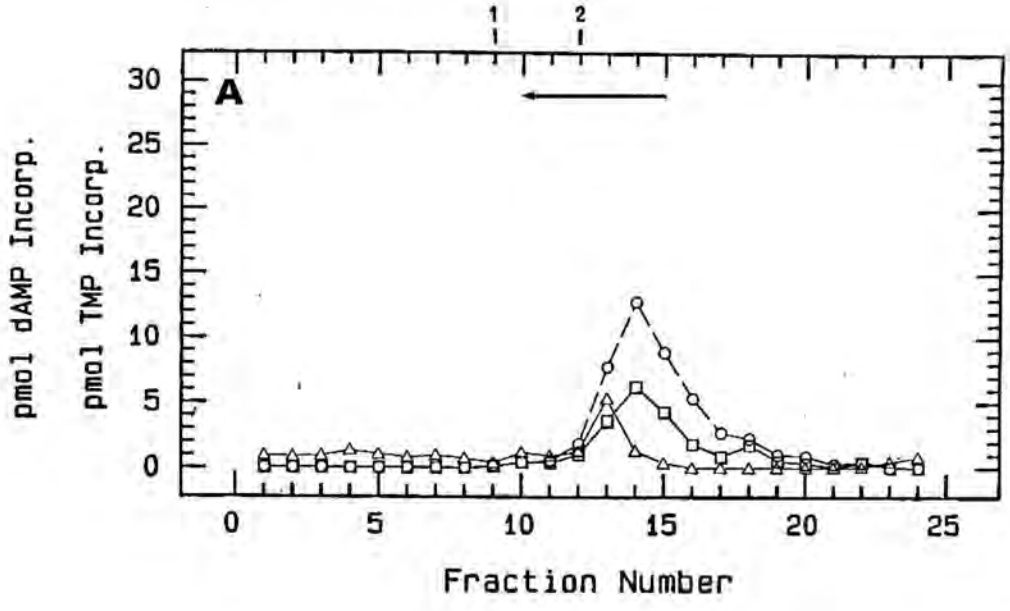

1
2
B
30
25
20
15
10
5
0
0
5
10
15
20
25
pmol dAMP Incorp.
pmol TMP Incorp.
Fraction Number

Figure 23. Glycerol Gradient Analyses - Mock Infected Extracts.

Velocity sedimentation profile of mock infected nuclear extracts on 12.5-30% glycerol gradients. High salt DNA polymerase activity (-□-), low salt DNA polymerase activity (-○-), and DNA primase determined via the indirect primase assay (-Δ-) are shown. Panel: A, untreated infected nuclear extract (38 high salt DNA polymerase units loaded, 96% recovered from the gradient); B, heat treated infected nuclear extracts (26 high salt DNA polymerase units loaded, 93% recovered from the gradient). Marker 1 indicates the sedimentation position of rabbit IgG and marker 2 indicates the sedimemtation position of E. coli DNA polymerase I.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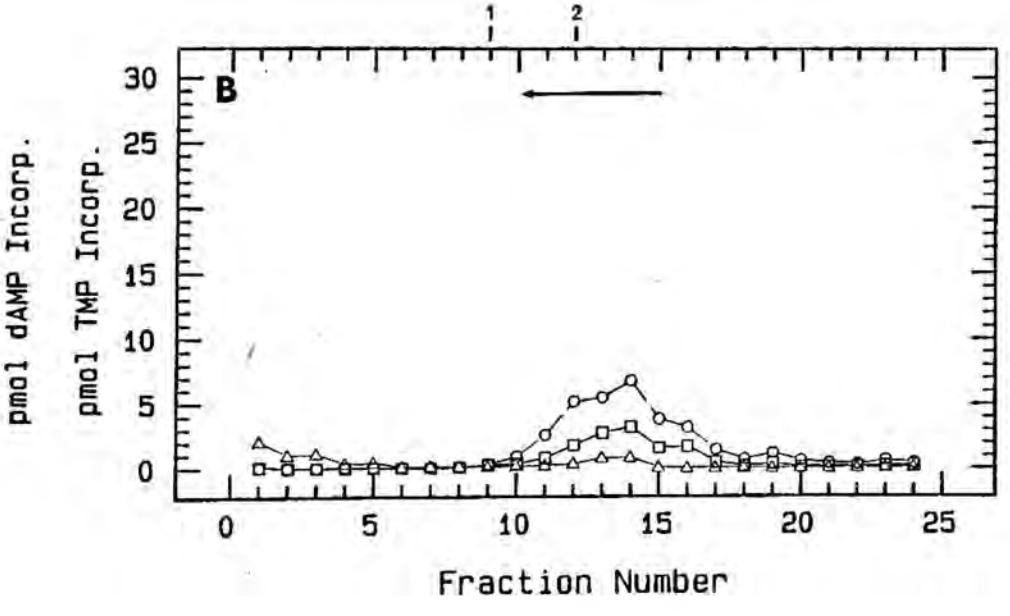
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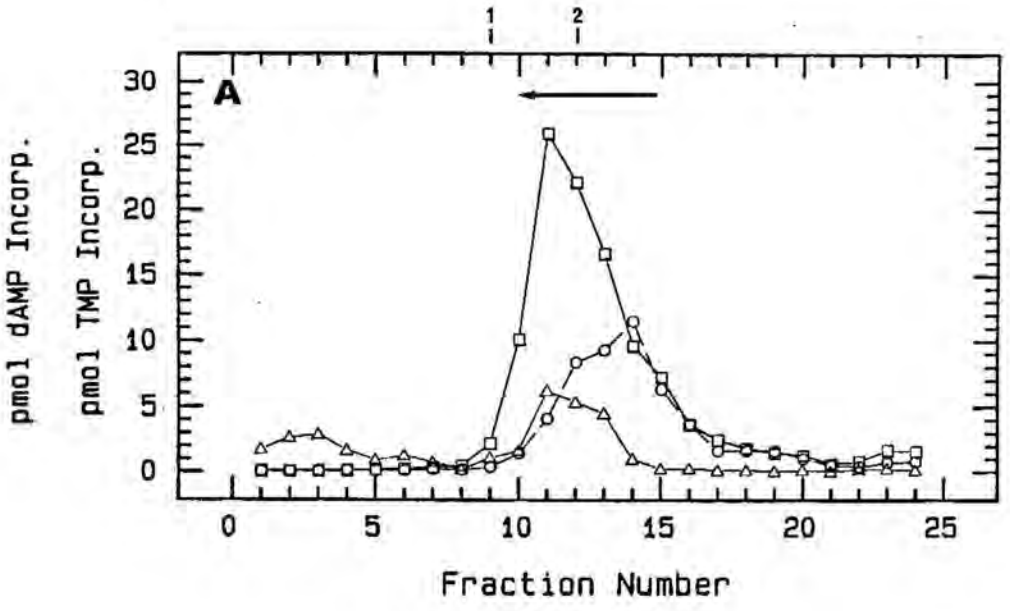
Figure 24. Glycerol Gradient Analyses - tsC4 Infected Extracts.

Velocity sedimentation profile of HSV tsC4 nuclear extracts on 12.5-30% glycerol gradients. High salt DNA polymerase activity (-□-), low salt DNA polymerase activity (-o-), and DNA primase determined via the indirect primase assay (-Δ-) are shown. Panel: A, untreated infected nuclear extract (202 high salt DNA polymerase units loaded, 91% recovered from the gradient); B, heat treated infected nuclear extracts (82 high salt DNA polymerase units loaded, 91% recovered from the gradient). Marker 1 indicates the sedimentation position of rabbit IgG and marker 2 indicates the sedimemtation position of E. coli DNA polymerase I.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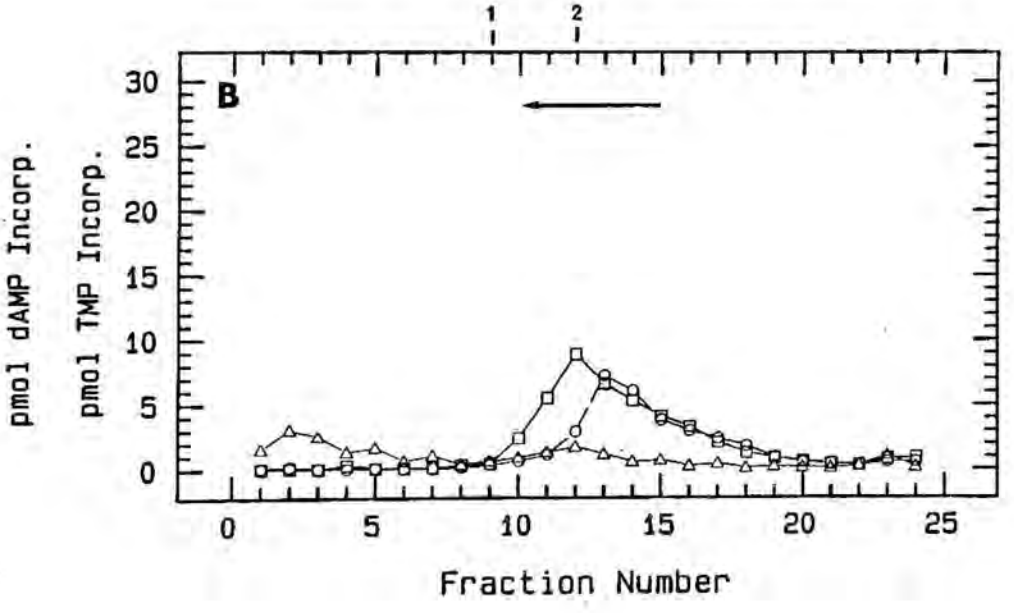
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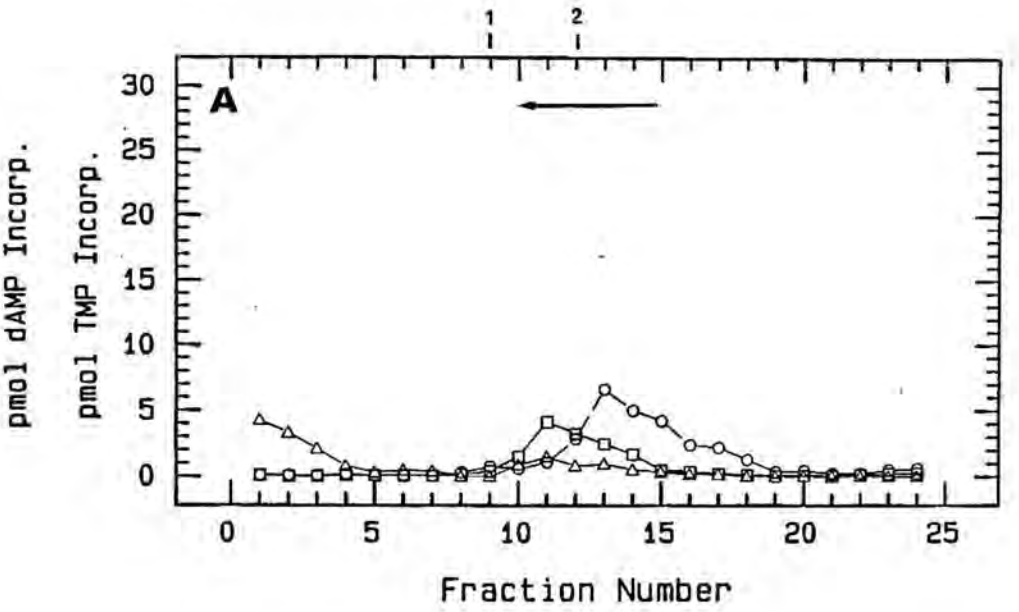
Figure 25. Glycerol Gradient Analyses - tsC7 Infected Extracts.

Velocity sedimentation profile of HSV tsC7 nuclear extracts on 12.5-30% glycerol gradients. High salt DNA polymerase activity (-□-), low salt DNA polymerase activity (-o-), and DNA primase determined via the indirect primase assay (-Δ-) are shown. Panel: A, untreated infected nuclear extract (29 high salt DNA polymerase units loaded, 94% recovered from the gradient); B, heat treated infected nuclear extracts (21 high salt DNA polymerase units loaded, 95% recovered from the gradient). Marker 1 indicates the sedimentation position of rabbit IgG and marker 2 indicates the sedimemtation position of E. coli DNA polymerase I.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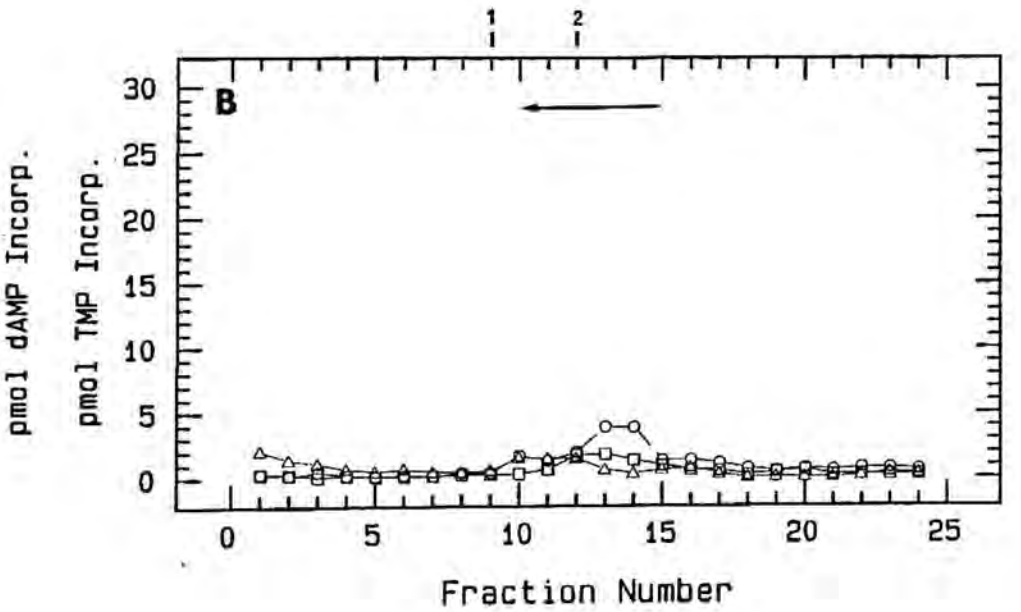
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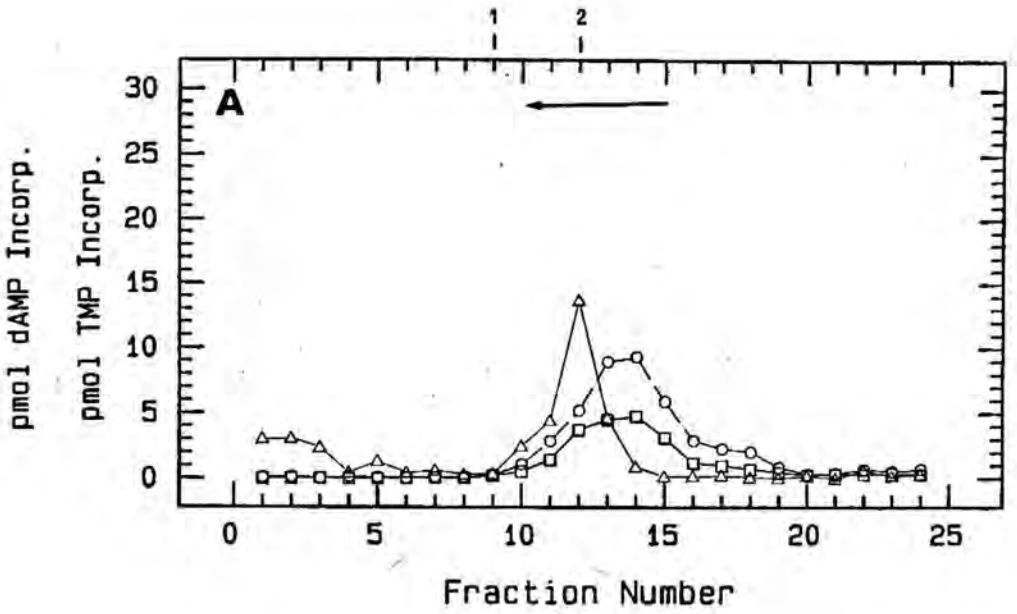
Figure 26. Glycerol Gradient Analyses - tsD9 Infected Extracts.

Velocity sedimentation profile of HSV tsD9 nuclear extracts on 12.5-30% glycerol gradients. High salt DNA polymerase activity (-□-), low salt DNA polymerase activity (-o-), and DNA primase determined via the indirect primase assay (-Δ-) are shown. Panel: A, untreated infected nuclear extract (42 high salt DNA polymerase units loaded, 95% recovered from the gradient); B, heat treated infected nuclear extracts (20 high salt DNA polymerase units loaded, 95% recovered from the gradient). Marker 1 indicates the sedimentation position of rabbit IgG and marker 2 indicates the sedimemtation position of E. coli DNA polymerase I.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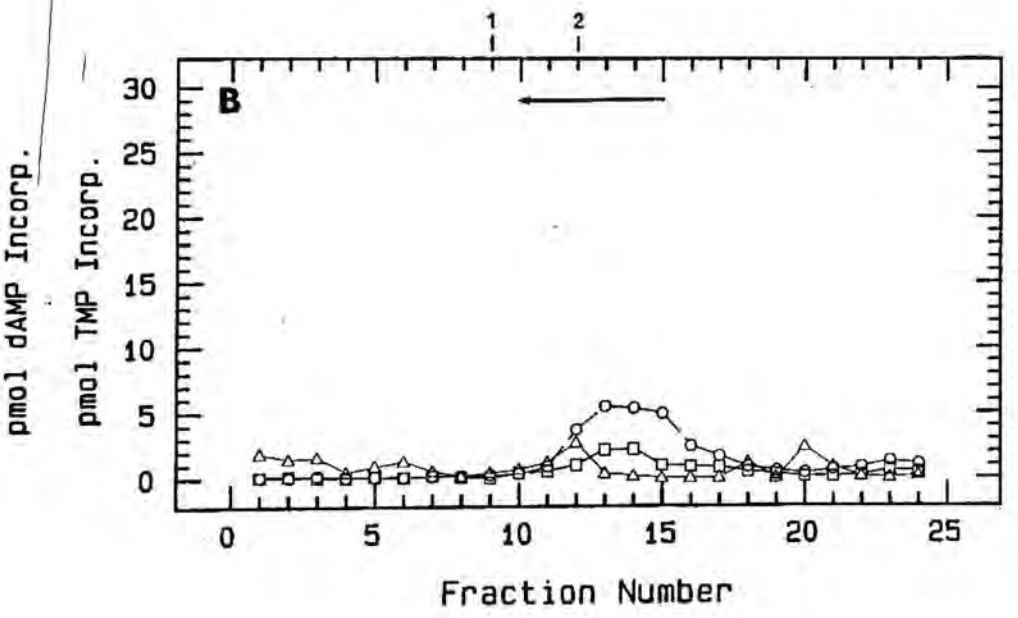
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changes were observed for HSV-1 tsC7 infected cells, one similarity to the other sedimentation profiles was observed. This was the shift in the sedimentation position of the high salt DNA polymerase activity after heating the nuclear extract.

Although the HSV tsD9 DNA polymerase in the untreated extracts sediments at 6 S, the change in the sedimentation position of the high salt DNA polymerase activity upon heating was also noted (Figure 26). The smaller S value noted for the HSV tsD9 DNA polymerase may be due to proteolytic degradation of the DNA polymerase, or may be due to a change in the conformation of the DNA polymerase resulting in a more asymmetrical molecule hence a change in the S value. Other investigators have shown that herpesvirus DNA polymerases isolated from other species are asymmetrical [Allen et al, 1977].

While the amount of high salt DNA polymerase activity was less in the HSV-1 tsD9 extracts, the amount of primase activity detected by the indirect primase assay was similar to the amounts that were obtained from KOS and tsC4 infected extracts. For HSV-1 KOS, tsC4, and tsD9 extracts, the amount of primase activity recovered was 2-3 times the amount obtained from the mock infected nuclear extracts (Figure 23). Although the mock infected extracts did show decreases in the amounts of primase and polymerase activities upon heating of the extracts, there was no change observed in the sedimentation position of the high salt DNA polymerase activity.

In order to determine if the increases in DNA primase activity observed in the infected nuclear extracts were due to increases in the amounts of host DNA primase or were due to the induction of a HSV coded DNA primase, antibody binding experiments were performed using anti-

calf DNA primase antibodies as described by Holmes et al. [1988]. HSV-1 KOS, and mock infected nuclear extracts were tested in this study as was HeLa DNA polymerase:primase, which was used as a control to determine the extent of DNA primase removed from solution. Duplicate samples, containing the same amount of DNA primase activity, were used. 70% of the HeLa DNA primase was removed from the solution, 40% of the DNA primase was removed from the mock infected nuclear extract, and only 25% of the DNA primase in the HSV-1 KOS infected nuclear extract was removed from solution. These results indicate that, although cross-reactivity of these antibodies to HeLa and Vero DNA primases are not identical, some of the DNA primase present in the infected nuclear extract is not Vero DNA primase.

The results of these experiments indicate that the HSV DNA polymerase could be: 1) participating in a protein:protein complex that is disrupted upon heating or 2) changing conformation upon heating resulting in a more asymmetrical DNA polymerase molecule. This is supported by the fact that the HSV DNA polymerase appears to shift to a slower sedimenting species when the crude extracts are heated prior to separation on glycerol gradients.

VI. DISCUSSION

The purpose of this study was to investigate the properties of HSV DNA polymerases purified from cells infected with HSV-1 wild-type strains $\bar{m}$P and KOS and the temperature sensitive mutants tsC4, tsC7, and tsD9 derived from HSV-1 KOS. The original characterization of HSV-1 tsC4, tsC7, and tsD9 by Coen et al. [1975] showed that these viruses were temperature sensitive in vivo based on virus titer assays. The results in Table VIII showed that the two wild-type viruses grew equally well at the permissive temperature of 34° and the non-permissive temperature of 39°C. In contrast, the mutant viruses replicated well at 34°C (titers of $\geq 10^{10}$ pfu/mL), but yielded low titers ($<10^{3}$ pfu/mL) at 39°C. Subsequent mapping of the temperature sensitive lesions in these viruses indicated that the mutations resulting in the temperature sensitive phenotype mapped within the HSV DNA polymerase gene. The question then remained as to whether the properties of the DNA polymerases encoded by the mutant viruses were significantly altered from those exhibited by the polymerase encoded by the parental and/or other wild-type strains.

In order to purify the HSV DNA polymerase from cells infected with the above strains of viruses, it was necessary to determine the time at which maximum DNA polymerase activity could be obtained under conditions of infection used in this study. Infected cells were harvested at various times after infections and were assayed for HSV DNA polymerase activity. Maximum HSV DNA polymerase activity was observed 8-12 hours post infection for cells infected with strains KOS and tsC4 (Figures 3A & 3B). These results agreed with other investigators who

have shown that the HSV DNA polymerase and other proteins involved in DNA metabolism are β proteins with maximum activity being detected from 7-10 hours after infection [Roizman & Batterson, 1985; Wagner 1985]. It was also noted that the HSV-1 KOS infected cell extracts had slightly more HSV DNA polymerase activity than did HSV-1 tsC4 cell extracts. The other mutant viruses, tsC7 and tsD9, both had maximal HSV DNA polymerase activity 18-20 hours post infection, but with noticeably less HSV DNA polymerase activity being evident in HSV-1 tsD9 than the other viruses (Figures 4A & 4B). tsC7 infected cells yielded approximately the same amount of DNA polymerase activity as tsC4 infected cells. In addition to determining the HSV DNA polymerase activity, DNA replication was also measured by the incorporation of [^{3}H]thymidine into DNA in the cell. It was found that increases in thymidine incorporation paralleled the increases in HSV DNA polymerase activity. The results of these experiments gave the optimal times for harvesting infected cells to yield the maximum amounts of HSV DNA polymerase activity.

The first scheme developed for the purification of the HSV DNA polymerase was based on the procedure described by Ruyechan & Weir [1984]. This method involved preparation of a high salt extract of HSV-1 $\bar{m}$P infected cells followed by precipitation of the nucleic acids with PEG. The resulting extract was dialyzed into a low salt buffer and chromatographed using ssDNA agarose. The fractions containing the salt activated HSV DNA polymerase were pooled, redialyzed and chromatographed on PC11. The resulting HSV DNA polymerase was once again redialyzed and chromatographed on a final ssDNA agarose column. A typical preparation of DNA polymerase had a specific activity of 2526 un/mg (Table IX), and showed multiple bands in SDS PAGE analysis of the prod-

uct with little of the 148 kDa HSV DNA polymerase peptide present (Figure 5). The specific activity of this preparation was at least five fold lower than that obtained by other investigators [Weissbach et al., 1973, Frank et al., 1984a, 1984b; O'Donnell et al, 1987a]. This purification method also required an extended period to complete. For these reasons, it was necessary to find a better purification method.

The second purification scheme was based on the procedures outlined by Allen et al. [1977] for the purification of the equine herpesvirus DNA polymerase. To compare the results of this purification scheme with the ones used previously in this laboratory, HSV-1 $\overline{m}$P infected HeLa cells were again used. This scheme involved preparation of infected cell extracts in a high ionic strength phosphate buffer, then removal of the nucleic acids by passing the extract through a DEAE-T-M column equilibrated with extraction buffer. The HSV DNA polymerase in the resulting crude extract was purified by sequential chromatography on DEAE-T-M, ssDNA agarose, PC11 and HAP columns. The HSV DNA polymerase from the HAP step had a specific activity of 35937 un/mg (Table IX). This was as good or better than the specific activity reported for HSV DNA polymerase by other investigators [Weissbach et al., 1973, Frank et al., 1984a, 1984b; O'Donnell et al, 1987a], however, the total recovery of HSV DNA polymerase using this method was low (1.8%). The purity of the enzyme was significantly better than that obtained using purification procedure I. SDS PAGE analysis indicated the presence of a major band at 148 kDa (Figure 5). Although this procedure yielded HSV DNA polymerase of relatively high specific activity, the low yield dictated the need to develop another purification scheme which would result in pure HSV DNA polymerase with a reasonable quantity of enzyme.

For this reason, purification procedure III was developed. This scheme employed the same buffers, extraction procedures, and two DEAE-T-M columns as purification scheme II. The HSV $\bar{m}$P DNA polymerase from the second DEAE-T-M column was adsorbed onto a ssDNA agarose column and eluted with a linear KCl gradient. Depending upon the purity of the HSV DNA polymerase at this stage as judged by SDS PAGE, additional purification steps were used. These steps included DEAE-T-M, PC11, and ssDNA columns in various combinations. A typical preparation of HSV-1 $\bar{m}$P DNA polymerase obtained by this purification procedure yielded an enzyme with a specific activity of 14178 un/mg (Table IX), and was judged to be pure based on SDS PAGE analysis (Figures 5 & 6). Once again, the specific activity of this preparation was comparable to the specific activities obtained for HSV DNA polymerase by other investigators [Weissbach et al., 1973, Frank et al., 1984a, 1984b; O'Donnell et al, 1987a]. This scheme gave a reasonable yield (~20%) of the initial HSV DNA polymerase activity in the crude extract. Depending upon the steps employed, purification procedure III could be accomplished in a total of 4 to 7 days. This method yielded satisfactory results and was therefore used to purify HSV-1 KOS, tsC4, and tsC7 DNA polymerases from Vero cells. Vero cells were used since variable amounts of HSV DNA polymerase activity were obtained from HeLa cells infected with these viruses.

A purification of HSV KOS DNA polymerase was accomplished by three chromatography steps (2 DEAE-T-M columns and a ssDNA agarose column) which yielded pure DNA polymerase as judged by SDS PAGE (Figure 7), and a specific activity of 4446 un/mg (Table X). This was almost a four fold reduction in the specific activity as compared to that ob-

tained for HSV $\overline{m}$P DNA polymerase. This may be due to inactive HSV DNA polymerase peptide present in the final product, or could be due to an intrinsically less active DNA polymerase. HSV-1 tsC4 and tsC7 DNA polymerases were also purified using the same procedure. Additional chromatography steps were not performed, since the specific activities of these preparations were similar to the specific activity obtained for typical parental KOS strain preparations, and attempts at further purification resulted in precipitous loss of DNA polymerase activity. Unlike the HSV KOS DNA polymerase, these preparations showed multiple bands on SDS PAGE (Figure 7), although, the total HSV DNA polymerase yield was approximately the same. Some of the other bands present on these gels may be proteolytic breakdown products of the HSV DNA polymerase.

In addition, the results presented in Figure 7 indicate that the HSV KOS DNA polymerase has an apparent molecular weight of approximately 150 kDa, whereas the HSV KOS derived polymerases have apparent molecular weights of 135 kDa. The HSV DNA polymerase has been shown to have a theoretical molecular weight of 136 kDa based on the sequence of the DNA polymerase gene [Quinn & McGeoch, 1985]. This discrepancy in molecular weight may have a couple possible explanations: 1) the HSV $\overline{m}$P DNA polymerase polypeptide may have an anomalous electrophoretic mobility on SDS PAGE or 2) there is an insertion of peptide sequences into the HSV $\overline{m}$P DNA polymerase polypeptide.

Although the HSV DNA polymerases from tsC4 and tsC7 infected cells could be purified, the DNA polymerase from HSV-1 tsD9 infected cells was not purified. Attempts to purify this polymerase were undertaken, but with no success. The polymerase activity could not be re-

covered from the second DEAE-T-M column. This could be due to the fact that the mutation in the polymerase, resulting in a -2 charge change on the polymerase, may result in an unstable enzyme, or in an enzyme that is more susceptible to proteolytic degradation.

An observation which can also be made, by comparing the purification tables derived from these various purification schemes (Tables IX & X), is that the total activity present in the crude extracts is highly variable, and often increases in subsequent purification steps. This observation has also been made by other investigators [Weissbach et al., 1973; Powell & Purifoy, 1977; Knopf, 1979b] and could have several explanations: 1) factors present in the crude extracts which inhibit the HSV DNA polymerase, 2) the presence of variable amounts of nuclease in the crude extracts which masks some of the DNA polymerase activity, and 3) variability in the infection characteristics (eg. host cell state - replicating or growth arrested cells, infection temperature, MOI, incubation temperature, and the time the infected cells are harvested after infection). The specific activity of the crude extracts also varied, probably reflecting the variability of the total activity in the crude extracts, since the total amount of protein was nearly identical for each of the preparations and for mock infected cells.

In order to determine that the DNA polymerases isolated were in fact HSV DNA polymerases, several characteristics of the purified enzymes were examined. These included salt stimulation, pH optimum, PAA sensitivity, and divalent cation use.

A number of investigators have shown that wild-type HSV DNA polymerases are activated by high ionic strength conditions with the

optimal salt concentration of 100-150 mM for dianionic salts and 150-250 mM for monoanionic salts. Salts tested have included potassium sulfate, potassium phosphate, ammonium sulfate, ammonium chloride, potassium chloride and sodium chloride [Weissbach et al., 1973; Aron et al., 1975; Powell & Purifoy, 1977; Knopf, 1979b; Ostrander & Cheng, 1980]. In addition, Weissbach et al. [1973], and Powell & Purifoy [1977] also noted differences in the maximal DNA polymerase activity obtained using the various salts. The salt optimum for KCl of all of the purified enzymes were obtained (Figure 8). The results show that the maximum HSV DNA polymerase activity was obtained between 200 and 300 mM KCl, with little detectable activity below 100 mM KCl. The lack of activity at low ionic strengths can help to distinguish the HSV DNA polymerase from the host DNA polymerases; since DNA polymerase α has maximum activity in the absence of salts, DNA polymerase β has optimum activity at 75-100 mM KCl and the mitochondrial DNA polymerase γ is maximally stimulated by 100-150 mM KCl [Haines et al., 1971; Knopf et al., 1976; Fry, 1983].

The salt activation curves for all of the HSV induced enzymes were nearly identical between 0 and 300 mM KCl, differences in the curves were noted at and above 400 mM KCl. HSV-1 $\bar{m}$P and tsC7 DNA polymerases were more active at 400 mM KCl than was the HSV KOS DNA polymerase; however, the HSV tsC4 DNA polymerase was much less active than HSV KOS DNA polymerase at this ionic strength (Figure 8).

The pH optima of the various HSV DNA polymerases were determined using several buffer systems over a broad pH range. All of the enzymes demonstrated maximal activity over the pH range 7.5 to 8.5 using Tris or HEPES buffers. These results confirmed the results obtained by

Weissbach et al. [1973] for the characterization of the HSV DNA polymerase. Although the enzymes showed identical pH optima, the HSV-1 KOS derived DNA polymerases demonstrated differing levels of activity at the same pH using different buffer systems (Figure 9). This was not the case for HSV-1 $\bar{m}$P DNA polymerase where identical activities were obtained at the same pH for the different buffers used.

Divalent cations are essential cofactors for DNA polymerases, including the HSV DNA polymerase. The divalent metals used in this study were Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+} and Co^{2+}. The maximum HSV DNA polymerase activity for all of the enzymes was obtained with Mg^{2+} as the divalent cation, although there was some variation in the concentrations of Mg^{2+} needed for maximum activity: $\bar{m}$P, 4-8 mM; KOS, 8-16 mM; tsC4, 4-12 mM; and tsC7, 12-16 mM (Figures 10 & 11). Other investigators have found that the HSV DNA polymerase is maximally stimulated by concentrations of Mg^{2+} between 2 and 8 mM [Weissbach et al., 1973; Aron et al., 1975; Powell & Purifoy, 1977; Knopf, 1979b; Ostrander & Cheng, 1980; O'Donnell et al., 1987a]. It should be noted that these investigators used a variety of wild-type strain for determining salt and pH optima. Knopf [1979b] showed that Mn^{2+} could be used in place of Mg^{2+} as the divalent cation in the DNA polymerase reaction. Mn^{2+} has been shown here to be used by the HSV DNA polymerases over a very narrow concentration range, with differing levels of activity: $\bar{m}$P, 1 mM at 60% maximum Mg^{2+} activity; KOS, 1 mM at 70% maximum Mg^{2+} activity; tsC4, 1 mM at 10% maximum Mg^{2+} activity; and tsC7, 1 mM at 50% maximum Mg^{2+} activity (Figures 10 & 11). The other divalent cation showing activity in these DNA polymerase assays was Co^{2+}: $\bar{m}$P, 1 mM at 90% maximum Mg^{2+} activity; KOS, 2 mM at 20% maximum Mg^{2+} activity; tsC4, 2 mM at 40%

maximum Mg^{2+} activity; and tsC7, 2 mM at 30% maximum Mg^{2+} activity (Figures 10 & 11). No HSV DNA polymerase activity was detected when Zn^{2+} and Ca^{2+} were used in place of Mg^{2+} in the DNA polymerase reaction mixture. Reports in the literature indicate that the HSV DNA polymerase is inhibited Zn^{2+} ions [Fridlender et al., 1978; Knopf, 1979b].

In addition to the differences seen in divalent cation use by the parental strain HSV KOS DNA polymerase and the mutant DNA polymerases, there are differences between the two wild-type HSV DNA polymerases. This may indicate differences in the active sites of the enzymes.

The HSV DNA polymerases have an absolute requirement for buffer, Mg^{2+} and template. As was discussed, maximal DNA polymerase activity was seen at high ionic strengths. A significant difference was noted in the reaction requirements for HSV $\bar{m}$P DNA polymerase (Table XI). This difference was the relatively high amount of DNA synthesis (60-90%) in the absence of one of the dNTPs which may suggest that this DNA polymerase has a high rate of misincorporation of the dNTPs. Additional experimentation using homopolymer templates and the various dNTPs could be used to determine if this DNA polymerase does in fact have a high rate of misincorporation. The result obtained for HSV $\bar{m}$P DNA polymerase was in contrast to that obtained for the other HSV DNA polymerases, which showed 15-40% maximal DNA synthesis when one of the dNTPs was omitted from the reaction mixture. Other laboratories have seen DNA synthesis in the range of 20-40% when one of the dNTPs was omitted from the reaction mixture [Weissbach et al., 1973; Powell & Purifoy, 1977].

A further observation regarding the DNA polymerase reaction re-

quirements was the need for thiol reducing agents by the HSV KOS DNA polymerase. This result may indicate that the HSV $\overline{m}$P, tsC4, and tsC7 DNA polymerases have a cysteine residue participating in the DNA synthesis reaction that is buried or better protected from oxidation than a corresponding cysteine in the KOS DNA polymerase. These results again suggest that there are differences in the active sites of the enzymes. All of the HSV DNA polymerases are NEM sensitive (data not shown). However, differential NEM studies could be performed on these DNA polymerases to determine if there are differences in the reactivity of the cysteines involved in the DNA polymerase reaction. These studies could yield some further information concerning the active site of these DNA polymerases.

Another characteristic of the HSV DNA polymerase is sensitivity to PAA [Shipkowitz et al., 1973; Overby et al., 1974; Mao et al, 1975; Mao & Robishaw, 1975; Ostrander & Cheng, 1980]. PAA has been shown to compete for pyrophosphate in the active site of the HSV DNA polymerase [Mao & Robishaw, 1975; Ostrander & Cheng, 1980]. The inhibition seen with PAA has also been shown to be noncompetitive with respect to dNTPs and uncompetitive with respect to template [Mao & Robishaw, 1975; Ostrander & Cheng, 1980].

The K_is for PAA were determined for HSV $\overline{m}$P, KOS, tsC4, and tsC7 DNA polymerases under conditions of saturating substrates for the DNA polymerase reaction. The Dixon plots (Figures 12 & 13) obtained for PAA yielded K_i values of 137 μM, 721 μM, 133 μM, and 360 μM for HSV $\overline{m}$P, KOS, tsC4, and tsC7 DNA polymerases, respectively. Derse et al. [1982] determined a K_i value for PAA of 970 ± 300 μM for the HSV KOS DNA polymerase. This is in good agreement with the value that obtained here.

Other investigators have defined HSV tsC4 DNA polymerase as being hypersensitive to PAA based on plaque reduction assays [Coen et al., 1982]. This correlates with the results obtained for HSV tsC4 DNA polymerase as this DNA polymerase is five fold more sensitive to PAA when compared with the parental strain HSV KOS DNA polymerase. The results of this series of experiments once again suggest that there are differences in the active sites of the HSV DNA polymerases and this allows for altered drug sensitivities.

Another characteristic that could be used to demonstrate differences in the active sites of these DNA polymerases is the apparent K_m values for the dNTPs. In order to demonstrate that the values obtained for the K_ms are valid, it was necessary to show that there are no nuclease or dNTPase activities functional in the DNA polymerase preparations under the conditions of DNA synthesis. While nuclease activities were present in the HSV DNA polymerase preparations based on assays conducted in the absence of dNTPs (Table XIII), no nuclease activity was demonstrated under conditions of DNA synthesis confirming the results of Knopf [1977], and O'Donnell et al. [1987a].

Since no nuclease or dNTPase activity was detected in the HSV DNA polymerase preparations under conditions of DNA synthesis, it was possible to determine apparent K_m values for the dNTPs and for activated DNA (see Figures 14, 15, & 16 for examples of Lineweaver-Burke plots obtained). These values were determined at 35°C, the permissive temperature for the mutant viruses. The two wild-type HSV DNA polymerases showed no variation in the apparent K_m values for the dNTPs (Table XIV), however, the values obtained in these studies are four to five fold higher than K_m values obtained by Frank et al. [1984b]. These

discrepancies may be due to differnces in the polymerase reaction substrates including the state of the activated DNA used. The values obtained here are similar to the K_ms for dNTPs observed with DNA polymerase α [Fisher et al., 1979]. The two mutant DNA polymerases, HSV-1 tsC4 and tsC7, both showed apparent K_m values five to six fold higher than the parental HSV-1 KOS DNA polymerase, indicating less affinity for the dNTP substrates. These experiments could be repeated at 40°C, to determine if there are any changes in the affinity for the dNTPs at the non-permissive temperature for the mutant viruses.

In addition to the apparent K_m values obtained for the dNTPs, the K_m values for activated DNA were also determined at 35°C. The values obtained for HSV-1 KOS, tsC4, and tsC7 DNA polymerases ranged from 77 to 105 μM nucleotide. These values were three to four fold greater than the value of 26 μM obtained for HSV-1 KOS DNA polymerase by Derse & Cheng [1981]. The other major difference noted in the apparent K_m value was for that of activated DNA for HSV-1 $\overline{m}$P DNA polymerase, which was five fold greater than the value obtained for HSV KOS DNA polymerase. These differences in the apparent K_m values once again suggest differences in the active sites of these enzymes which allow for differences in the way that the HSV DNA polymerase can interact with the substrates of the DNA synthesis reaction. Again, these experiments could be repeated at 40°C to determine if the HSV DNA polymerases demonstrate changes in the affinity for activated DNA at the non-permissive temperature for the temperature sensitive viruses.

The results presented to this point indicate that there are a number of differences between the mutant HSV-1 tsC4 and tsC7 DNA polymerases and the parental HSV-1 KOS DNA polymerase. In addition, there

are differences of apparently equal magnitude between the wild-type HSV-1 $\overline{m}$P and KOS DNA polymerases. The results presented in this study of the wild-type and temperature sensitive HSV DNA polymerases are summarized in Table XV.

The DNA polymerase activity of the purified HSV DNA polymerases was determined at 34° and 40°C. For all of the DNA polymerases, there was a 5-10% increase in the DNA polymerase activity at 40°C when compared to the activities obtained at 34°C. These results suggest that the HSV DNA polymerase activity may not be thermolabile under conditions of DNA synthesis.

Although the mutant HSV viruses tsC4, and tsC7 have been shown to be temperature sensitive in vivo [Aron et al., 1975; Coen et al., 1984], with the mutations mapping within the DNA polymerase gene [Coen et al., 1984; Gibbs et al., 1985, 1987], the DNA polymerases produced by these viruses have not been definitely shown to be temperature sensitive [Aron et al., 1975]. For this reason, the thermolability of the HSV KOS, tsC4 and tsC7 DNA polymerases was tested at 34°, 40°, and 45°C. At all of these temperatures, it was found that the putative temperature sensitive HSV DNA polymerases were no more thermolabile than the parental HSV DNA polymerase (Figure 17), and in fact, HSV tsC7 DNA polymerase seemed somewhat more thermostable than the parental DNA polymerase.

Another possibile function that could potentially be thermolabile in the HSV DNA polymerase is the ability of the DNA polymerase to interact with DNA. Alternatively, the interaction with DNA could make the DNA polymerase more or less thermolabile. To test these possibilities, activated DNA was added to the thermal inactivation buffer prior

TABLE XV

CHARACTERISTICS OF THE HSV DNA POLYMERASES

	mP	KOS	tsC4	tsC7
Salt (KCl) Optima	- - - - - - - - - 200 - 300 mM - - - - - - -			
% Max. Act. at 400 mM KCl	80%	40%	10%	70%
pH Optima	- - - - - - - - - 7.5 - 8.5 - - - - - - - -			
Mg^{2+} ion optima (mM)	4-8	8-16	8	12-16
Mn^{2+} ion optima (mM) (% max. activity)	1 (60%)	1 (70%)	1 (12%)	1 (55%)
Co^{2+} ion optima (mM) (% max. activity)	1 (90%)	2 (25%)	2 (40%)	2 (30%)
PAA (K_i (μM))	137	721	133	360
Apparent K_ms (μM)				
dATP	2.4	2.1	14.3	11.1
dCTP	2.4	3.4	11.1	10.0
dGTP	4.3	2.6	12.5	14.3
TTP	2.1	1.9	14.2	11.1
Activated DNA (μM nucleotide)	500	105	77	95

to the addition of the HSV DNA polymerases. Interaction with DNA resulted in decreased heat sensitivity for all of the HSV DNA polymerases, at the permissive and non-permissive temperatures (Figure 18). This shows that: 1) interaction of the mutant DNA polymerases with DNA is not thermosensitive, because the decrease in heat sensitivity observed would have been lost at the non-permissive temperature, and 2) the interaction with DNA at the non-permissive temperature did not increase the thermolability of any of the DNA polymerases.

One other possibile explanation to account for the temperature sensitivity seen in the HSV tsC4 and tsC7 viruses in vivo is that the HSV DNA polymerase may interact with other proteins in a DNA replication complex, and this interaction may be temperature sensitive. In order to test this hypothesis out, crude extracts were prepared from HSV KOS, tsC4 and tsC7 infected cells. Also, the other putative HSV temperature sensitive DNA polymerase mutant, tsD9, was studied. These extracts were used in place of the purified HSV DNA polymerase in the thermal inactivation studies. The results of these studies showed that the DNA polymerase activity in HSV tsC7 and tsD9 crude extracts were slightly more thermolabile at 34°C than the other two DNA polymerase activities (Figure 19). At 40°C, there were no differences between the HSV polymerase activities (Figure 20). This was not the case at 45°C where the polymerase activity of tsD9 was more thermolabile than the parental strain KOS. Within limits of error, the other two mutant enzymes appeared to be no more thermolabile than HSV KOS (Figure 21). Except for the HSV tsD9 HSV DNA polymerases, the HSV DNA polymerase mutants showed no differences in thermolability when compared to the parental strain. Also, the HSV KOS, tsC4, and tsC7 enzymes were less

thermolabile under these conditions than the partially purified enzymes. At this point it should be noted that protein:protein interactions may have several effects: 1) increasing the thermostability of the HSV DNA polymerase, 2) decreasing the thermostability, and/or 3) affecting the DNA polymerizing activity of the enzyme.

Although crude extracts were used in the above studies, it is still possible that some potentially thermosensitive protein:protein interactions involving the HSV DNA polymerase may have been disrupted by the high salt extraction procedure used to prepare the extracts. For this reason, the thermal inactivation studies could be repeated using infected nuclei. These results may show that the temperature sensitive HSV DNA polymerases are either more thermolabile or thermostabile than the parental HSV KOS DNA polymerase.

The results using crude extracts indicate that there are no thermolabile protein:protein interactions that affect HSV DNA polymerase activity under the conditions tested here. However, this still does not rule out the possibility that there are other protein-protein interactions necessary for DNA replication in the infected cell that may be thermolabile. Systems in which the DNA polymerase can be isolated in a complex with other proteins include eukaryotic DNA polymerase:primase complexes [Campbell, 1986] and the T4 bacteriophage replication complex [Nossal & Alberts, 1983]. In addition, recent reports suggest that the HSV DNA polymerase can be isolated as a complex with other proteins involved in HSV DNA metabolism including $\beta_1 8$ and the 65 kDa DPB [Vaughan et al., 1984; Gallo et al., 1988]. Also, there is a unique DNA primase activity that can be found in HSV infected cells and not in mock infected cells [Holmes et al., 1988]. This DNA primase ac-

tivity copurified with the HSV DNA polymerase suggesting that these proteins may be involved in a complex.

To test the possibility that the HSV DNA polymerase participates in a replication complex and that this interaction is thermosensitive in the temperature sensitive mutants, nuclear extracts were prepared from cells infected with the HSV KOS derived enzymes and were analyzed by velocity sedimentation on neutral glycerol gradients. The gradients were fractionated and analyzed for high and low salt DNA polymerase activities. The untreated HSV KOS, tsC4, and tsC7 DNA polymerase activities were found to have an S value of approximately 7.5 while the HSV DNA polymerase activity from tsD9 infected cells had an S value of 6 indicating that the HSV tsD9 DNA polymerase has been partially proteolyzed or is more asymmetrical than the other HSV DNA polymerases. Upon heating the nuclear extracts, all of the HSV DNA polymerase activities were decreased and appeared to shift to slightly smaller S values (Figures 22 to 26). This suggests that either a protein:protein interaction is being disrupted, or the enzymes are changing shape.

The gradient fractions were also assayed for DNA primase activities. Although the high and low salt primase assays utilizing the intrinsic HSV DNA polymerase yielded no detectable activity in any of the gradients, the indirect DNA primase assay, using E. coli DNA polymerase I, did detect some DNA primase activity in the gradients of the untreated nuclear extracts from HSV KOS, tsC4, and tsD9 DNA polymerases (Figures 22, 24, 26). Almost no DNA primase activity was detected in the untreated nuclear extract of HSV tsC7 infected cells (Figure 25). In addition, very little DNA primase activity could be detected in gradients of any heat treated nuclear extracts. This indicates that

the primase activity appears to be relatively thermolabile. Also, the amount of DNA primase activity observed was greater in the fractionation profile of those gradients which had nuclear extracts from cells infected with HSV KOS, tsC4, and tsD9 loaded on them than the gradient which had extract from mock infected cells loaded (Figure 23).

Anti-calf DNA primase antibodies were used to try to estimate the amount of host cell DNA primase activity present in the crude nuclear extracts in antibody binding experiments. The results showed that while the crossreactivity of the Vero cell DNA primase and the HeLa DNA primase (used as a control) with the antibodies are not identical, not all of the DNA primase present in the infected nuclear extracts was Vero DNA primase. Although there is no direct evidence for a HSV encoded DNA primase, there is evidence to indicate that a DNA primase activity is increased in HSV infected cells and is different from the host cell DNA primase [Holmes et al., 1988].

The results of the gradient analysis experiments indicate that the HSV DNA polymerases may be present in a replication complex in the infected cell nucleus, or the HSV DNA polymerases are changing shape (i.e. becoming more asymmetrical). This is supported by the observation that the HSV DNA polymerase appears to be shifting to a slower sedimenting species when the nuclear extracts are heated prior to separation on glycerol gradients. Additional experiments will have to be performed to distinguish between the possibilities indicated above. These experiments may include gel filtration studies of untreated and heat treated nuclear extracts to determine Stoke's radii for the HSV DNA polymerases which could then be used to determine molecular weights of the DNA polymerases using the data obtained from the velocity sedi-

mentation studies. The results from the velocity sedimentation studies indicate that the HSV DNA polymerases are undergoing macromolecular changes upon heating of the nuclear extracts, but what those changes are unclear at this time.

There is some evidence from other laboratories suggesting that the HSV DNA polymerase may be interacting with other proteins involved in HSV DNA replication, such as $\gamma_1 8$ and the 65K DNA binding protein [Vaughan et al., 1984; Gallo et al., 1988]. Other proteins that may be involved in a HSV DNA replication complex have yet to be identified, but these will include a combination of the seven proteins identified by Wu et al. [1988] that are necessary for the replication of plasmids that contain the HSV ori_L or ori_S origin of replication. There may be other proteins in addition to the seven essential proteins, such as the HSV ribonucleotide reductase, that are involved in the replication of HSV DNA in vivo. These proteins may be encoded for by the virus or may be host proteins recruited by the virus. Further study will have to be undertaken to determine the other proteins involved in viral DNA replication.

The proteins involved in a replication complex could potentially be identified through the use of immunological methods. Antibodies raised against the HSV DNA polymerase could be used to immunoprecipitate the HSV DNA polymerase from crude nuclear extracts of infected cells. In addition to precipitating the HSV DNA polymerase, the immunoprecipitation may also reveal other proteins involved in the DNA replication complex. Antibodies to other HSV DNA metabolism proteins, such as $\beta_1 8$ and the 65 kDa BDP, have been shown to coprecipitate HSV DNA polymerase [Vaughan et al., 1984; Gallo et al., 1988]. A further

extension of this experiment would be to use untreated and heat treated nuclear extracts from cells infected with the mutants HSV-1 tsC4, tsC7 and tsD9. These studies may show changes in the polypeptides associated with the HSV DNA polymerase after heat treatment of the extracts. These anti-DNA polymerase antibodies could also be used to prepare an immunoaffinity column which could be used to enrich the HSV DNA polymerase and other proteins that may be complexed to the DNA polymerase. A procedure such as this, using monoclonal antibodies columns, has been used to purify polymerase:primase complexes from other eukaryotic sources including yeast [Plevani et al., 1984, 1985], and calf thymus [Chang et al, 1984]. The proteins retrieved from this purification could be tested for various enzymatic activities to determine the activities involved in the replication complex. It is to be expected that some of the potential activities will include DNA primase, topoisomerase, nuclease, helicase, an origin of replication DNA binding protein, and $\beta_1 8$.

Another system that could be used to determine if the HSV DNA polymerase mutants are temperature sensitive in protein-protein interactions is the assay developed by Challberg [1986] for determining the viral genes required for HSV DNA replication. In this assay, the viral genes for $U_L 5$, $U_L 8$, $U_L 9$, $U_L 42$, $U_L 52$, and $U_L 29$ ($\beta_1 8$) are transfected into Vero cells together with the gene for the HSV DNA polymerase, and a plasmid containing one of the two HSV origins of replication. The HSV tsC4, tsC7 and tsD9 DNA polymerase genes could be cloned and substituted for the HSV KOS DNA polymerase gene in the assay. The transfected cells could be incubated at the permissive and nonpermissive temperature and the extent of replication of the ori containing plasmid deter-

mined.

The sequences of the seven essential replication genes are known [McGeoch et al, 1988] and could be used to prepare synthetic peptides to produce antibodies to these proteins. These antibodies could then be used to determine if the HSV DNA polymerase can be coprecipitated with any of these proteins implicating the protein in a complex with the HSV DNA polymerase. Once again, untreated and heat treated extracts from HSV-1 KOS, tsC4, tsC7, and tsD9 infected cells could be analyzed with the antibodies prepared to the seven essential replication proteins to determine if there is a thermolable protein-protein interaction involved in the replication complex.

The studies proposed above may implicate other gene products in addition to the seven essential replication gene products. The studies described may also yield information as to the nature and functions of the proteins involved in the HSV DNA replication complex. The information gained by these studies may also yield insights into the eukaryotic DNA replication complex.

As was mentioned earlier, the results shown in Figure 7 indicate that the HSV $\bar{m}$P DNA polymerase appears to have a molecular weight of 148 kDa, while the HSV KOS DNA polymerase appears to be slightly smaller (130 kDa). The HSV KOS DNA polymerase gene has been sequenced and has been shown to encode for a DNA polymerase of 136 kDa [Quinn & McGeoch, 1985]. In order to determine if the apparent difference in molecular weight based on SDS PAGE analysis is real, the sequence of the HSV $\bar{m}$P DNA polymerase gene could be determined. This will also reveal other sequence differences between the two wild-type HSV DNA polymerase genes. In addition, the sequences for the mutant HSV tsC4 and

tsC7 DNA polymerase genes could also be determined to show the position of the mutation present in the DNA polymerase genes.

If the experiments proposed above do not yield any insights into the temperature sensitive nature of these mutant HSV DNA polymerases, further studies could be undertaken to determine if the mutation result in changes in the levels of HSV DNA polymerase mRNA or its stability. These results may indicate that the temperature sensitive phenotype observed with the mutant HSV DNA polymerase viruses may be due to changes at the level of transcription.

Although the studies undertaken in this work did not yield the expected result based on the mapping of the temperature sensitive mutation, information has been obtained on the putative temperature sensitive DNA polymerase mutants and on the overall heterogeneity of the wild-type HSV DNA polymerases. It was possible to partially purify these enzymes, and make some determination of their DNA synthesis requirements. This study also showed that the temperature sensitive phenotype observed with these viruses was not due to thermal inactivation of the DNA polymerase activity, or the ability of the HSV DNA polymerases to interact with DNA at the nonpermissive temperature, but may be due to thermolabile protein-protein interactions in a replication complex. Further investigation will be required to show the proteins involved in the HSV replication complex and the role that the HSV DNA polymerase plays in this complex.

VII. Appendices

VII. A. Appendix A

BASIC Computer Program "ASSAY"

```
5       DIMSTR=0
7       LPRINT CHR$(27);CHR$(15)
8       WIDTH "LPT1:",132
10      CLS
20      PRINT:PRINT "                    Polymerase Assay Data Handling
        Programs"
30      PRINT:PRINT "Select the appropriate module from the following
        table:"
40      PRINT:PRINT "                    1 - Data Table Initialization"
50      PRINT:PRINT "                    2 - Data Entry"
60      PRINT:PRINT "                    3 - Data Printing"
70      PRINT:PRINT "                    4 - Data Output to Disk Files"
75      PRINT:PRINT "                    5 - Exit program"
80      PRINT:PRINT
90      PRINT:INPUT "       Enter the number of the selected module:
        ",MODNUM
95      CLS
100     ON MODNUM GOTO 1000,2000,3000,4000,32766
110     GOTO 10
1000    INPUT "Assay Title";TITLE$
1010    INPUT "Specific Activity of 3H-TTP (DPM/pmol)";SAN
1020    INPUT "Total Assay Volume (uL)";TAV
```

```
1030    INPUT "Volume of polymerase fraction used in assay (uL)";VPF
1040    INPUT "Total Volume Spotted (uL)";TVS
1050    INPUT "Length of assay (min)";LA
1060    INPUT "Background Counts (DPM)";BKG
1990    GOTO 10
2000    CLS:PRINT "                                    Data Entry Module"
2010    PRINT:PRINT "Select the data entry option from the table
        below:"
2020    PRINT:PRINT "                    1 - Enter High Salt Data"
2030    PRINT:PRINT "                    2 - Enter Low Salt Data"
2040    PRINT:PRINT "                    3 - Enter Kinetic Assay Data"
2050    PRINT:PRINT "                    4 - Enter Protein
        Concentrations for "
2051    PRINT "                            Specific Activity
        Calculations"
2060    PRINT:PRINT "                    5 - Prepare a Purification
        Table"
2070    PRINT:PRINT "                    6 - Return to Main Menu"
2080    PRINT:INPUT "       Enter the number of the selected option:
        ",ENTRYOPT
2085    CLS
2090    ON ENTRYOPT GOTO 2100,2300,2500,2700,8000,10
2095    GOTO 2000
2100    OPT1=1:GOSUB 10000
2105    GOSUB 15000
2110    GOSUB 20000
2115    IF HSF<>1 THEN 2120
```

```
2116    ERASE HSL$,HSDPM,HSUN,HSUNML,HSPM,HSTVP,HSTUN,HSUNMG
2120    DIM HSL$(NF), HSDPM(NF), HSUN(NF), HSUNML(NF), HSPM(NF),
        HSTVP(NF), HSTUN(NF), HSUNMG(NF):HSF=1
2125    NFHS=NF
2130    FOR I = 1 TO NFHS
2140    HSL$(I)=L$(I):HSDPM(I)=DPM(I):HSUN(I)=UN(I):HSUNML(I)=UNML(I)
2150    HSPM(I)=PM(I):HSTVP(I)=TVP(I):HSTUN(I)=TUN(I)
2160    NEXT I
2170    GOTO 2000
2300    OPT2=1:GOSUB 10000
2305    GOSUB 15000
2310    GOSUB 20000
2315    IF LSF<>1 THEN 2320
2317    ERASE LSL$,LSDPM,LSUN,LSUNML,LSPM,LSTVP,LSTUN,LSUNMG
2320    DIM LSL$(NF), LSDPM(NF), LSUN(NF), LSUNML(NF), LSPM(NF),
        LSTVP(NF), LSTUN(NF), LSUNMG(NF):LSF=1
2325    NFLS=NF
2330    FOR I=1 TO NFLS
2340    LSL$(I)=L$(I):LSDPM(I)=DPM(I):LSUN(I)=UN(I):LSUNML(I)=UNML(I)
2350    LSPM(I)=PM(I):LSTVP(I)=TVP(I):LSTUN(I)=TUN(I)
2360    NEXT I
2370    GOTO 2000
2500    INPUT "How many kinetic data sets (8 max)";KDS:OPT3=1
2505    IF KDS>8 THEN 2500 ELSE 2510
2510    INPUT "How many time points per data set";PTS
2515    IF KF<>1 THEN 2520
2516    ERASE DPMK,TIM,PTC
```

```
2520    DIM DPMK(8,PTS),TIM(PTS),PTC(8):KF=1
2521    INPUT "Assay Title";T$
2522    INPUT "Specific Activity of 3H-TTP (DPM/pmol)";SAN
2523    INPUT "Total Assay Volume (uL)";TAV
2524    INPUT "Volume of polymerase fraction used in assay (uL)";VPF
2525    INPUT "Total Volume Spotted (uL)";TVS
2530    FOR J=1 TO PTS
2540    PRINT "Time (min) at point";J;:INPUT TIM(J)
2550    NEXT J
2560    FOR I=1 TO KDS
2572    PRINT "Protein Concentration (mg/ml) for data set";I;:
        INPUT;PTC(I): PRINT
2574    PRINT "Protein Concentration (mg/ml) = ";PTC(I)
2575    INPUT "Is this value correct (Y/N)",W$
2576    IF W$="n" OR W$="N" THEN 2575
2579    PRINT "DPM for data set";I;"at time:"
2580    FOR J=1 TO PTS
2590    PRINT TIM(J);:INPUT DPMK(I,J)
2600    NEXT J
2605    NEXT I
2610    CLS
2615    FOR I=1 TO KDS
2618    CLS:PRINT "Data Set";I:PRINT "Protein Concentration (mg/ml) =
        ";PTC(I):PRINT "index","time","DPM"
2620    FOR J=1 TO PTS
2625    PRINT J,TIM(J),DPMK(I,J)
2630    NEXT J
```

```
2640    PRINT:INPUT "Are these values correct"; OK$
2645    IF OK$="n" OR OK$="N" THEN 2650 ELSE 2660
2650    INPUT "Which row is wrong";J
2655    INPUT "New DPM value for this data point is";DPMK(I,J)
2657    GOTO 2618
2660    NEXT I
2665    GOSUB 31000
2690    GOTO 2000
2700    GOSUB 10000
2705    OPT4=1
2708    NFP=NF
2710    FOR I = 1 TO NFP
2714    CLS
2715    PRINT "Protein concentration of polymerase used (mg/ml) for
        assay";I;:INPUT PC(I)
2716    PRINT:PRINT "[Protein] for assay";I;"=";PC(I);:INPUT "  Is
        this correct";OK$
2717    IF OK$="n" OR OK$="N" THEN 2714
2719    NEXT I
2720    IF OPT1=1 THEN 2730 ELSE 2745
2730    FOR J=1 TO NFP
2735    HSUNMG(J)=HSUNML(J)/PC(J)
2740    NEXT J
2745    IF OPT2=1 THEN 2755 ELSE 2770
2755    FOR J=1 TO NFP
2760    LSUNMG(J)=LSUNML(J)/PC(J)
2765    NEXT J
```

```
2770    GOTO 2000
3000    Q$="":IF OPT1<>1 THEN 3280
3050    Q$="High Salt Assay"
3100    GOSUB 25000
3130    FOR I=1 TO NFHS
3140    LPRINT USING "###";I;
3145    LPRINT TAB(4);HSL$(I);TAB(21);
3150    LPRINT USING "###.#";HSTVP(I);
3160    LPRINT TAB(28);:LPRINT USING "########";HSDPM(I);
3170    LPRINT TAB(39);:LPRINT USING "####.##";HSPM(I);
3180    LPRINT TAB(49);:LPRINT USING "###.##";HSUN(I);
3190    LPRINT TAB(57);:LPRINT USING "######.##";HSUNML(I);
3200    LPRINT TAB(69);:LPRINT USING "########.##";HSTUN(I);
3210    LPRINT TAB(83);:LPRINT USING "##.###";PC(I);
3220    LPRINT TAB(89);:LPRINT USING "########.##";HSUNMG(I)
3230    NEXT I
3250    GOSUB 30000
3280    IF OPT2<>1 THEN 3400
3285    Q$="Low Salt Assay"
3290    GOSUB 25000
3300    FOR I=1 TO NFLS
3310    LPRINT USING "###";I;
3315    LPRINT TAB(4);LSL$(I);TAB(21);
3320    LPRINT USING "###.#";LSTVP(I);
3330    LPRINT TAB(28);:LPRINT USING "########";LSDPM(I);
3340    LPRINT TAB(39);:LPRINT USING "####.##";LSPM(I);
3350    LPRINT TAB(49);:LPRINT USING "###.##";LSUN(I);
```

```
3360    LPRINT TAB(57);:LPRINT USING "######.##";LSUNML(I);
3370    LPRINT TAB(69);:LPRINT USING "########.##";LSTUN(I);
3380    LPRINT TAB(83);:LPRINT USING "##.###";PC(I);
3390    LPRINT TAB(89);:LPRINT USING "########.##";LSUNMG(I)
3392    NEXT I
3395    GOSUB 30000
3400    IF OPT3<>1 THEN 3800
3410    CLS
3420    PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
        "PRINTNG IN PROGRESS"
3430    PRINT:PRINT "                                    PLEASE WAIT"
3460    LPRINT "            Kinetic Assay Data"
3465    D$=DATE$
3470    LPRINT D$
3475    LPRINT T$
3480    FOR I=1 TO KDS
3481    LPRINT "        Data Set ";I;TAB(40);"Protein Concentration
        (mg/ml) = ";PTC(I):LPRINT
3482    LPRINT "The data fits the line :  Y = ";
3483    LPRINT USING "########.###";M(I);
3484    LPRINT " X ";B$ ;:LPRINT USING "########.###";ABS(B(I))
3485    LPRINT:LPRINT "with the Sum of Deviations Squared = ";
3486    LPRINT USING "##########.####";D2(I):LPRINT
3490    LPRINT:LPRINT "index","time","DPM","Corr DPM"
3500    FOR J=1 TO PTS
3510    LPRINT J,TIM(J),DPMK(I,J),DPMK(I,J)-B(I)
3520    NEXT J
```

```
3522    LPRINT
3524    SAP=(((M(I)/SAN)/TVS)/PTC(I))*(TAV/VPF)*1000
3526    LPRINT "The specific activity for the polymerase = ";
3527    LPRINT USING "########.##";SAP;
3528    LPRINT " un/mg":LPRINT
3530    NEXT I
3540    LPRINT CHR$(12)
3800    GOTO 10
4000    INPUT "What is the name of the output file to be created";OF$
4010    OPEN OF$ FOR OUTPUT AS #1
4020    IF OPT1<>1 THEN 4200
4030    FOR I=1 TO NF
4040    PRINT #1, I;HSUNML(I)
4050    NEXT I
4060    PRINT #1, "";""
4200    IF OPT2<>1 THEN 4400
4210    FOR J=1 TO NF
4220    PRINT #1, J;LSUNML(J)
4230    NEXT J
4240    PRINT #1, "";""
4400    INPUT "Do you want to enter salt gradient data";SED$
4410    IF SED$="n" OR SED$="N" THEN 4900
4420    IF SED$="y" OR SED$="Y" THEN 4430 ELSE 4400
4430    CLS:INPUT "Step or Linear gradient (s/l)";SLG$
4440    IF SLG$="L" OR SLG$="l" THEN  4670
4450    IF SLG$="s" OR SLG$="S" THEN  4460 ELSE 4430
4460    INPUT "How many steps are there in the gradient";NOSTP
```

```
4465    DIM FRAC$(2*NOSTP),SCS(2*NOSTP)
4470    FOR J=1 TO NOSTP
4480    CLS:PRINT "Fraction number that step";J;:INPUT "began
        on";FRAC$((2*J)-1)
4490    PRINT "Fraction number that step";J;:INPUT "ended
        on";FRAC$(2*J)
4500    PRINT "Salt concentration of step";J;:INPUT SCS$((2*J)-1)
4510    SCS(2*J)=SCS((2*J)-1)
4520    NEXT J
4530    CLS  :PRINT "step      begin      end      salt"
4540    PRINT "          frac       frac     conc"
4550    FOR I=1 TO NOSTP
4560    PRINT TAB(2);J;TAB(12);FRAC$((2*J)-1);TAB(20); FRAC$(2*J);
        TAB(30);SCS((2*J)-1)
4570    NEXT J
4580    PRINT:INPUT "Are these values correct";CV$
4590    IF CV$="n" OR CV$="N" THEN 4600 ELSE 4660
4600    INPUT "Step to correct";CS
4610    CLS:PRINT "Fraction number that step";CS;:INPUT "began on";
        FRAC$((2*CS)-1)
4620    PRINT "Fraction number that step";CS;:INPUT "ended
        on";FRAC$(2*CS)
4630    PRINT "Salt concentration of step";CS;:INPUT SCS$((2*CS)-1)
4640    SCS(2*CS)=SCS((2*CS)-1)
4650    GOTO 4530
4660    FOR J=1 TO NOSTP
4662    PRINT #1, FRAC$(J);SCS(J)
```

```
4664    NEXT J
4665    GOTO 4900
4670    INPUT "First fraction of salt gradient";FRAC$(1)
4680    INPUT "Last fraction of salt gradient";FRAC$(2)
4690    INPUT "Initial salt concentration";SCS(1)
4700    INPUT "Final salt concentration";SCS(2)
4710    CLS:PRINT "First fraction of gradient = ";FRAC$(1)
4720    PRINT "Last fraction of gradient = ";FRAC$(2)
4730    PRINT "Initial salt concentration = ";SCS(1)
4740    PRINT "Final salt concentration = ";SCS(2)
4750    PRINT:INPUT "Are these values correct";P$
4760    IF P$="N" OR P$="n" THEN 4670
4770    PRINT #1, FRAC$(1);SCS(1)
4775    PRINT #1, FRAC$(2);SCS(2)
4780    PRINT #1, "";""
4900    CLOSE #1
4990    GOTO 10
8000    INPUT "Assay Title";TITLE$
8001    INPUT "Specific Activity of 3H-TTP (DPM/pmol)";SAN
8002    INPUT "Total Assay Volume (uL)";TAV
8003    INPUT "Volume of polymerase fraction used in assay (uL)";VPF
8004    INPUT "Total Volume Spotted (uL)";TVS
8006    INPUT "Length of assay (min)";LA
8007    INPUT "Background Counts (DPM)";BKG
8010    INPUT "How many points are in this table";HMP
8015    IF KFS<>1 THEN 8020
8016    ERASE KSL$,KSDPM,KSUN,KSUNML,KSPM,KSTVP,KSTUN,KPC,KUNMG
```

```
8020    DIM KSL$(HMP), KSDPM(HMP), KSUN(HMP), KSUNML(HMP), KSPM(HMP),
        KSTVP(HMP), KSTUN(HMP), KPC(HMP), KUNMG(HMP):KFS=1
8030    FOR J=1 TO HMP
8040    CLS:PRINT "Label of Assay";J;:INPUT KL$(J)
8050    PRINT "DPM 3H-TTP incorporated into assay";J;:INPUT KDPM(J)
8060    PRINT "Total volume of polymerase fraction";J;:INPUT KTVP(J)
8070    KDPM(J)=KDPM(J)-BKG
8080    IF KDPM(J)<0 THEN 8090 ELSE 8100
8090    KDPM(J)=0
8100    KPM(J)=(KDPM(J)/SAN)*(TAV/TVS)
8110    KUN(J)=KPM(J)/LA:KUNML(J)=(KUN(J)*(1000/VPF))
8120    KTUN(J)=KUNML(J)*KTVP(J)
8130    PRINT "Protein concentration of polymerase used (mg/ml) for
        assay";J;:INPUT KPC(J)
8140    KUNMG(J)=KUNML(J)/KPC(J)
8150    CLS
8160    PRINT "                                                Polymerase
        Assay Data"
8170    PRINT:PRINT TITLE$:D$=DATE$:PRINT D$
8230    PRINT
8235    PRINT KL$(J)
8240    PRINT "Ttl Vol of Pol Fraction";TAB(46);:PRINT USING
        "###.#";KTVP(J)
8250    PRINT "DPM";TAB(41);:PRINT USING "########";KDPM(J)
8260    PRINT "Pmol incorporated";TAB(45);:PRINT USING
        "####.##";KPM(J)
8270    PRINT "Units Pol per reaction";TAB(46);:PRINT USING
```

```
        "###.##";KUN(J)
8280    PRINT "Units per mL";TAB(43);:PRINT USING "######.##";KUNML(J)
8290    PRINT "Units in pol fraction";TAB(41);:PRINT USING
        "########.##";KTUN(J)
8300    PRINT "Protein Concentration (mg/ml)";TAB(47);:PRINT USING
        "##.###"; KPC(J)
8310    PRINT "Un/mg";TAB(41);:PRINT USING "########.##";KUNMG(J)
8320    PRINT:INPUT "Are these values correct";KCS$
8330    IF KCS$="n" OR KCS$="N" THEN 8040 ELSE 8340
8340    NEXT J
8345    CLS
8350    PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
        "PRINTING IN PROGRESS"
8360    PRINT:PRINT "                                    PLEASE WAIT"
8390    LPRINT "                                          Polymerase
        Assay Data"
8400    LPRINT:LPRINT TITLE$:D$=DATE$:LPRINT D$:LPRINT Q$
8410    LPRINT:LPRINT TAB(20);"Fract";TAB(72);"Total
8420    LPRINT TAB(5);"Fraction";TAB(21);"Vol";TAB(31);"DPM";TAB(41);
8430    LPRINT "pmol";TAB(48);"un/samp";TAB(60); "un/ml";TAB(72);
        "units";
8440    LPRINT TAB(83);"mg/ml";TAB(94);"un/mg";TAB(103);"% rec";
        TAB(114); "Purif"
8450    LPRINT TAB(21);"(ml)";TAB(47);"(pmol/min)":LPRINT
8460    FOR I=1 TO HMP
8470    LPRINT USING "###";I;
8475    LPRINT TAB(4);KL$(I);TAB(21);
```

```
8480    LPRINT USING "###.#";KTVP(I);
8490    LPRINT TAB(28);:LPRINT USING "########";KDPM(I);
8500    LPRINT TAB(39);:LPRINT USING "####.##";KPM(I);
8510    LPRINT TAB(49);:LPRINT USING "###.##";KUN(I);
8520    LPRINT TAB(57);:LPRINT USING "######.##";KUNML(I);
8530    LPRINT TAB(69);:LPRINT USING "########.##";KTUN(I);
8540    LPRINT TAB(83);:LPRINT USING "##.###";KPC(I);
8550    LPRINT TAB(89);:LPRINT USING "########.##";KUNMG(I);
8560    PCT=(KTUN(I)/KTUN(1))*100
8570    LPRINT TAB(102);:LPRINT USING "###.##";PCT;
8580    PUR=KUNMG(I)/KUNMG(1)
8590    LPRINT TAB(113);:LPRINT USING "######.#";PUR
8600    NEXT I
8610    GOSUB 30000
8900    GOTO 2000
10000   PRINT "How many fractions (";NF;")";:INPUT NFTEMP
10010   IF NFTEMP=0 THEN 10020 ELSE NF=NFTEMP
10020   RETURN
15000   IF DIMSTR=1 THEN 15005 ELSE 15010
15005   ERASE L$,DPM,UN,UNML,PM,TVP,TUN,PC
15010   DIM L$(NF), DPM(NF), UN(NF), UNML(NF), PM(NF), TVP(NF),
        TUN(NF), PC(NF)
15020   DIMSTR=1
15030   RETURN
20000   CLS
20010   INPUT "Do you want fraction labels";FLAG$
20020   IF FLAG$="y" OR FLAG$="Y" THEN GOTO 20040
```

```
20030   IF FLAG$="n" OR FLAG$="N" THEN GOTO 20050 ELSE 20010
20040   FLAG=1:GOTO 20060
20050   FLAG=0
20060   FOR I = 1 TO NF
20070   PRINT
20080   IF FLAG=0 THEN 20100
20090   PRINT "Label of Assay";I;:INPUT L$(I)
20100   PRINT "DPM 3H-TTP incorporated into assay";I;:INPUT DPM(I)
20110   PRINT "Total volume of polymerase fraction";I;:INPUT TVP(I)
20120   NEXT I
20121   FOR I= 1 TO NF
20122   CLS:PRINT "Label for fraction";I;"=";L$(I)
20123   PRINT DPM(I)
20124   PRINT TVP(I)
20125   PRINT:INPUT "Are these values correct";CORR$
20126   IF CORR$="n" OR CORR$="N" THEN 20127 ELSE 20133
20127   PRINT "Label of Assay";I;:INPUT L$(I)
20128   PRINT "DPM 3H-TTP incorporated into assay";I;:INPUT DPM(I)
20129   PRINT "Total volume of polymerase fraction";I;:INPUT TVP(I)
20130   GOTO 20122
20133   NEXT I
20135   FOR J=1 TO NF
20140   DPM(J)=DPM(J)-BKG
20150   IF DPM(J)<0 THEN 20160 ELSE 20170
20160   DPM(J)=0
20170   PM(J)=(DPM(J)/SAN)*(TAV/TVS)
20180   UN(J)=PM(J)/LA:UNML(J)=(UN(J)*(1000/VPF))
```

```
20190   TUN(J)=UNML(J)*TVP(J)
20215   NEXT J
20220   RETURN
25000   CLS
25010   PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
        "PRINTING IN PROGRESS"
25020   PRINT:PRINT "                                        PLEASE WAIT"
25050   LPRINT "                                    Polymerase Assay Data"
25060   LPRINT:LPRINT TITLE$:D$=DATE$:LPRINT D$
25065   LPRINT Q$
25070   LPRINT:LPRINT TAB(20);"Fract";TAB(72);"Total
25080   LPRINT TAB(5);"Fraction";TAB(21);"Vol";TAB(31);"DPM";TAB(41);
25090   LPRINT "pmol";TAB(48);"un/samp";TAB(60);"un/ml"; TAB(72);
        "units";
25100   LPRINT TAB(83);"mg/ml";TAB(94);"un/mg"
25110   LPRINT TAB(21);"(ml)";TAB(47);"(pmol/min)":LPRINT
25120   RETURN
30000   LPRINT:LPRINT "Specific Activity of 3H-TTP = ";
30010   LPRINT USING "####";SAN;
30020   LPRINT " DPM/pmol":LPRINT "Assay length = ";LA;" min"
30030   LPRINT "Total assay volume = ";:LPRINT USING
        "####.#";TAV;:LPRINT " uL"
30040   LPRINT "Volume polymerase fraction used per assay = ";
30050   LPRINT USING "###.#";VPF;:LPRINT " uL"
30060   LPRINT "Volume spotted on GF/C paper = ";:LPRINT USING
        "###.#";TVS;
30070   LPRINT "uL"
```

```
30080   LPRINT "Background = ";BKG;" DPM":LPRINT CHR$(13):LPRINT
        CHR$(12)
30090   RETURN
30130   GOTO 20122
31000   FOR I= 1 TO KDS
31010   B$="-"
31020   X1=0:Y1=0:XY=0:X2=0
31025   FOR J=1 TO PTS
31030   X1=X1+TIM(J)
31040   Y1=Y1+DPMK(I,J)
31050   XY=XY+(TIM(J)*DPMK(I,J))
31060   X2=X2+(TIM(J)*TIM(J))
31070   NEXT J
31080   R=PTS*X2-X1*X1
31090   IF J<>0 THEN 31110
31100   LPRINT "WARNING -- NO SOLUTION FOR LINEAR LEAST SQUARES
        SUBROUTINE"
31105   GOTO 31190
31110   M=(PTS*XY-X1*Y1)/R
31115   M(I)=M
31120   B=(Y1*X2-X1*XY)/R
31125   B(I)=B
31130   IF ABS(B)=B THEN B$="+"
31140   D2=0
31150   FOR J=1 TO PTS
31160   D2=D2+(DPMK(I,J)-M*TIM(J)-B)^2
31170   NEXT J
```

```
31175    D2(I)=D2
31180    NEXT I
31190    RETURN
32766    LPRINT CHR$(28);"@";CHR$(27);"M";CHR$(28);"S";CHR$(1):SYSTEM
32767    END
```

VII. B. Appendix B

BASIC Computer Program "dNTPase"

```
5   CLS
8   WIDTH LPRINT 132
10  INPUT "How many samples";N
20  INPUT "Title";T$
30  INPUT "Subtitle";ST$
40  D$=DATE$
50  TM$=TIME$
60  DIM
    CPMT(N),CPMNTP(N),CPMNDP(N),CPMNMP(N),NDP(N),NTP(N),NMP(N),L$(N)
70  FOR I=1 TO N
75  CLS
80  PRINT "Label for Sample ";I;:INPUT L$(I):PRINT
90  PRINT "Input the CPM for the information requested for sample ";I
100 PRINT:INPUT "CPM for dNTP region";CPMNTP(I)
110 PRINT:INPUT "CPM for dNDP region";CPMNDP(I)
120 PRINT:INPUT "CPM for dNMP region";CPMNMP(I)
130 PRINT:PRINT:PRINT
140 PRINT "CPM for dNTP = ";CPMNTP(I)
150 PRINT "CPM for dNDP = ";CPMNDP(I)
160 PRINT "CPM for dNMP = ";CPMNMP(I):PRINT
170 INPUT "Are there any corrections to this data (Y/N)",C$
180 IF C$="Y" OR C$="y" THEN 75
190 CPMT(I)=CPMNTP(I)+CPMNDP(I)+CPMNMP(I)
```

```
200 NTP(I)=(CPMNTP(I)/CPMT(I))*100
210 NDP(I)=(CPMNDP(I)/CPMT(I))*100
220 NMP(I)=(CPMNMP(I)/CPMT(I))*100
230 NEXT I
235 CLS:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
236 PRINT "                    PRINTING IN PROGRESS - PLEASE WAIT"
240 LPRINT CHR$(27);"(s16.6H";CHR$(27);"&l1L"
250 LPRINT TAB(50);"HSV DNA POLYMERASE":LPRINT
260 LPRINT T$:LPRINT ST$:LPRINT D$:LPRINT TM$
270 LPRINT "     Label";TAB(50);"CPM dNTP";TAB(60);"CPM
    dNDP";TAB(70);"CPM dNMP";TAB(80);
280 LPRINT "Total CPM";TAB(90);"% dNTP";TAB(100);"% dNDP";TAB(110);"%
    dNMP"
285 FOR I=1 TO N
290 LPRINT:LPRINT L$(I);TAB(50);
300 LPRINT USING "#######";CPMNTP(I);:LPRINT TAB(60);
310 LPRINT USING "#######";CPMNDP(I);:LPRINT TAB(70);
320 LPRINT USING "#######";CPMNMP(I);:LPRINT TAB(80);
330 LPRINT USING "#######";CPMT(I);:LPRINT TAB(90);
340 LPRINT USING "###.##";NTP(I);:LPRINT TAB(100);
350 LPRINT USING "###.##";NDP(I);:LPRINT TAB(110);
360 LPRINT USING "###.##";NMP(I)
370 NEXT I
375 CLS
380 LPRINT CHR$(12);CHR$(27);"E"
383 ERASE L$,CPMNTP,CPMNDP,CPMNMP,CPMT,NTP,NDP,NMP
385 CLS:INPUT "Are there more analyses to do";Y$
```

```
386 IF Y$="Y" OR Y$="y" THEN 5
387 SYSTEM
32767     END
```

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