DNA REPLICATION AND TELOMERE RESOLUTION IN VACCINIA VIRUS

A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Master of Science

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Department of Human Genetics

University of Manitoba

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DNA REPLICATION AND TELOMERE RESOLUTION IN VACCINIA VIRUS

BY

XUEKUN XING

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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TABLE OF CONTENTS

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Abbreviations

bp	base pair
BSA	bovine serum albumin
cpm	counts per minute
DEPC	diethypyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
h	hour
ITR	inverted terminal repeat
kDa	kilodalton
moi	multiplicity of infection
mRNA	messenger RNA
OD	optical density
ORF	open reading frame
PFGE	pulsed-field gel electrophoresis
pfu	plaque forming unit
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
ss	single stranded
SSC	standard saline citrate
TBE	tris borate/EDTA buffer
TE	Tris EDTA
TRT	telomere resolution target
ts	temperature sensitive
VETF	vaccinia early transcription factor
VITF	vaccinia intermediate transcription factor
VLTF	vaccinia late transcription factor
VP-16	etoposide

LIST OF FIGURES

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Abstract

The telomere of the linear double-stranded DNA genome of vaccinia virus strain WR consists of a covalently-closed hairpin end and two sets of near-terminal 70 base pair (bp) tandem repeats which are interrupted by a 325-bp non-repeat region called NR2 (Baroudy et al., 1982). During viral DNA replication, the linear genome is transiently converted to a high molecular weight linear concatemer, which is then resolved to a linear unit-length genome by cleavage and rejoining of replicated hairpin intermediates, a process called telomere resolution (reviewed in DeLange and McFadden, 1990). Previous studies in a monkey kidney cell line (BSC40) indicated that etoposide, an inhibitor of the cellular type II topoisomerase, inhibited telomere resolution of the newly replicated vaccinia virus genome, but had little or no effect on the initiation and elongation phases of DNA replication (DeLange et al., 1995). A drug resistant mutant was isolated and mapped to the vaccinia virus encoded DNA ligase gene. Three independent mutants were sequenced and each had a single point mutation. Pulsed-field gel electrophoresis (PFGE) along with restriction enzyme digestion and Southern blot analysis also demonstrate that the addition of etoposide to cells infected with the vaccinia virus strain WR29, a plaque purified isolate from strain WR with several sets of the NR2/tandem repeat sequence, strongly inhibited replication of the viral genome. Interestingly, a 1.6-Kb fragment which contains the NR2 and tandem repeat sequences was present in large amounts and had apparently been selectively amplified. Transfection of the plasmid which contains this 1.6-Kb sequence into vaccinia WR-infected rabbit cornea (SIRC) cells indicated that plasmid replication in these

cells is not inhibited under conditions where replication of the viral DNA genome is strongly suppressed. These observations suggest that the selective amplification of the 1.6-Kb fragment in monkey kidney cells was caused by selective replication of a plasmid that was generated in strain WR29 by homologous recombination between two successive NR2 regions. It is not clear why etoposide inhibits viral replication in some conditions but not in others.

Using pulsed-field gel electrophoresis (PFGE) along with restriction enzyme analysis, the vaccinia virus conditional lethal temperature-sensitive mutant ts793 was demonstrated to be defective in telomere resolution at the non-permissive temperature (40°C). It has the same phenotype as another ts mutant, ts9383 (Carpenter and DeLange, 1991). In contrast to other mutants of this phenotype, late protein synthesis was normal in both ts mutants. Each of the ts mutants had a single point mutation in the virus-encoded mRNA capping enzyme. Mutant ts9383 has been mapped to the small subunit of the mRNA capping enzyme (D12) and ts793 to the large subunit of the mRNA capping enzyme (D1). RNA analysis indicated that one of the late promoters which was located in NR1, the unique region distal to the tandem repeats, was poorly utilized by these two ts mutants at 40°C. This suggests that the vaccinia virus encoded mRNA capping enzyme has a direct role in the process of telomere resolution, possibly through the control of telomeric transcription.

Introduction

Understanding of mechanisms of DNA replication and recombination in prokaryotes and eukaryotes has always been a major goal for biological scientists. Several different models have been used to study these mechanisms. Much of our current knowledge of the mechanism of DNA synthesis derives from the prokaryote, *Escherichia coli (E.coli)*. The situation in higher eukaryotes is more complicated and more difficult to study. Virus research has opened a window for this eukaryotic research area. Depending on the host cells, a virus may use virus-encoded and /or host-derived replication factors. Therefore, virus research has proven to be a useful tool for understanding mechanisms of DNA replication in eukaryotes.

Poxviruses

The poxviruses comprise a large family of DNA viruses which infect both invertebrate and vertebrate hosts. They are the only DNA viruses which replicate entirely in the cytoplasm of infected animal cells (Moyer, 1987; Moss, 1990). These viruses are characterized by their large genome size, a complex virion, a linear double-stranded DNA genome and cytoplasmic site of DNA replication. Vaccinia virus, the prototypal member of the *Orthopoxvirus* genus, was the first animal virus to be seen under microscope, grown in tissue culture, accurately titered, physically purified and chemically analyzed (reviewed in Moss, 1990). Because of the cytoplasmic replication site, it is believed that most of the

proteins needed for the viral life cycle are virus-encoded (for example, vaccinia virus encodes its own DNA and RNA polymerases). Therefore, it is a very attractive model system for studying the mechanism of DNA replication, repair and recombination. In addition, the recent development of vaccinia virus as a gene expression vector may provide a powerful tool in the area of vaccines and gene therapy (Mackett and Smith, 1986; Moss and Flexner, 1987).

Morphology

Poxviruses are the largest animal virus that can be seen under the light microscope. Electron microscopic studies show that the virus particle is brick-shaped or ellipsoid, approximately 200-400 nanometers (nm) in length. The structure of the virus is complex since it is neither icosahedral nor has it helical symmetry as displayed by most other viruses. There is an outer envelope which encloses a dumbbell-shaped core structure and two structures of unknown function, the lateral bodies (Fig. 1). The core includes the double-stranded genomic DNA and proteins required for the early stage of the virus life cycle.

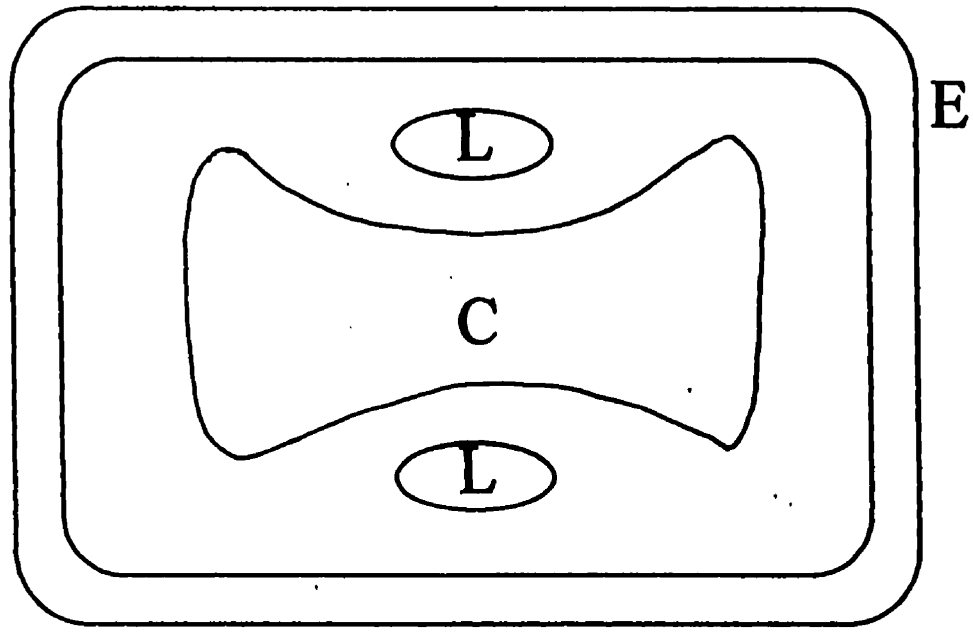

Figure 1. Illustration of vaccinia virus structure. C, core; L. lateral body; E, outer membrane (Modified from Moss, 1990a).

Genome structure

Vaccinia virus has a large double-stranded DNA genome which is 185-200Kb (1-2x10^5 kDa) in length. The Copenhagen strain has been completely sequenced and was found to be AT-rich (66.6%) and 191,636bp in length (Goebel et al., 1990). The vaccinia virus genome is characterized by: 1) terminal hairpin loop structures; 2) tandem repeats; 3) 10 Kb inverted terminal repeats; and 4) a highly conserved central region (Fig. 2). A terminal cross-link between the two complementary strands was demonstrated by denaturation of the viral genome followed by neutral gradient sedimentation (Szybalski et al., 1963; Jungwirth and Dawid, 1967) and by the failure of separation of the complementary DNA strands during alkaline gradient sedimentation (Berns and Silverman, 1970). The ability of sheared vaccinia DNA to "snap back" after denaturation definitely suggested a cross-link structure (Geshelin and Berns, 1974). The hairpin loops in vaccinia virus are A+T-rich (92%), 104 bp in length, and unable to form a completely base-paired structure. They exist in two isomeric forms, called "flip" and "flop" (Baroudy and Moss, 1982). A region close to the hairpin containing the essential sequences for telomere resolution is called telomere resolution target (TRT) (reviewed in DeLange and McFadden, 1990). Each end of the viral genome contains an identical, approximately 10 Kb, sequence which is called the inverted terminal repeat (ITR) (Garon et al., 1978; Wittek et al., 1978). Within this sequence, there exist several sets of tandem repeats. The number of repeats is variant in the poxvirus family. For example, this region in the vaccinia virus strain WR contains a set of thirteen 70-bp tandem repeats which are located very close to the hairpin structure and are followed by a unique region, 325 bp in length.

Adjacent to this unique sequence, there is the second set of eighteen 70-bp tandem repeats, two 125-bp repeats and eight 54-bp repeats (Baroudy and Moss, 1982: Baroudy et al., 1982) (Fig. 2).

Although the functions of the ITR are unknown, 37 of 38 *Orthopoxvirus* strains show cross-hybridization with the vaccinia virus 70-bp repeat. This suggests that the highly conserved region must have significant roles in the life cycle of the virus. Most of the essential genes were mapped to the central region of the genome where the gene density is very high with most coding regions separated by only a few base pairs (Esposito and Knight, 1985; reviewed in Moss, 1990). Non-essential genes which are believed to play roles in host range and tropism were mapped near the ends (reviewed in Turner and Moyer, 1990).

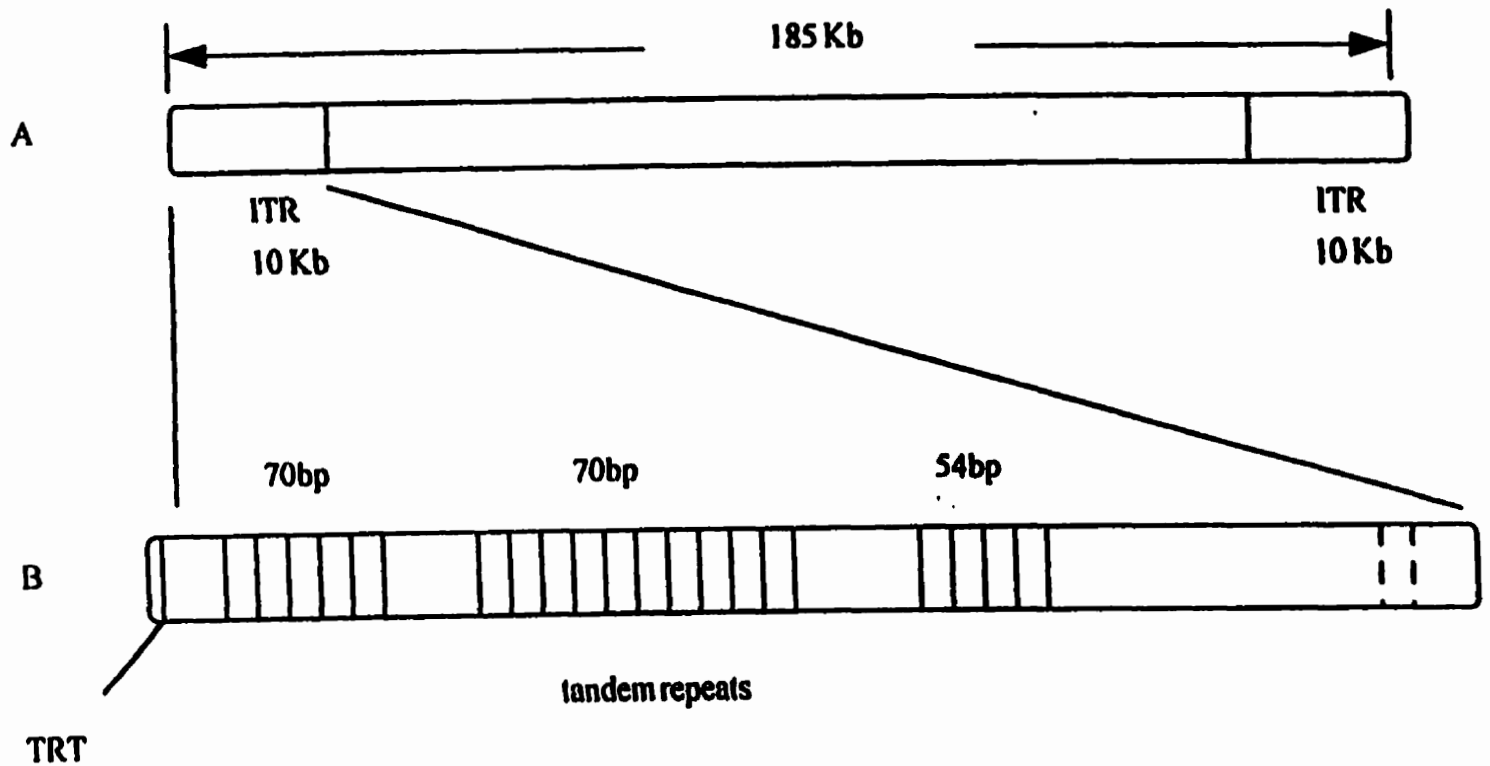

Figure 2. Vaccinia virus genome structure. (A) represents the whole 185-Kb length of the genome. The inverted terminal repeat (ITR) is located at both ends of the genome. (B) shows the sequence feature within the ITR. Close to the hairpin structure is the essential region for telomere resolution, the telomere resolution target (TRT). The two sets of 70 bp tandem repeats and the 54 bp tandem repeat are shown by the vertical lines (Modified from Moss, 1990a).

The life cycle of vaccinia virus

Members of the poxvirus family are unique among animal DNA viruses in that the entire multiplication cycle takes place in the cytoplasm of the host cells. In order to start the cycle, the virus particle makes contact with the cell surface and is then taken up by the cell (Fig. 3). The penetration process includes fusion of viral and cellular membranes (Dales and Kajioka, 1964; Chang and Metz, 1976). Following uncoating, the core of the virus is released into the cytoplasm of the host cell. Viral cores initiate early transcription and produce functional capped and polyadenylated mRNA within minutes post infection (reviewed in Moss, 1990). Almost half of the genome is transcribed at this early stage of the virus life cycle. The enzymes required for early transcription are present in the viral core and include RNA polymerase, mRNA capping enzyme and poly(A) polymerase (reviewed in Moss, 1990; Gershon and Moss, 1990). After the initiation of early gene expression, viral DNA replication starts. Many proteins involved in the process of replication are the products of early genes. The time of DNA replication varies within different members of the poxvirus family. DNA replication triggers the next step of gene expression, transcription of the intermediate class genes (Vos and Stunnenberg, 1988). At least three intermediate genes are required for the transcriptional activation of late gene expression (Keck et al., 1990). The products of two late genes that are packaged into the viral core serve to transcribe early genes in the next infection cycle. The last step of infection involves assembly in the cytoplasm, maturation and release of mature virion particles from the host cells (Fig. 3).

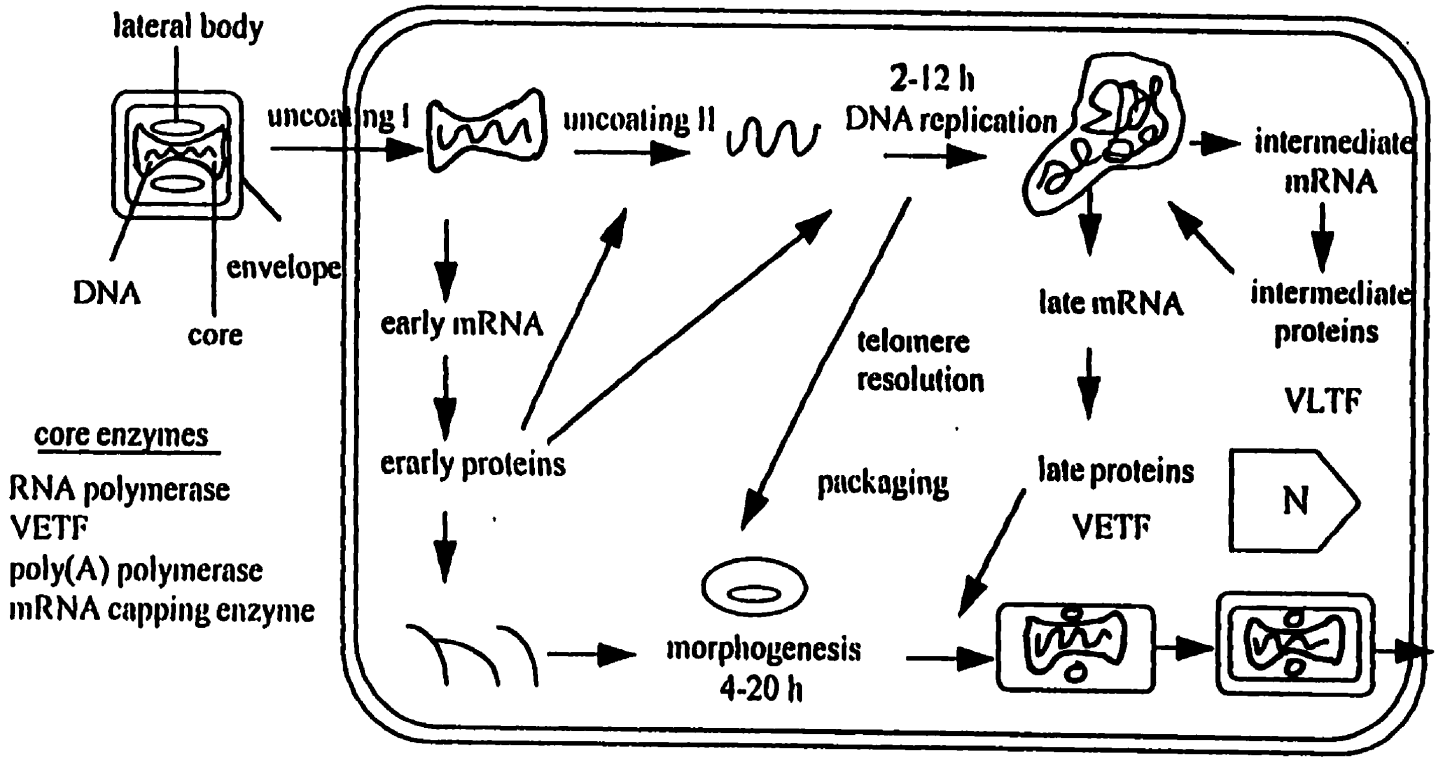

Figure 3. Life cycle of vaccinia virus. Arrows indicate the general order of each step. Abbreviations: VETF, VITF, VLTF, vaccinia early, intermediate and late transcription factors; N, host cell nucleus (Modified from Moss, 1990a).

Vaccinia virus DNA replication

DNA replication is a critical step in the life cycle of vaccinia virus as it makes the transition from early to intermediate/late gene expression. Much of our current knowledge of the mechanism of DNA replication is derived from prokaryotes, such as *E.coli*. The prokaryotic process requires an RNA primase, a DNA polymerase, a helicase, a DNA binding protein and a DNA ligase (reviewed in Nossal, 1992). For eukaryotes, these studies are generally more difficult because DNA replication is much more complex and it is difficult to isolate suitable mutants in diploid organisms. Vaccinia virus provides an excellent model for such studies because it appears to encode most or all factors required for transcription and replication of the viral genome (reviewed in Moss, 1990; Traktman, 1990).

The time of DNA replication varies within the poxvirus family and is dependent on the multiplicity of infection and the host cell type. In a synchronous infection of vaccinia virus, DNA replication takes place after 2-3 hours infection and can continue until 12 hours post infection after which DNA synthesis starts to slow down and a plateau in the amount of replicated DNA is achieved (reviewed in Traktman, 1990).

Because of the large genome size, it is not surprising that the detailed process of DNA synthesis is not completely understood. Several models for vaccinia virus DNA replication have been proposed: 1) the currently favored self-priming model (Cavalier-

Smith, 1974; Bateman, 1975) (Fig. 4) and 2) the de novo synthesis model (reviewed in Holowczak, 1982; reviewed in Baroudy et al., 1982). These models are discussed in detail below.

1) Self-priming model

Replication of the viral genome is believed to be initiated and terminated at or near the end of the genome (Esteban et al., 1977; Pogo et al., 1981, 1984). The model proposes that a nick is introduced at one end of the genome and a 3' OH end is produced for DNA polymerase access (Fig. 4, step 1). This 3' OH end acts as a primer (self-priming) and the complementary strand is used as the template for primer extension (Fig. 4, step 2). The precise sequence needed for the initiation of DNA replication is unknown. Elongation involves synthesis of a small stretch of DNA which contains the hairpin sequence. Because of the self complementarity, the elongated strand folds back on itself and again allows priming (Fig. 4, step 3). The elongation step is repetitious, allowing the formation of concatemers. It has been shown that the predominant form of poxvirus DNA during DNA replication contains the concatemeric structures (Moyer and Graves, 1981; DeLange 1989; Merchlinsky and Moss, 1989a) and that these structures can be detected by pulsed-field gel electrophoresis (DeLange 1989; Merchlinsky and Moss, 1989a). The last step of DNA replication is called telomere resolution (Fig. 4, step 5). This is a site-specific recombination event which occurs at the inverted repeat concatemer junction.

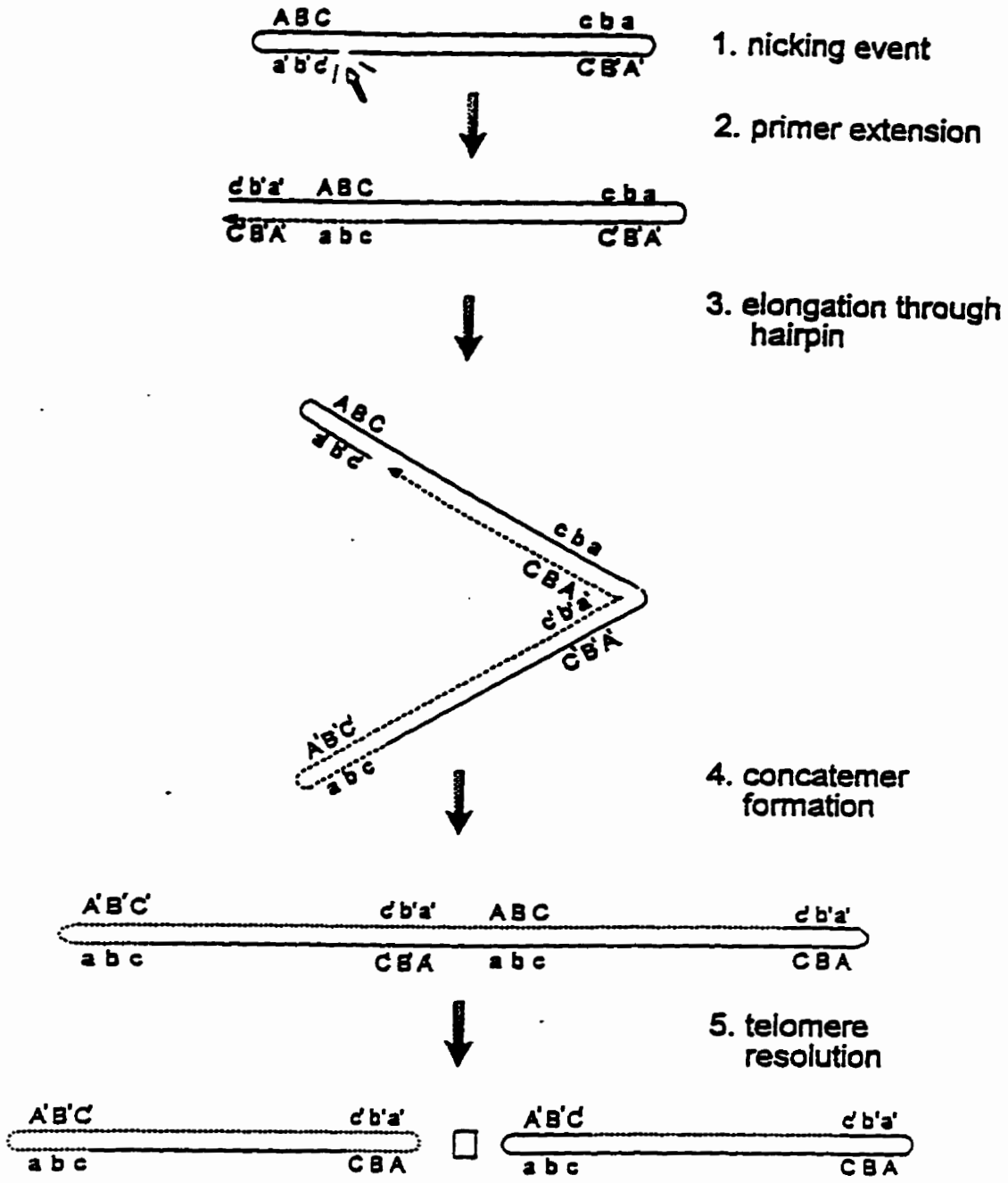

Figure 4. Self-priming model proposed for poxvirus DNA replication. ABC/A'B'C' are complementary to abc/a'b'c' and they are the sequences located near the hairpin termini. Arrows show the order of the replication process. Dotted lines represent the newly synthesized DNA. For simplicity, step 4 shows dimer formation although higher molecular weight concatemers are also produced during DNA replication (Modified from Traktman, 1990).

2) *de novo* synthesis model

The critical difference between the self-priming and the *de novo* synthesis models is the utilization of an RNA rather than a DNA primer for the initiation of DNA replication in the *de novo* synthesis model. In each case, the initiation site of replication is proposed to be at or near the end of the genome. Esteban et al. (1977) suggested that DNA synthesis starts at one end of the genome but Pogo et al. (1981) proposed bi-directional replication from both ends.

In both replication models, there is no need for lagging strand synthesis and telomere sequences are essential for initiation, elongation and termination of DNA replication.

Several vaccinia virus encoded enzymes are thought or known to be essential in the process of DNA synthesis. They are DNA polymerase, DNA ligase and topoisomerase I (reviewed in Traktman, 1990).

Action of etoposide on vaccinia virus DNA replication

Epipodophyllotoxin etoposide (VP-16), an antitumour drug which targets the cellular type II topoisomerase, has recently been shown to inhibit vaccinia virus telomere resolution in BSC40 cells. This drug does not appear to affect the initiation and elongation

phases of viral DNA replication (DeLange et al., 1995). This non-intercalating drug affects mammalian DNA topoisomerase II in a specific manner. It inhibits the breakage-rejoining reaction of cellular topoisomerase II by stabilizing the cleaved reaction intermediate (Fig. 5). Topoisomerase II is required for releasing intertwined double-stranded DNA molecules during segregation of replicated daughter chromosomes. Etoposide acts by binding to and stabilizing the DNA-topoisomerase II complex and thereby preventing the resealing step of this complex. This block is lethal to proliferating cells (reviewed in Liu, 1989).

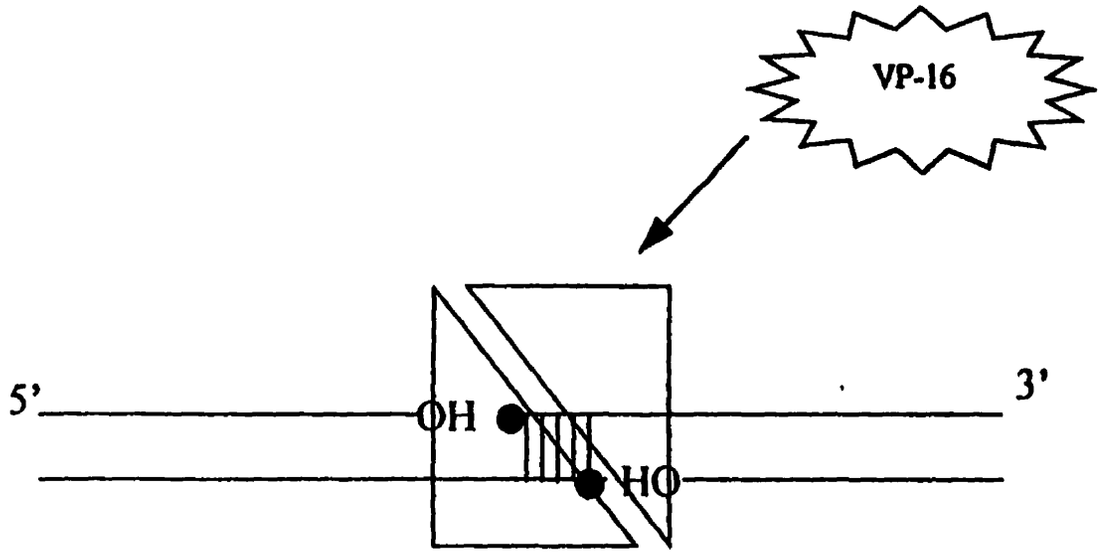

Figure 5. A partial reaction of mammalian DNA topoisomerase II (topoII) and double-stranded DNA and the effect of VP-16. Mammalian DNA topoisomerase II is a homodimer which is indicated by two identical triangles. This enzyme catalyzes double-stranded DNA breaks and VP-16 is proposed to stabilize the topoII/DNA "cleavable complex" and blocks the strand-passing reaction (Modified from Liu, 1989).

Vaccinia virus telomere

At least two families of eukaryotic DNA viruses have hairpin termini: parvoviruses and poxviruses (reviewed in Berns, 1990; DeLange and McFadden, 1990). Telomeres have been functionally defined as that region of DNA at the molecular end of a linear eukaryotic chromosome that is required for replication and stability of that chromosome (Blackburn and Szostak, 1984). The telomere of poxvirus is defined as the noncoding region which is close to the end of the genome. It includes three parts: 1) the terminal cross-link which is an AT-rich, incompletely base-paired hairpin structure; 2) the telomere resolution target (TRT) region which has the function of segregation into replicated daughter hairpin-terminated molecules; 3) several sets of 70-bp tandem repeats. Understanding the mechanism of telomere resolution has been one of the elusive goals of poxvirus scientists and the detailed steps of this process are still unknown.

Poxvirus telomeres are cross-linked at the end of the genome. Figure 6 shows the sequence of "flip" and "flop". The terminal cross-link has been identified in every vertebrate poxvirus tested, one or more members of orthopoxviruses, parapoxviruses, avipoxviruses, leporipoxviruses, and several unclassified poxviruses. The terminal cross-link is indeed a universal feature in the poxvirus family (reviewed in DeLange and McFadden, 1990).

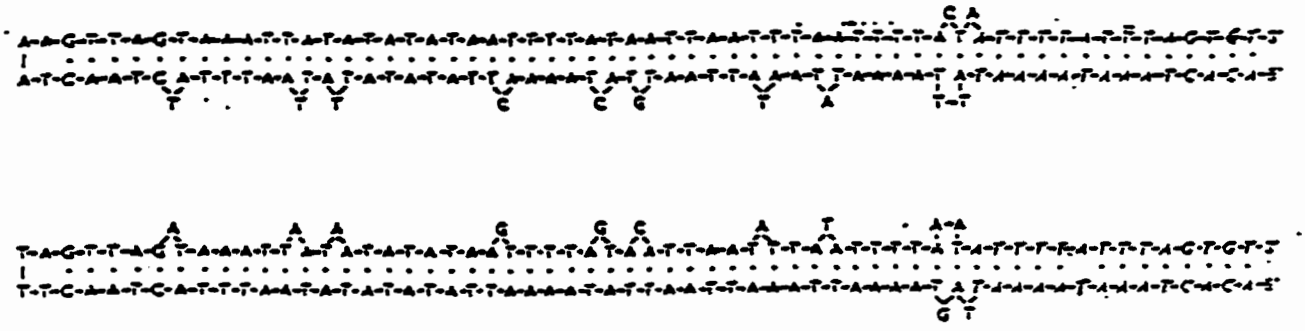

Figure 6. DNA sequence of the vaccinia virus telomere. The upper and lower sequences indicate two inverted and complementary conformations which are called "flip" and "flop". Base-paired sequences are connected by a dot (Baroudy et al., 1982).

The conformation of poxvirus telomeres changes during DNA synthesis. The mature virus genome contains a hairpin structure at both ends. During DNA replication, this hairpin is converted to an inverted repeat conformation which has its axis of symmetry at the original hairpin end (Fig. 7). This inverted repeat conformation was first found in rabbitpox virus (Moyer and Graves, 1981) and was also identified in vaccinia virus (Baroudy et al., 1982; Moss et al., 1983) and Shope fibroma virus (SFV) (DeLange et al., 1986). This telomere junction could be generated by either the self-priming or de novo synthesis models.

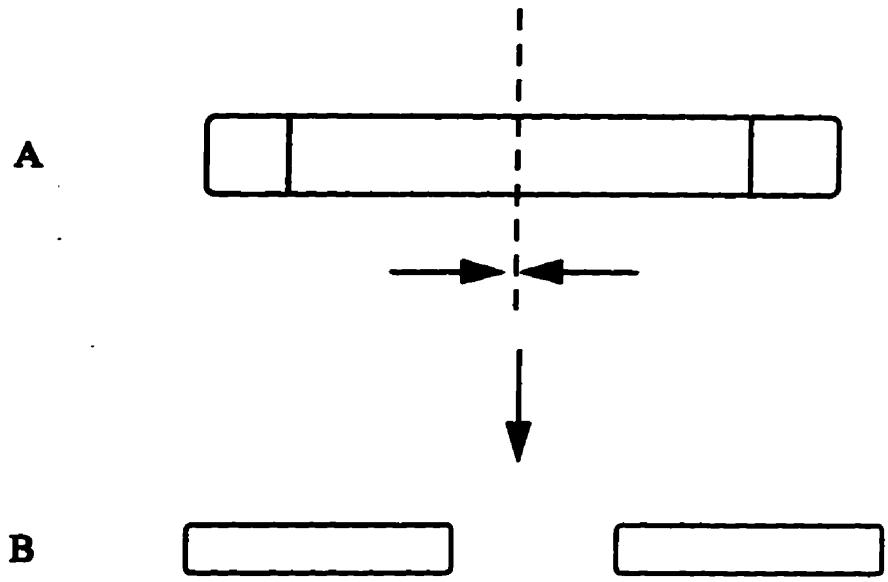

Figure 7. Genome of the telomere junction of vaccinia virus. Panel "A" represents the inverted repeat of the telomere fusion which is present in the high molecular weight replicative intermediate. The dashed line represents the axis of the symmetry. Panel "B" indicates the two resolved mature monomers.

In *vivo* resolution of cloned poxvirus telomere fusion

Understanding the mechanism of telomere resolution of poxviruses would be greatly facilitated by cloning of the telomeric region. This is a difficult process because of the "snap-back" structure of the hairpin termini. DeLange et al. (1984, 1986) used the ability of yeast to maintain long inverted repeats within plasmid vectors. Gel-purified hairpin-containing restriction fragments were ligated into a linearized yeast cloning vector and transformed into a recipient yeast strain. During replication in yeast, the hairpin end was replicated into the inverted repeat conformation but was unable to resolve back into hairpin ends. This replicated intermediate conformation was then subcloned into a high copy number bacterial plasmid and transformed into a recombination-deficient *E.coli* strain (DeLange et al., 1986). These cloned replicative intermediates are called telomere fusions or concatemer junctions. They have provided the substrates for in *vivo* testing of telomere resolution.

Replication of plasmids in the nuclei of mammalian cells requires an origin of replication (Kucherlapati and Skoultchi, 1984). The observation that plasmids replicate in poxvirus infected mammalian cells in a sequence non-specific manner (DeLange and McFadden, 1986) provided a powerful tool for understanding the mechanism of poxvirus telomere resolution. It was shown that telomere fusion containing plasmids transfected into poxvirus infected mammalian cells first replicated into head-to-tail high molecular weight concatemers and then resolved into linear hairpin-terminated minichromosomes (DeLange et al., 1986) (Fig. 8). Plasmids failed to replicate in the cytoplasm of uninfected

cells and the cloned telomere fusions were not resolved to mature monomers. This observation suggested that the factors required for replication and resolution are virus-encoded. Furthermore, the mechanism of telomere resolution must be highly conserved since a plasmid containing the Shope fibroma virus telomere fusion could also be resolved in vaccinia virus infected mammalian cells (DeLange et al., 1986).

Using the above transfection assay, the telomere resolution target (TRT) sequence was identified (DeLange et al., 1986; DeLange and McFadden, 1987; Merchlinsky amd Moss, 1986; 1989). The telomere fusion of vaccinia virus and SFV were subjected to unidirectional deletion from either end or bi-directional deletion from the *Afl*II site at the axis of symmetry (DeLange et al., 1986; DeLange and McFadden, 1987). The deletion constructs were used to transfect poxvirus infected cells. Replicated plasmids were then analyzed. In addition, Merchlinsky (1990) used point mutation analysis to determine the critical sequences for telomere resolution. It was shown that in SFV, a domain of 58-76 bp which is close to but does not include the nonpalindromic nucleotides in the viral hairpin, is essential for virus telomere resolution (Fig. 9). The sequence in this domain must be present in the inverted repeat conformation. Although a 20-bp region (region I and IA) is enough to direct some of the telomere resolution, the efficiency of this event increases with the additional regions (region II and III), especially for SFV. For vaccinia virus, a 20-bp sequence representing regions I and IA appears to be sufficient to promote telomere resolution (Merchlinsky and Moss, 1989b; Merchlinsky, 1990).

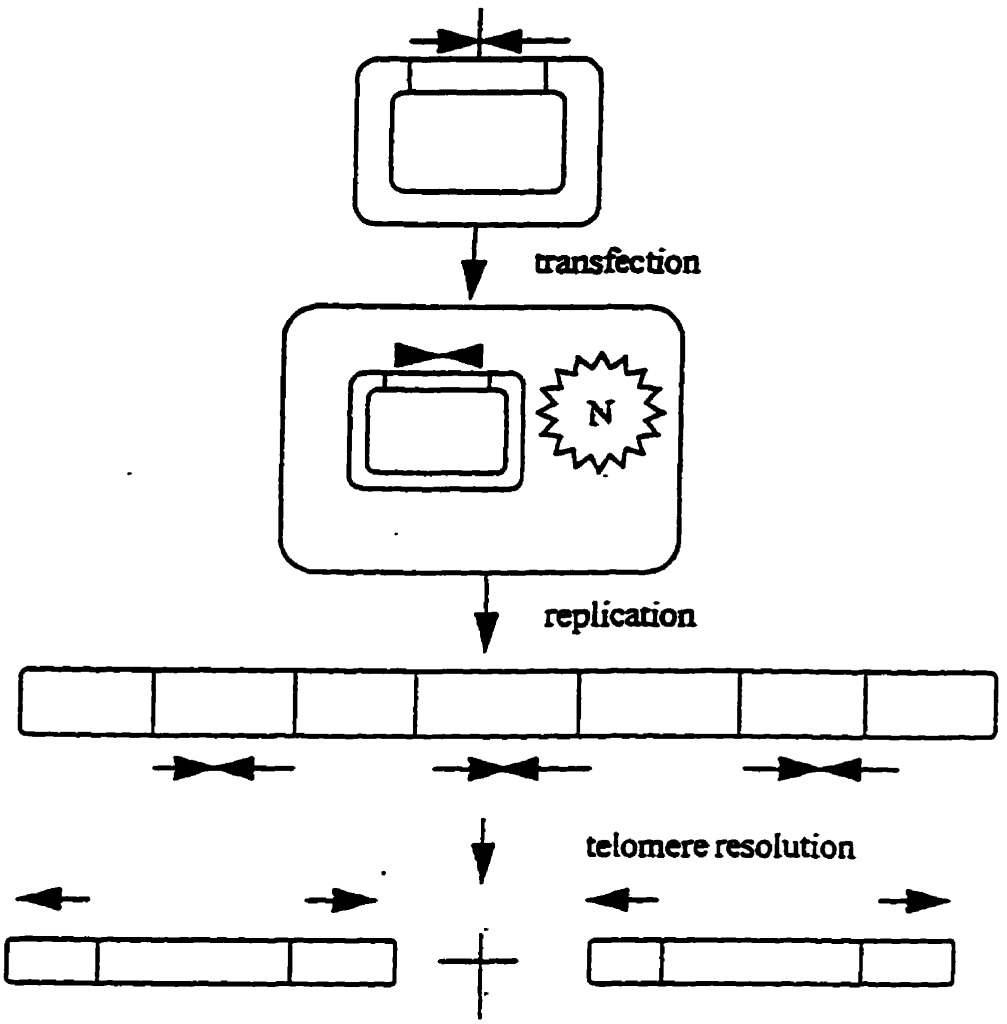

Figure 8. Plasmid transfection assay of poxvirus telomere resolution. The TRT region of vaccinia virus and Shope fibroma virus were cloned into a PUC vector and transfected into poxvirus-infected mammalian cells. The plasmid replicated into high molecular weight concatemers and then resolved to mature monomers. N indicates the nucleus of the mammalian cell.

The nucleotide sequences in regions I/IA in vaccinia virus, Shope fibroma virus, raccoon poxvirus and cowpox virus are exactly identical. Regions II and III in the above viruses show a high degree of similarity (Fig. 9). This suggests that the mechanism of telomere resolution in the poxvirus family is conserved.

Mutational analysis by Merchlinsky (1990) indicated that the critical sequence for telomere resolution in regions I and IA is (A/T)TTT(A/G)$N_{7-9}A_7$, where N represents any nucleotide. Davison and Moss (1989) found that the TRT domain showed sequence similarity with vaccinia virus late promoters. Parsons and Pickup (1990) also found that although the sequence composition of the *Orthopoxvirus* telomeres is highly conserved, these regions do not appear to encode any proteins. At late time of infection, the telomeres of vaccinia virus are transcribed and the initiation of the transcription is located in two non-repeated regions, NR1 and NR2. Sequence analysis showed that in both the two NR1 and NR2 regions there is a late promoter element (5'-ATTTA-3') which could be a potential transcriptional starting site. The length of these late transcripts suggests that the transcription of telomeres is toward the hairpin-loop and that some transcripts cross the junction (Parsons and Pickup, 1990). The functions of these late transcripts are unknown. They may have a role in vaccinia virus telomere resolution.

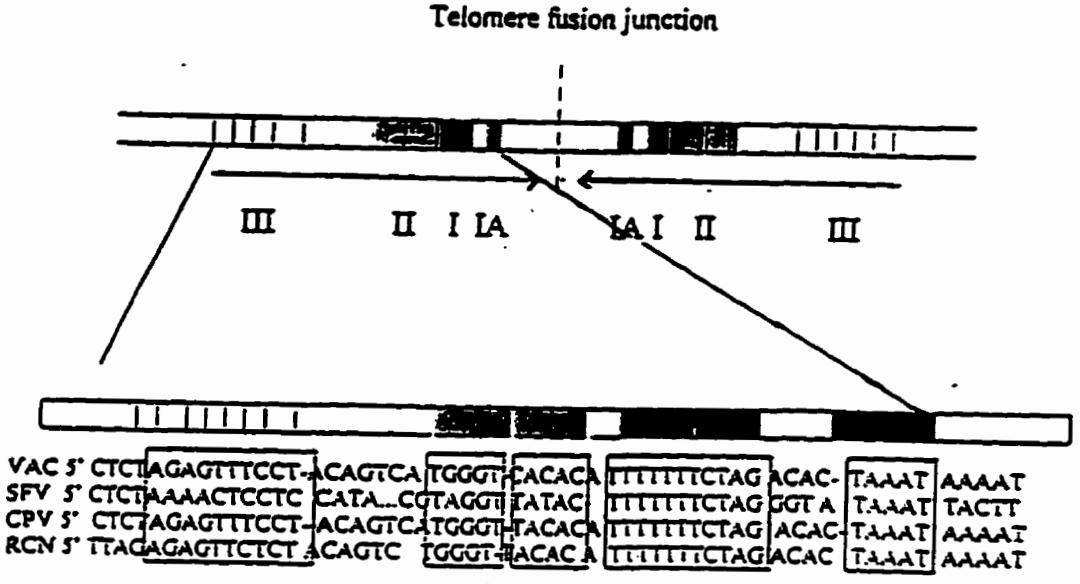

Figure 9. Boundaries and DNA sequence of the telomere resolution target (TRT) region: IA to III. The axis of symmetry is represented by the dotted line. One entire TRT region is drawn at the bottom, and the DNA sequence of vaccinia virus (VAC), Shope fibroma virus (SFV), cowpox virus (CPV) and raccoon poxvirus (RCN) is shown. Conserved regions in the poxvirus family are boxed (modified from DeLange and McFadden, 1990).

Mutants defective in telomere resolution

A combination of genetic and molecular approaches would be powerful tools to study the mechanism of poxvirus telomere resolution. The characterization of temperature-sensitive and drug-resistant mutants has the potential to identify the genes and proteins that are involved in the resolution process. DeLange (1989) identified six temperature-sensitive mutants which are defective in telomere resolution. Those six mutants are divided into three functional groups (DeLange, 1989).

1) TsC21, tsC53 and tsC7 (C means that the mutants are from the Condit collection) are defective in both intermediate and late protein synthesis. The proteins responsible for the tsC53 and tsC7 are known as the 147-kDa and 22-kDa subunits of the virus-encoded RNA polymerase (Thompson et al., 1989; Hooda-Dhingra et al., 1989). TsC21 is defective in the 21-kDa subunit of the virus encoded RNA polymerase (Seto et al., 1987).

2) TsC22 has a normal switch from early to late gene expression. However, shortly after initiation of late gene expression, protein synthesis suddenly stops. Studies have shown that this "abortive late" phenotype was due to the rapid degradation of RNA at late times of virus infection (Pacha and Condit et al., 1985). The gene which is responsible for this phenotype was identified as ORF A18R (Pacha et al., 1990) and is now known to encode a protein with DNA helicase (Simpson and Condit, 1995) and DNA-dependent ATPase (Bayliss and Condit, 1995) activities.

There is a common feature in groups (1) and (2): defective post-replicative protein synthesis. This observation suggests that post-replicative gene expression is necessary for telomere resolution (DeLange, 1989). However, mutants in the group described below are definitely different from those in group (1) and (2).

3) TsC63 and ts9383 display a normal switch from early to post-replicative protein synthesis. TsC63 has been mapped to ORF A1L, which encodes a late transcription factor (Keck et al., 1990; Wright and Coroneos, 1993) and ts9383 was mapped to ORF D12(Carpenter and DeLange, 1991),which codes for the small subunit of the mRNA capping enzyme (Niles et al., 1989). Mutant tsC63 was shown to have normal intermediate gene expression but very much reduced late RNA and protein synthesis (Carpenter and DeLange, 1992). Mutant ts9383 was unique since both intermediate and late gene expression were essentially unaffected. Therefore, the protein involved in this phenotype, the mRNA capping enzyme, may have a direct role in the process of telomere resolution.

Models for telomere resolution

Three models have been proposed for telomere resolution (Fig. 10).

1) <u>Nicking and refolding model.</u> This model is a version of the terminal palindrome model of Cavalier-Smith (1974), as modified for hairpin termini (Bateman, 1975). In this model, telomere resolution begins when two specific nicks are introduced at the terminal

region of the viral genome on opposite strands. Because of the self-complementarity, the separated strands can refold and DNA ligase seals the nicks to form two monomers.

2) **Nicking/strand exchange/branch migration model.** In this model, the initiation nicking event also occurs on each strand, close to the hairpin termini. It is followed by strand exchange and branch migration to produce the "flip" and "flop" conformation (McFadden, et al., 1988; reviewed in DeLange and McFadden, 1990). Two important factors required by this model are a branch migrating activity necessary to segregate the two supercoiled strands in the process of branch migration and the symmetrical nicking at or near the TRT region.

3) **Cruciform extrusion model.** The requirement for helix unwinding is the first step in this model. This unwinding happens at the central axis of the hairpin terminus region to generate Holliday junctions (McFadden and Morgan, 1982; McFadden et al., 1988). This Holliday junction can then be cleaved by an enzyme which has the activity of T4 endonuclease VII or T7 endonuclease I

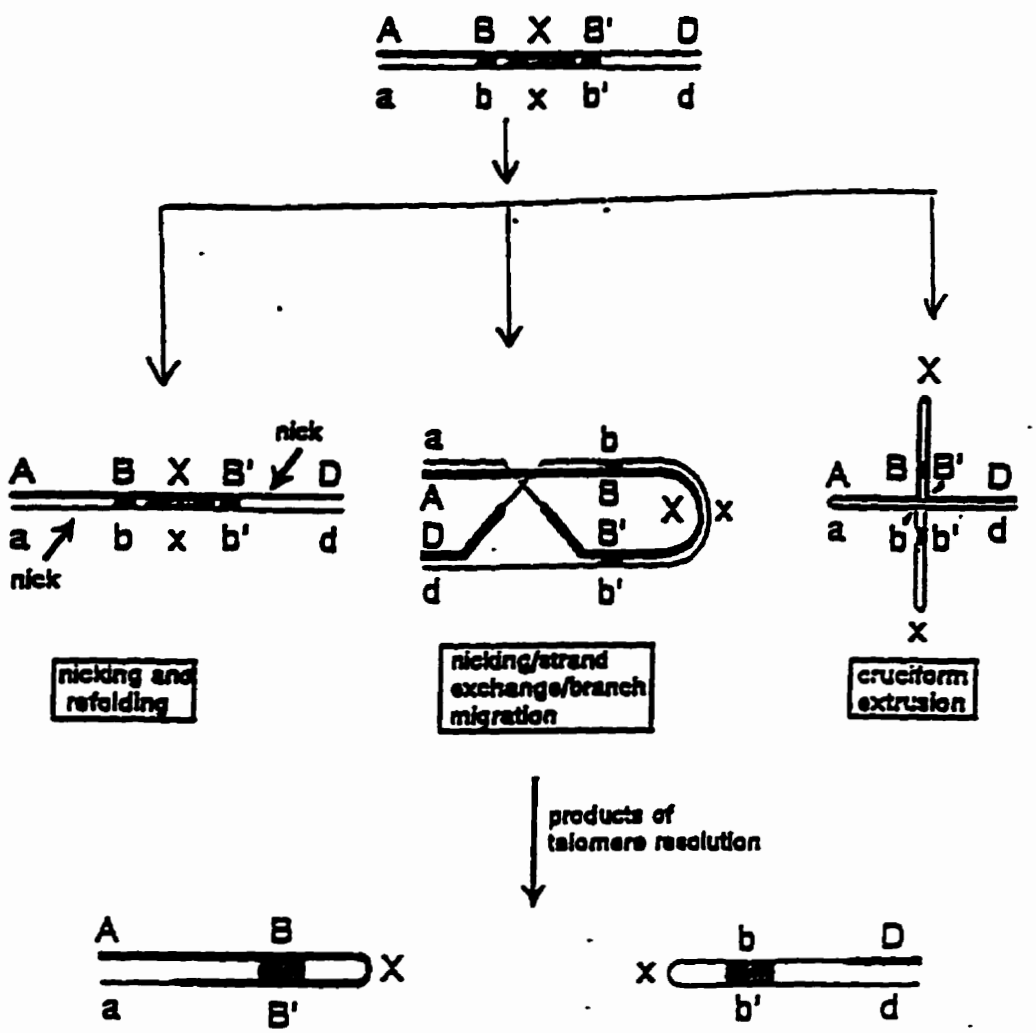

Figure 10. Three models proposed for telomere resolution. The upper portion of the figure represents the replicative intermediate of the vaccinia virus telomeric junction. The axis of symmetry of the fusion is indicated by "X". The complementary strands are represented by either A, B, B', D or a, b, b', d. The TRT sequence is shaded. The middle portion of this figure shows the three models of telomere resolution: nicking and refolding (left), nicking/strand exchange/branch migration (center) and cruciform extrusion (right). Nicks are indicated by the arrows. The lower portion shows the products of telomere resolution: mature monomers which have the complete loop at both ends of the genome (Modified from Merchlinsky, 1990).

Goals

To gain a better understanding of the mechanism of poxvirus telomere resolution.

Telomere resolution can be affected indirectly by inhibition of late gene expression. In an attempt to identify trans-acting resolvase proteins, we have screened ts mutants and drugs inducing a defect in telomere resolution without concomitant inhibition of late gene expression. The drug etoposide displayed this phenotype as does mutant ts9383 when grown at the nonpermissive temperature.

Part I describes studies aimed at gaining a better understanding of the effect of etoposide on vaccinia virus replication and telomere resolution. I have used Southern blotting to examine its effect on DNA replication of two viral strains along the length of the viral genome. I have also used DNA sequencing to further characterize etoposide resistant mutants of vaccinia virus.

Part II describes characterization of telomere resolution and transcription of telomeric transcripts of ts9383 and of another recently isolated mutant, ts793.

PART I

STUDY OF THE EFFECT OF ETOPOSIDE ON DNA REPLICATION OF TWO VACCINIA VIRUS STRAINS

Materials and Methods

Source of Materials

All the restriction enzyme, T4 ligase, T7 RNA polymerase, SP6 RNA polymerase, Klenow enzyme and Taq polymerase which were used in this study were purchased from either Boehringer Mannheim Canada (Laval, Quebec), Gibco/BRL (Burlington, Ontario) or Pharmacia Canada Inc. (Baie d'Urfe, Quebec). Radioisotope α^{32}P-dATP, α^{32}P-dCTP and α^{32}P-CTP were obtained from Dupont/New England Nuclear (Mississauga, Ontario). Etoposide (VP-16) powder was a gift from Bristol-Myers/Squibb. Nylon membrane for DNA and RNA transfer was from BioRad (Mississauga, Ontario) and the Riboprobe kit was from Promega (Madison, Wisconsin).

Africa green monkey kidney (BSC40) cells were originally obtained from Dr. Richard Condit (University of Florida, Gainesville, Florida), and rabbit cornea cells (SIRC cells) were kindly donated by Dr. David Evans (University of Guelph, Ontario).

1. Cell culture

A. Media and reagents

Dulbecco's Modified Eagle Medium (DMEM)

DMEM was made by dissolving 20 g Dulbecco's Modified Eagle (DME) powder (Sigma) and 7.4 g sodium bicarbonate in 2 liters of sterilized deionized water and pH was adjusted to 7.0. 50 IU/ml penicillin (Flow), 50 mcg/ml streptomycin (Flow) and 15 mg/ml Phenol red were added to the medium. The medium was sterilized by passing through a 0.22 um filter and stored at 4°C. A specific percentage of fetal bovine serum (FBS) from Gibco/BRL was added to the medium just before use.

Phosphate buffered saline (PBS)

1 liter of PBS was made up by dissolving 2 g NaCl, 1.14 g Na_2HPO_4, 0.2 g KCl and 0.2 g KH_2PO_4. The pH was adjusted to 7.0 and the medium was autoclaved and stored at 4°C.

Phosphate buffered saline with Ethylenediamine tetraacetic acid (PBS+1 mM EDTA)

PBS was made up with autoclaved EDTA at a finial concentration of 1 mM.

Etoposide (VP-16) stock solution

Etoposide (VP-16) powder was stored at -20°C and was dissolved in sterile, autoclaved dimethyl sulfoxide (DMSO) immediately before use to get a stock concentration of 25 mg/ml.

B. Maintenance of tissue culture cells

BSC40 cells

BSC40 cells were maintained in DMEM+5% FBS. When the cells were confluent in a T150 (150 mm) flask (Corning), the medium was aspirated and the flask was washed twice with 10 ml of PBS+EDTA. 5 ml PBS along with 0.5 ml 2.5% trypsin (Gibco) was added to the flask, the cap of the flask was sealed tightly and the flask was incubated at 37°C for 2 minutes. Cells were checked under a microscope to make sure they were rounded. Five ml of pre-warmed DMEM+5% FBS was then added to the flask and the flask was shaken several times, followed by trituration of remaining cell clumps. At this time, BSC40 cells were ready for laying down plates for further experiments. Also, 0.5 ml of cells were left in the flask and 30 ml pre-warmed fresh DMEM+5% FBS was added. The flask was then incubated at 37°C in a 5% CO_2/95% air incubator.

SIRC cells

The method used for maintaining SIRC cells was the same as that for BSC40 cells except that DMEM+10% FBS+ 1x non-essential amino acids (Gibco) was used instead of DMEM+5% FBS.

C. Preparation of plates and dishes

BSC40 cells and SIRC cells were maintained a described above. Cells were ready to split when they were complete monolayers. One T150 flask was able to make three 150 mm plates, four 100 mm plates, three 6-well dishes, or four 24-well dishes by the method described above.

D. Virus

Vaccinia virus strain WR, designated WR6 in this thesis, was provided by Dr. Richard Condit. Both the virus stocks of WR6 and WR29 were stored in a -80°C freezer.

Crude Virus Preparation

This method was described previously by DeLange (1989). Briefly, when BSC40 cells were confluent, virus stocks were taken from the -80°C freezer and thawed in a 37°C waterbath. After 1 minute indirect sonication (the virus stocks were floated on ice-cold water and put into a Sonifier cell disrupter, model W140 with sonic power above 80), virus was diluted in pre-warmed Hanks balanced salt solution (Gibco)+0.1% bovine serum albumin (BSA). The medium from 6-well dishes was removed and the BSC40 cells were infected at a low multiplicity (0.05 to 0.1 pfu per cell). The 6-well dishes were incubated at 37°C for 30 to 60 minutes with gentle rocking every 15 minutes. The virus was then

aspirated and 2 ml of pre-warmed DMEM+5% FBS was added to each well. Incubation continued for 48 to 72 hours until a noticeable cytopathic effect had taken place. The cells were then scraped off with an autoclaved rubber policeman, transferred to a clean 15 ml Corning tube, and centrifuged for 5 minutes at 2,000 rpm in a Beckman JA-10 rotor at room temperature. The supernatant was aspirated and the pellet was resuspended in 1 ml of ice-cold PBS by briefly vortexing. This suspension was subjected to three cycles of freeze/thawing to lyse the cells and release the virus. Each cycle included freezing at -80°C for 30 minutes to overnight and rapid thawing in a 37°C waterbath. After the last cycle of thawing, cellular debris was spun down by centrifugation for 5 minutes at 2,000 rpm in a JA-10 rotor. The supernatant was aliquoted into a 1.5 ml microcentrifuge tube. This aliquot was now ready for titration. The virus stock was stored at -80°C.

Virus Titres

Virus titres were determined in 24-well or 6-well dishes (6-well dishes were used if a high accuracy was needed). When BSC40 cells had grown into a monolayer, the virus stock was taken from -80°C freezer, melted in a 37°C waterbath, and sonicated for 1 minute. Serial dilutions, usually ranging from 10^{-1} to 10^{-6} were made in Hanks balanced salt solution + 0.1% BSA. The medium was removed from the cell monolayers and the diluted virus solution was added to each well (150 ul per well for a 24-well dish and 330 ul per well for a 6-well dish). After 30 to 60 minutes incubation at 37°C with gently rocking each 15 minutes, the virus was aspirated and pre-warmed DMEM+5% FBS was

added to each well. Incubation was continued for 48 hours at 37°C. After 48 hours of infection, the medium was removed and the infected cell monolayers were fixed with neutral buffered formalin (Sigma) for 5 to 10 minutes at room temperature and stained with 0.1% crystal violet. The number of plaques in each well were counted and the results were expressed as plaque forming units/ml (pfu/ml) of virus preparation.

Viral DNA isolation and purification

Isolation and purification of the viral DNA according to the method described by Esposito et al. (1981). Briefly, eight 150 mm tissue culture plates were used for the infection. Monolayers of BSC40 cells were infected at a multiplicity of 0.1 pfu/cell. The plates were incubated at 37°C for 2-3 days until a severe cytopathic effect was observed. The cells were then harvested with a rubber policeman into 50 ml Coring tubes. Cells were pelleted by centrifugation at 2,000 rpm for 15 minutes and resuspended in 20 ml of ice-cold isotonic buffer (10 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA). They were re-pelleted by centrifugation at 2,000 rpm for 5 minutes and resuspended in 9 ml of ice-cold hypotonic buffer (10 mM Tris pH 8, 10 mM KCl, 5 mM EDTA). The cell suspension was then left to incubate on ice for 10 minutes to allow swelling of the cells. Twenty-five ul β-mercaptoethanol and 1 ml Triton X-100 were then mixed in by gentle pipetting. The above mixture was put on ice for 10 minutes and subjected to centrifugation at 2,000 rpm for 5 minutes at 4°C (Beckman JA-20 rotor). The supernatant was transferred to a fresh tube and spun in a Beckman JA-20 rotor at 1,600 rpm for 1 hour at 4°C. The pellet was

resuspended in 0.8 ml ice-cold core buffer (100 mM Tris pH 8, 1 mM EDTA) with 15 ul β-mercaptoethanol, 50 ul Proteinase K (10 mg/ml), 200 ul of 20% (w/v) N-lauroyl sarcosine. The mixture was incubated at 4°C for 30 minutes and 1.4 ml 54% (w/v) sucrose solution was added. Incubation lasted 2 hours at 55°C after which 400 ul of 5 M NaCl was added to the solution to prevent non-specific binding of proteins to viral DNA. The solution was extracted once each with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (49:1). The viral DNA was precipitated with one tenth the volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol at -20°C for several hours. The pellet was then resuspended in 100 ul of TE.

2. Pulsed-Field Gel Electrophoresis (PFGE)

A. Preparation of plugs

This method was described by DeLange (1989). Briefly, monolayers of BSC40 cells in 6-well dishes were infected with vaccinia virus at a multiplicity of 10 pfu/cell. After 30 minutes of infection, virus was removed and pre-warmed DMEM+5% FBS with different concentrations of VP-16 was added to each well. After 24 hours of infection at 37°C in a 5% CO_2 incubator, the BSC40 cells were scraped off with an autoclaved rubber policeman and transferred into 15 ml Coring tubes. Cells were spun for 5 minutes at 2,000 rpm (Beckman JA-10 rotor) at room temperature and the supernatant was removed by aspiration. The pellet was resuspended in 100 ul ice-cold PBS/40 mM EDTA. After briefly vortexing, the pellet was incubated at 42°C for 5 minutes and then mixed with 125 ul of

1.4% low-melting-point (LMP) agarose gel. The above mixture was triturated with a cut-off Eppendorf pipet tip. The mixture was then transferred to a plastic mold (about 110 ul/well). The agarose was allowed to set at room temperature for 15 minutes and the plugs were then transferred into tubes containing 3 ml of a Proteinase K solution (100 ug/ml Proteinase K, 10 mM Tris-HCl, pH 7.5, 0.18 M EDTA and 1% sarkosyl). After overnight incubation in a 50°C waterbath, the Proteinase K solution was removed and the plugs were equilibrated in 0.1 M TBE (0.1 M Tris-HCl, 0.1 M boric acid, 0.2 mM EDTA, pH 8.3-8.5) with gently shaking at 4°C. The 0.1 M TBE was changed three times every eight hours. After the last buffer change, the plugs were ready for PFGE. Plugs were stored in 0.1 M TBE at 4°C.

B. Gel formation and electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed in an LKB 2015 Pulsaphor electrophoresis apparatus as described by DeLange (1989). Half of the plugs were sealed with 1% low-melting-point (LMP) agarose gel in the wells of a 1.5% 0.1 M TBE agarose gel. First, electrophoresis was performed in a uniform field of 5 V/cm for 30 minutes in 0.1 M TBE buffer. The gels were then pulsed for 20 hours at 25 or 100second intervals and 200 mA with an hexagonal attachment. The cooling water circulation set at 8°C was connected to the gel running apparatus, maintaining the running buffer at 15°C. After 20 hours of electrophoresis, gels were stained with 0.5 ug/ml ethidium bromide in 0.1 M TBE, photographed and then transferred to a nylon membrane.

3. Polymerase chain reaction (PCR)

Two PCR products were generated in this study. The method used for PCR was as described by Ausubel et al. (1987). The Taq DNA polymerase was purchased from Pharmacia. In the first PCR, the two primers were primer 1 (29 mer): 5' AAGGGTTAGAACAGTATTTGTGTGTGATC and primer 2 (29 mer): 5' GAATAGTACCCCCATTACAAGAGCTACA. The use of these primers resulted in amplification of a 2.8 Kb region within the inverted terminal repeat but outside the second set of tandem repeats of the vaccinia virus genome. There was a single *SalI* digestion site within the PCR product. The PCR conditions were 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 47°C for 2 minutes, 72°C for 3 minutes and 1 cycle of 72°C for 7 minutes. In the second PCR, the vaccinia virus DNA ligase gene was amplified from plasmid pJB333S. The two primers used in this reaction were LigN (26 mer): 5' GATAACATATGACGTCACTTCGCGAA (A was substituted for G to disrupt palindromic structure of this DNA region) and LigC (29 mer): 5' GTTTCCTCGAGAGATTTAGTCAAGTTTAC. LigN and LigC generated a *NruI* and a *XhoI* site respectively (restriction recognition sites are underlined). The PCR conditions were 95°C for 5 minutes, 25 cycles of 95°C for 1.5 minutes, 61°C for 2 minutes, 72°C for 2 minutes and 1 cycle of 72°C for 7 minutes. The PCR products were checked on a 0.7% agarose gel with 0.5% ug/ml ethidium bromide stain.

4. Restriction enzyme digestion, DNA purification and plasmid construction

A. Restriction enzyme digestion

Restriction enzymes were used according to the manufacturer's instruction. By definition, 1 U of enzyme digests 1 ug of DNA in 1 hour under appropriate conditions. Excess enzyme was added in order to obtain complete digestion.

For PFGE, half of the plug was put into a 1.5 ml microcentrifuge tube and quickly melted in a 70°C waterbath. The tube was then transferred to a 42°C waterbath where it was left to cool down for at least 5 minutes. A mixture of restriction enzyme plus buffer was simultaneously incubated at 37°C for 5 minutes. The enzyme mixture and the melted plug were mixed and the digestion was allowed to proceed at 40°C for 2 to 4 hours. After digestion, the DNA fragments were separated by electrophoresis in a 0.7% agarose gel.

B. DNA purification

Following electrophoresis, the band of interest was stained by 0.5 ug/ml ethidium bromide and visualized with a hand-held long-wavelength UV lamp. The band of interest was excised with a clean scalpel, and placed in a plastic mold which was sealed with 1% low-melting-point agarose and left for 10-15 minutes in the dark. A layer of Spectrapor-4 cellulose dialysis membrane (MW cutoff of 12-14 kDa; Spectrum Medical Industries, Inc,

Los Angelos) was then placed between the mold and a second plastic mold which were then held together with two rubber bands. The mold unit was immediately placed in the electrophoresis chamber and the dialysis membrane was never allowed to dry. A small amount of 1x TAE buffer was added into a space between the membrane and the plug. Electrophoresis was carried out in the dark room for 5 to 15 minutes (depending on the size of the band) at 150 V. Progress was monitored by detection of the ethidium bromide stained DNA band with a hand-held long-wavelength UV lamp. When all of the DNA ran out of the agarose gel, the current was reversed for 15 seconds to remove DNA from the membrane. The liquid between the membrane and the plug was collected into a 1.5 ml microcentrifuge tube. After a phenol/chloroform and chloroform extraction, DNA was precipitated by addition of 1 in 10th volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol followed by incubation at -80°C for 15 minutes. DNA was washed once with 75% ethanol and lyophilized. The pellet was dissolved in sterile water.

C. Plasmids

Plasmids p1369 and p1322, obtained from Dr. David Pickup (Duke University), contain the tandem repeat region and NR2, respectively (Parsons and Parsons, 1990; Hu and Pickup, 1991).

Three plasmids were constructed. First, the 1.6-Kb etoposide amplified fragment was cloned into pGEM-7Z. The 1.6-Kb insert was obtained by digestion of purified viral DNA with *Alu*I and a linear pGEM-7Z vector (obtained from Dr. E.V.Vrotakis) was

obtained by digestion with *Sma*I. Blunt end ligation was set up by standard methods: a final reaction volume of 10 ul contained insert and vector (6:1 molar ratio with approximately 100 ng of vector), 1 ul of 10x ligase buffer (200 mM Tris-HCl pH 8, 50 mM $MgCl_2$, 50 mM dithiothreitol), 1 ul of 5 mM ATP and 0.8 U of T4 DNA ligase. The ligation was set up at room temperature for 16-24 hours. The resulting plasmid was named pGEM-1.6.

A second plasmid was generated by ligation of the 2.8-Kb PCR product, from the terminal inverted repeat region, directly into the PCR™ II vector using the TA cloning kit from Invitrogen. This system takes advantage of the non-template dependent activity of the thermostable polymerase used in PCR which adds single deoxyadenosines to the 3'-end of the vectors. Because of A-T complementary, this facilitated the ligation efficiency. The ligation was carried out at room temperature for 16 hours. The resulting plasmid was called pPCR2.8.

A third plasmid was generated by ligation of the second PCR product (vaccinia virus DNA ligase gene) into pET21a. Both were digested with *Nru*I and *Xho*I. Sticky end ligation was set up according to standard methods: a final reaction volume of 10 ul contained insert and vector (3:1 molar ratio with approximately 100 ng of vector), 1 ul of 10x ligase buffer, 0.5 ul of 5 mM ATP and 0.1U of T4 DNA ligase. The ligation was set up at 16°C for 4-16 hours. The construct was named pXK.

The first subclone was used for transfection assays and the second and third clones were used as hybridization probes.

5. Bacterial transformation

A. Solutions

SOB consisted of 2 g Bacto-tryptone, 0.5 g Bacto-yeast extract, 0.06 g NaCl and 0.018 g KCl in 100 ml water. This solution was autoclaved, and stored at 4°C. A separate solution of 1 M $MgCl_2$ plus 1 M $MgSO_4$ was made, filter sterilized, and stored at 4°C. Just before use, a 100x dilution of the magnesium stock was added to the tryptone solution.

SOC was the same as SOB except that it also contained 20 mM glucose.

Luria Bertani (LB) medium was made up with 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl in 1 L water, and adjusted to pH 7.5. The medium was autoclaved and stored at 4°C.

LB plates consisted of 15 g of agar for every 1 L of LB medium. The solution was autoclaved and cool in a 60°C waterbath. Antibiotic (usually carbenicillin) was added to the medium at a final concentration of 40 ug/ml. The plates were poured in a hood, set at room temperature for 30 minutes and stored at 4°C. Just before use, the plates were incubated at 37°C upside down for 20 minutes.

B. Preparation of competent cells

E.coli strain DH5α was used in the transformation. Briefly, a single colony of DH5α was cultured in 5 ml LB medium at 37°C with moderate shaking for 5 hours to overnight. Two and a half ml of the culture was transferred into 500 ml of LB medium. Growth was continued to an OD of 0.5 to 0.7. The cells were chilled in an ice-cold waterbath for 10 to 15 minutes and centrifuged at 4,200 rpm for 20 minutes at 2°C in a Beckman J-6M centrifuge. The supernatant was removed and the pellet was washed with 5 ml of ice-cold water. The pellet was resuspended in 500 ml of ice-cold water and the above centrifugation step was repeated twice. After the last step of centrifugation, the pellet was suspended in 4 ml of ice-cold 10% glycerol, mixed well, distributed in 50 ul aliquots, flash frozen in liquid nitrogen and stored at -80°C.

C. Transformation by electroporation

For each transformation, 50 ul of competent DH5α cells were used. The competent cells were taken out from -80°C, thawed at room temperature and put on ice. For each transformation, a 0.1 cm gap cuvette was treated with UV light for 10 minutes and placed on ice for another 10 minutes. One ul of the ligation mixture and 50 ul of competent cells were transferred to a pre-treated cuvette, triturated, and pulsed with the BIORAD Gene Pulser set at 25 uF capacitance, 1.25 kV and 400 Ω. The cells were resuspended in 1 ml SOC medium at room temperature and transferred to a Falcon 2059 snap cap tube. To allow phenotypic lag, the tube was gently shaken at 37°C for 1 hour.

Generally, 10 ul aliquots with 200 ul SOC were used to plant the LB plates. The planted plates were incubated at 37°C overnight. The transformants were then picked and colonies containing the desired recombinant plasmids were identified.

D. Plasmid DNA preparation

Plasmid DNA was prepared according to the alkaline lysis method of Birnboim and Doly (1979). Briefly, 5 ml of overnight culture medium was collected in a 1.5 ml microcentrifuge tube by 20 second centrifugation. The supernatant was removed and the pellet resuspended in 100 ul ice-cold solution I (50 mM glucose, 50 mM Tris-HCl pH 8, 10 mM EDTA) with vortexing and set at room temperature for 5 minutes. Two hundred of fresh solution II (1% SDS in 0.2 M NaOH) was added and the tube was gently inverted 6-8 times and placed on ice for 5 minutes. This was followed by the addition of 150 ul ice-cold solution III (3 M potassium acetate, pH 4.8). The tube was mixed well by inversion and put on ice for 5 minutes. After 10 minutes centrifugation at room temperature, the clear supernatant was carefully transferred to a new eppendorf tube and an equal volume of pheno/chloroform/isoamyl alcohol (25:24:1) was added. The solution was mixed by vortex, and the two layers were separated by centrifugation at room temperature for 5 minutes. The supernatant was transferred to a fresh eppendorf tube and plasmid DNA was precipitated by adding two volumes of absolute ethanol. The liquid was mixed by vortexing and placed at room temperature for 5 minutes, followed by centrifugation at room temperature for 10 minutes. The supernatant was removed and the pellet was washed with 70% ethanol, dried under vacuum and dissolved in 100 ul of TE+ RNaseA

(10 mM HCl pH 7.5, 0.1 mM EDTA, 10 ug/ml RNaseA). The concentration of DNA was determined by comparison to a *Hind*III digested λ marker stained with 0.5 ug/ml ethidium bromide in a 0.7% agarose gel. The plasmid DNA was stored at 4°C.

6. Southern blotting

This method was based on Southern (1975). After restriction enzyme digestion, DNA samples were separated by electrophoresis in a 0.7% agarose gel. The gel was stained with 0.5 ug/ml ethidium bromide in TAE and photographed prior to transfer. The gel was irradiated with short wavelength UV light for 4 minutes and soaked in 0.25 M HCl for 10 minutes with gentle agitation. The gel was rinsed once with water and denatured for 30 minutes in 0.2 N NaOH/ 0.6 M NaCl prior to transfer to Zetaprobe (Bio-Rad) membrane. The gel was transferred upside-down in 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for approximately 16 hours. The nylon membrane was washed in 2x SSC for 10 minutes at room temperature with gentle shaking and air dried for 10 minutes. The filter was baked for 30 minutes at 80°C in a vacuum oven and stored at 4°C in a plastic bag. At this stage, the membrane was ready for hybridization.

7. DNA probe and hybridization

DNA probes were labeled according to the random primer method of Feinberg and

Vogelstein (1983). A 14 ul mixture of random primer and 25 to 250 ng of probe DNA was boiled for 3 minutes and placed on ice. Klenow buffer, dGTP and dTTP mixture, α^{32}P-dATP and α^{32}P-dCTP with 3 to 8 U Klenow were added to the boiled solution for a final volume of 25 ul. The reaction was carried out at room temperature for 2 to 4 hours and stopped by the addition of 1 ul of 0.5 M EDTA. The reaction was placed in a 65°C waterbath for 10 minutes. The probe was passed through a Sephadex G-50 Nick column (9x20 mm, Pharmacia) to remove unincorporated radioactive nucleotides. The efficiency of labeling was determined by the Beckman LS 1801 liquid scintillation system.

Pre-hybridization and hybridization were carried out as recommended by the manufacturer. The nylon membrane was sealed in a plastic bag in a prehybridization solution (7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4). Any air bubbles were removed. Pre-hybridization was carried out at 65°C for 1 hour. After pre-hybridization, the solution was removed and replaced with hybridization solution [fresh hybridization solution (150 ul/cm^2) containing an appropriate amount of probe (1x 10^6 cpm/ml)]. Hybridization was performed at 65°C overnight with gentle agitation.

Following hybridization, the nylon membrane was washed in 40 mM $NaHPO_4$ (pH 7.2)/ 1 mM EDTA/ 5% SDS at room temperature for 30 minutes. The membrane was rewashed at 65°C for 30 minutes. After washing, the membrane was air-dried briefly on Whatman 3 MM filter paper, resealed in a fresh plastic bag and exposed to X-ray film. Exposures were at -80°C for 1 hour to overnight, dependent on the signal intensity.

8. Transfection assay

The transfection method was as described by DeLange (1989). SIRC cells were cultured with DMEM+10% FBS+1x nonessential amino acids. Twenty-four hours before transfection, the medium was removed and fresh medium was added to each 6-well plate. Cell monolayers were used at 60-80% confluence for the virus infection. Plasmid DNA was prepared as described above. Plasmid DNA (1 ug/well) was precipitated by the addition of 150 ul of 2x NNH (50 mM HEPES [N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic, pH 7.1], 280 mM NaCl, 1.5 mM Na_2HPO_4). and an equal volume (150 ul) of 250 mM $CaCl_2$ (fresh stock prepared just before each experiment). This mixture was placed at room temperature for 1 hour. At the same time, SIRC cells were infected with vaccinia virus at a multiplicity of 1 pfu per cell at 37°C using the method described above. After 1 hour of precipitation and infection, the virus was removed and the precipitated DNA (300 ul) was added to each well. The 6-well plates were placed at 37°C for 30 minutes. This was followed by the addition of 3.3 ml of culture medium, with or without different concentrations of etoposide, to each well. Incubation was continued for 3 to 4 hours at 37°C. Medium was aspirated and 2 ml of fresh medium, with or without etoposide, was added to each well. After 24 hours infection, DNA was harvested for PFGE or restriction enzyme digestion.

9. DNA sequencing

Sequencing of cloned vaccinia DNA ligase genes from three independent etoposide resistant mutants (pDLL15, pDLL23, pDLL31) was performed by the Sanger dideoxy chain-termination method (Sanger et al., 1977). Plasmid pDLL17, which contains a point mutation in the previously sequenced DNA ligase gene, was used as a control. Plasmid DNA was purified as described above. The Sequenase kit (US Biochemical version 2.0) was used for all sequencing reactions. The primers used in sequencing were

Lig-L: 5' GGAATTCTAATGATATCCGCGAACG 3'

Lig-6R: 5' GTCCTAATGCCTATGATGTG 3'

Lig-6L: 5' CATGGGATTAATTGGAGTC 3'

Lig-9R: 5' GTCTATCGTATTGGATTCTG 3'

Lig-15R: 5' CATCGTTAATTGGTCTTGC 3'

Lig-R: 5' GGCGACCACCAACTAAAT 3'

The location of the primers and relative to the DNA ligase gene is shown in Figure 11. and Appendix.

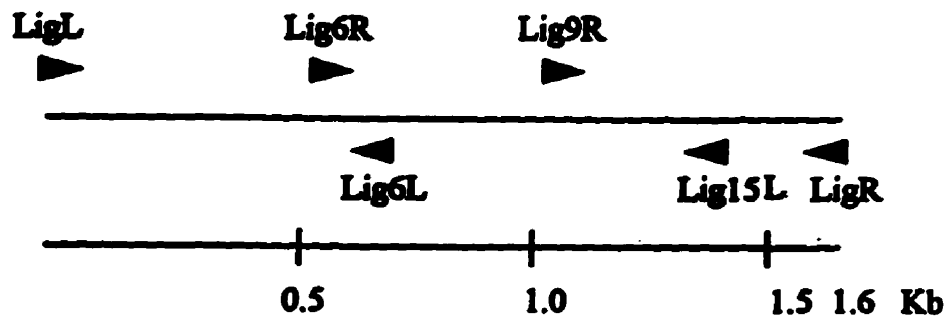

Figure 11. Primer locations in vaccinia virus DNA ligase gene. The ligase gene is 1.6 Kb in length. The name of each primer and the location is indicated by the arrows.

Results

It has been shown previously that etoposide, a cellular type II topoisomerase inhibitor, inhibits vaccinia virus telomere resolution in BSC40 cells. It has no apparent effect on the rate of virus genome DNA replication (DeLange et al., 1995). This is the situation for the standard vaccinia virus strain WR, here called WR6. We had a variant vaccinia virus strain (WR29) which was plaque-purified from WR6. We noticed that, in contrast to WR6, DNA replication of WR29 appeared to be inhibited by etoposide. Pulsed-field gel electrophoresis (PFGE) along with restriction enzyme digestion, Southern blotting and transfection assay have provided us with some understanding of the genome structure of this variant strain and the effect of etoposide on its genome replication. At the same time, sequencing results indicated that drug-resistance was due to point mutations in the DNA ligase gene.

Etoposide inhibits vaccinia virus genome replication of WR29 but not WR6

Epipodophyllotoxin etoposide (VP-16), an inhibitor of the cellular type II topoisomerase (reviewed in Liu, 1989) can inhibit telomere resolution of vaccinia virus but has little effect on the rate of DNA replication and gene expression (DeLange et al., 1995).

WR29 is a recently plaque purified isolate of vaccinia virus WR (WR6). We

adapted the technique of pulsed-field gel electrophoresis (PFGE) to evaluate the quality and quantity of vaccinia virus DNA replication of both strains. This is a rapid, accurate and convenient method to detect virus genome replication and the conversion of high molecular weight concatemers to mature monomers (DeLange, 1989; Merchlinsky and Moss, 1989a).

In this study, four different probes were used for the Southern blot hybridization after PFGE (Fig. 12). Three correspond to loci within the 10-Kb inverted terminal repeat (ITR) region and the other to a locus in the internal region of the viral genome. The sequence in the first probe is homologous to non-repeat region 2 (NR2) which is present in plasmid p1322 (Parsons and Pickup, 1990). The plasmid for the second probe contains a set of 70 base pair tandem repeats and is named p1369. The third probe within the ITR region is a 2.8-Kb PCR product cloned into the pGEM-7Z vector and called pPCR2.8. The probe to the internal region of the genome is called pXK and contains the sequence of vaccinia virus DNA ligase gene.

To ascertain the effects of etoposide on replication of the viral DNA genome, monolayers of BSC40 cells were infected with either WR6 or WR29 at a multiplicity of 10. After 24 hours of infection, replicated viral DNA was analyzed by PFGE and hybridization to the four probes described above (Fig. 13). The patterns of DNA replication with etoposide were identical for WR6 when the near-terminal probes p1322 and p1369 were tested (Fig. 13A). In the absence of drug, the newly replicated 185-Kb

mature monomer was detected. When etoposide was added, it strongly inhibited monomer formation. At ≥ 50 ug/ml of etoposide, detection of the mature monomer was negligible. At the same time, a strong hybridization signal corresponding to replicated viral DNA was present in the well. To detect viral DNA replication in the central region of the genome, this filter was reprobed with the internal pXK probe which is located about 33 Kb from the right end of the genome (Fig. 12). Using this internal probe, we again detected inhibition of monomer formation and replicated DNA remained in the well. At higher concentrations, efficiency of replication was somewhat reduced (Fig. 13A). Therefore, in the presence of etoposide, the vaccinia virus genome does replicate although with a reduced efficiency, but it forms high molecular weight replicative intermediates which fail to migrate into the gel. This is consistent with results previously obtained with the tandem repeat probe (DeLange et al., 1995).

The replication patterns of WR29 are totally different than those of WR6 (Fig. 13B). Probes p1322 and p1369 gave the same results with WR6 and WR29. However, hybridization of these blots with the internal pXK probe demonstrated that neither mature monomer nor well DNA was produced, suggesting that DNA replication in this region was strongly inhibited by etoposide. Vaccinia virus DNA replication is thought to start at the end of the genome and to proceed toward the interior (Fig. 4; self-priming model). We wanted to determine whether the elongation step of DNA replication in WR29 was blocked with etoposide. We therefore used a probe, pPCR2.8, which is located about 2 Kb outside of the tandem repeat region. Interestingly, this probe gave the same result as

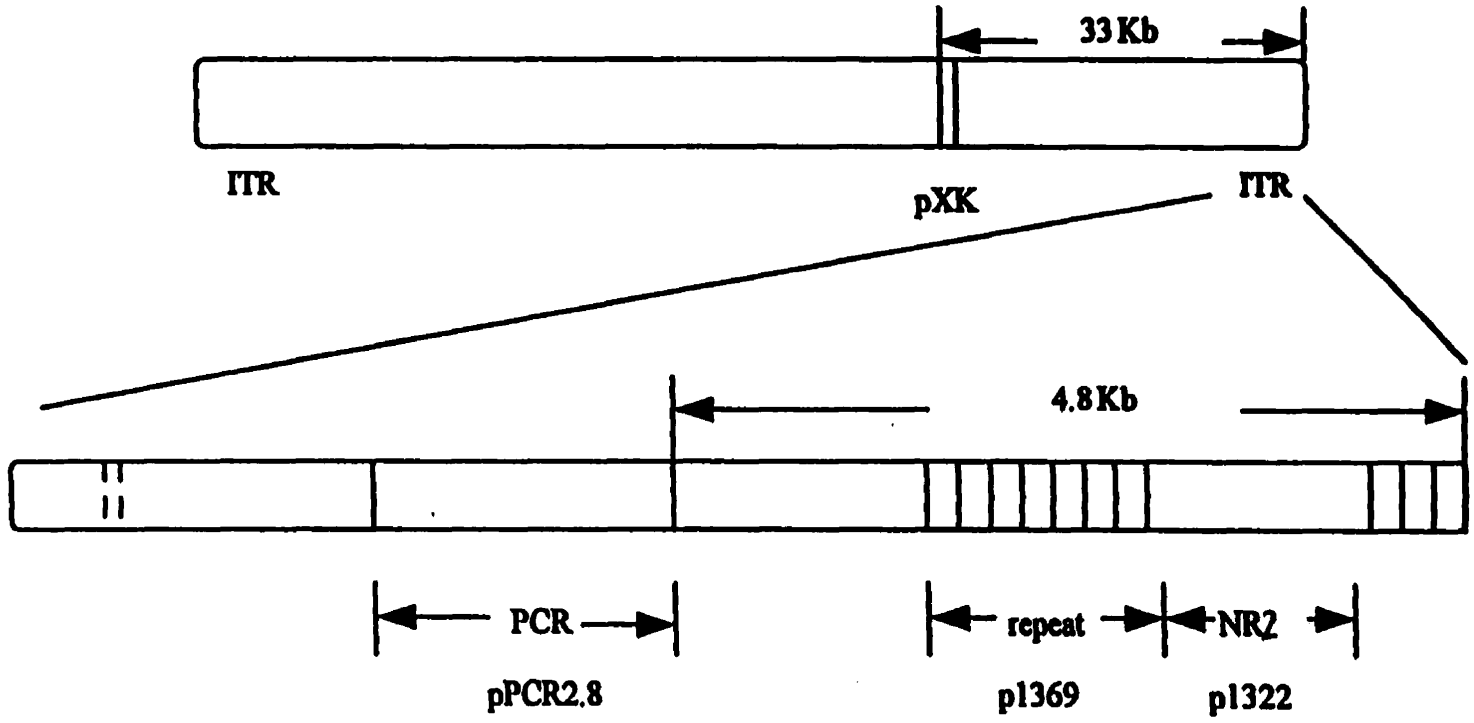

Figure 12. Locations of the four probes corresponding to loci in the vaccinia virus genome. Three loci are within the 10-Kb inverted terminal repeat (ITR) region. The corresponding probes are: p1322 (NR2), p1369 (tandem repeat) and pPCR2.8. The probe corresponding to the internal region of the genome is named pXK and consists of the vaccinia virus DNA ligase gene cloned into the pET21a vector. The distance of pPCR2.8 from the hairpin end is 4.8 Kb and the distance of pXK from right hairpin end is 33 Kb (diagram not to scale).

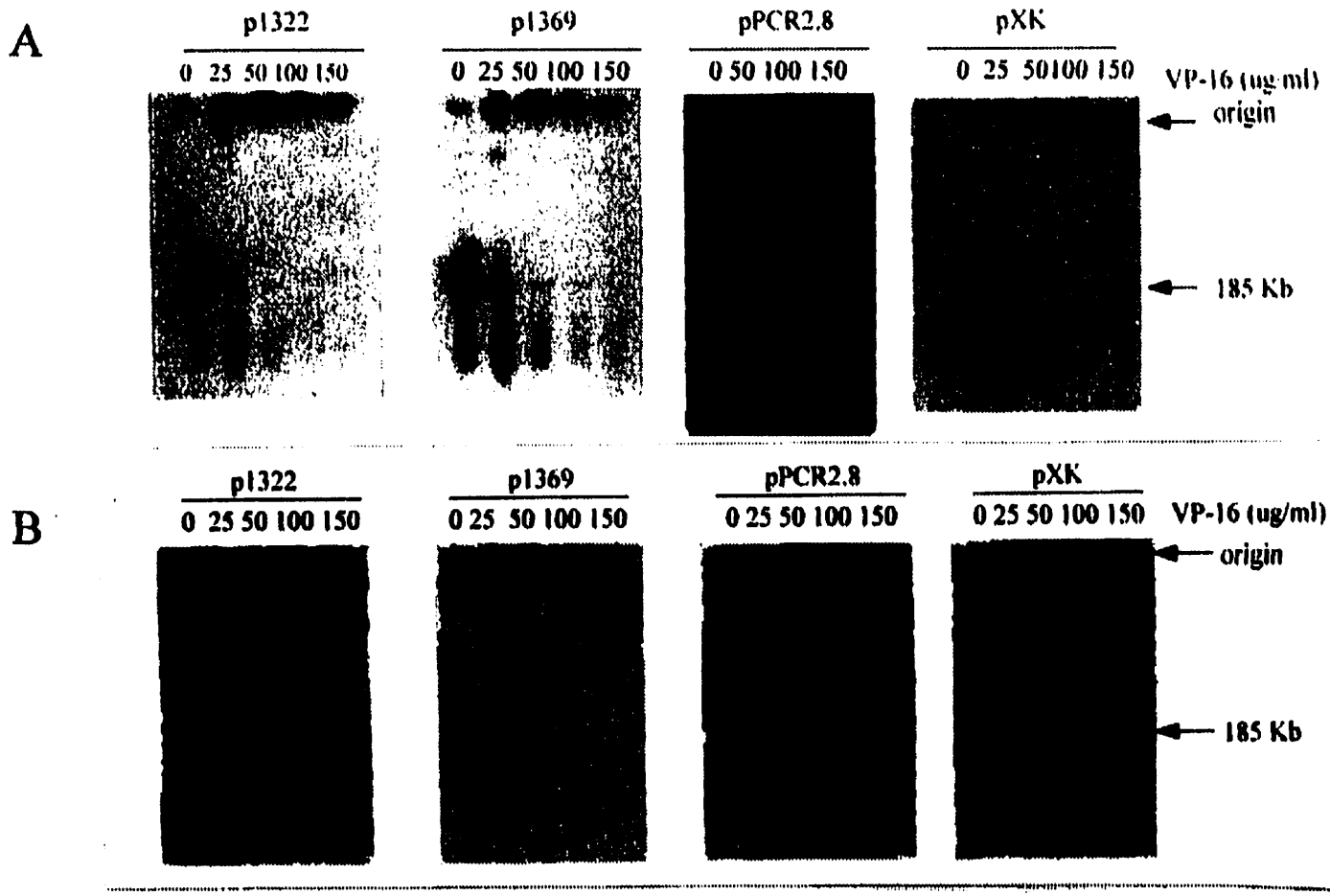

Figure 13. Effects of etoposide on DNA replication of two vaccinia virus strains. Pulsed-field gel electrophoresis (PFGE) of vaccinia virus DNA from BSC40 cells infected with WR6 (A) or WR29 (B) with different concentrations of etoposide ranging from 0 to 150 ug/ml, as indicated at the top of each panel. Confluent BSC40 cells were infected at a multiplicity of 10 pfu/cell. After 24 hours of infection, monolayers were harvested to make plugs and subjected to PFGE. DNA was then transferred to nylon membrane and hybridized with ^{32}P-labeled p1322 (NR2), p1369 (tandem repeat), pPCR2.8 or pXK probes. The migration of the mature monomer (185 Kb) and the gel origin are indicated.

that observed with the pXK probe (Fig. 13B). Little or no replicated or well-DNA was detected in the presence of etoposide. This suggests that, in the presence of etoposide, the replicated portion of the WR29 genome includes NR2 and tandem repeats, but not the rest of the viral genome. This was an unexpected finding since previous studies with WR6 had demonstrated that etoposide had little or no effect on the initiation and elongation phases of vaccinia virus DNA replication. In order to further confirm the effect of VP-16 on DNA replication of these two viral strains, the DNA was digested with one of three restriction enzymes, *Alu*I, *Sal*I, or *Xho*I and analyzed by Southern blotting (Fig. 14). Hybridization of WR29 DNA with pXK showed a dramatic decline of both large (Fig. 14A; *Xho*I and *Sal*I) and small DNA fragments (Fig. 14A; *Alu*I). For WR6, there was no significant difference with or without drug, especially for the small restriction fragments (Fig. 14B). There was some visible decrease in the intensity of the large restriction fragments. This decrease could be due to fragmentation of viral DNA fragments in the presence of etoposide (Fig. 14). The difference of DNA replication patterns between WR6 and WR29 will be covered in "Disscussion" section.

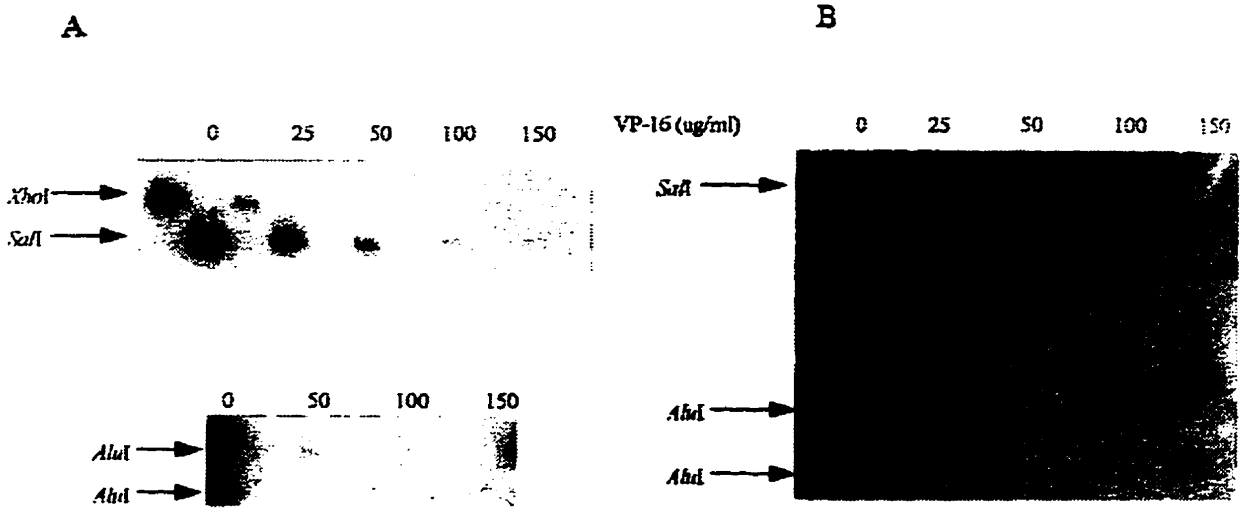

Figure 14. Southern blot analysis of DNA from vaccinia strains WR29 (A) and WR6 (B). DNA from the experiment described in Figure 13 was digested with indicated restriction enzymes (*Xho*I, *Sal*I, or *Alu*I), subjected to agarose gel electrophoresis, transferred to a membrane and hybridized with the internal pXK probe. Concentrations of VP-16 are as indicated.

Heterogeneous population of vaccinia virus in WR29

To further analyze differences between WR6 and WR29, we determined the structure of the terminal inverted repeats of these vaccinia strains. This was done by infection of confluent monolayers of BSC40 cells either with WR6 or WR29. After 24 hours of infection, viral genomic DNA was isolated by the same method as used for PFGE. After equilibration with 0.1 M TBE buffer, half the plug was digested with *Xho*I which has a recognition site about 6 Kb from the end of the viral genome (Fig. 15A). The digested fragments were separated in a 0.7% agarose gel followed by transfer to a nylon filter and hybridization. For the standard vaccinia virus strain WR6, hybridization with either tandem repeat probe p1369 (Fig.15B) or NR2 probe p1322 (data not shown) gave a single band of approximately 6 Kb. When these same probes were used to determine the end structure of WR29, a ladder of terminal fragments was detected. The smallest of these bands was 6 Kb and each band was 1.6 Kb longer than the preceding band. These results suggest that WR29 consists of a heterogeneous population of vaccinia virus which has a repeated structure at the end of the genome, which includes NR2 and the tandem repeats.

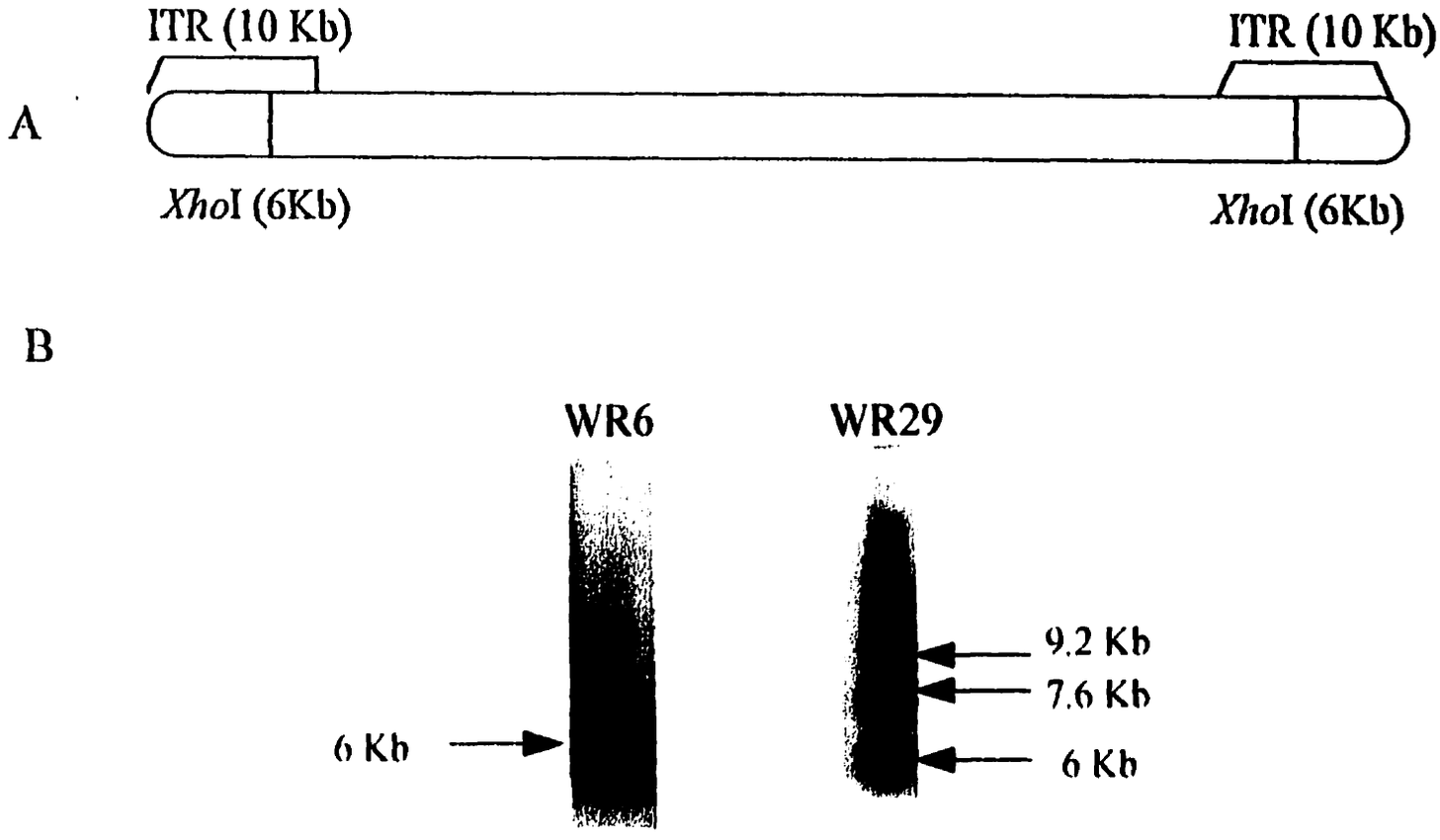

Figure 15. End genome structure of WR6 and WR29. Monolayers of BSC40 cells were infected either by WR6 or WR29 at a multiplicity of 10 pfu/cell and incubated at 37°C for 24 hours. DNA from the two strains was embedded in 1% low melting agarose gel. Half the plug was digested with *Xho*I at 40°C for 2 hours. Digested DNA fragments were separated on the 0.7% agarose gel and transferred to a nylon filter. The p1369 (tandem repeat) probe was used for the hybridization.

A 1.6-Kb fragment of the WR29 DNA genome was amplified in the presence of etoposide

As previously mentioned, PFGE of WR29 DNA allowed detection of a large amount of well-DNA that hybridized to NR2 and tandem repeat probe (Fig. 13). Previous studies (DeLange et al., 1995) suggest that replicative intermediates containing irregular conformations consistent with loop structures or branched DNA molecules were unable to enter the gel. We were particularly interested in the structure of WR29 DNA since this virus had a different terminal genomic structure. In an attempt to determine whether the well-DNA could be released into the gel, several restriction enzyme digests were attempted. Figure 16 illustrates restriction enzyme digestion sites at the end of the vaccinia virus genome. First, *Alu*I which has a recognition site within the NR2 region, was used to determined if well-DNA could be released into the gel. Hybridization with the NR2 probe showed a decrease of viral genome DNA replication, with concatemer amplification of the etoposide-induced 1.6-Kb fragment (Fig. 17). The same result was obtained with the tandem repeat probe (data not shown). To definitively prove that NR2 digestion was required for release of well-DNA and detection of the 1.6-Kb band, *Xba*I, which also has an NR2 recognition site (Fig. 16), was used. The same result was obtained (*F*ig 17). Another restriction enzyme, *Cla*I which has an NR1 and an NR3 but no NR2 site was used as a control (Fig. 17). Although the reduction of genomic DNA could be detected with *Cla*I digestion, the 1.6-Kb fragment could not be detected. Furthermore, there was still a large amount of well-DNA that could not be released into the gel (Fig. 17). Definitive evidence that this 1.6-Kb fragment consisted of NR2/tandem repeat sequences

was obtained by cloning into the pGEM-7Z vector and subsequent hybridization with NR2 and repeat probes (data not shown).

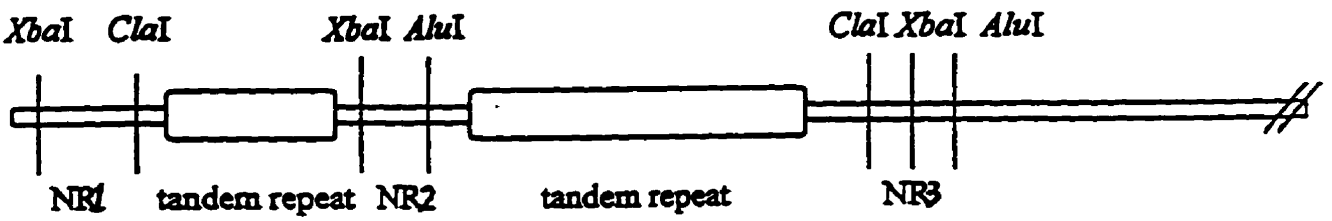

Figure 16. Restriction enzyme recognition sites at the end of the vaccinia virus genome. *Xba*I and *Cla*I have a site in NR1, and *Alu*I and *Xba*I have sites in NR2. *Cla*I, *Xba*I and *Alu*I also sites in NR3.

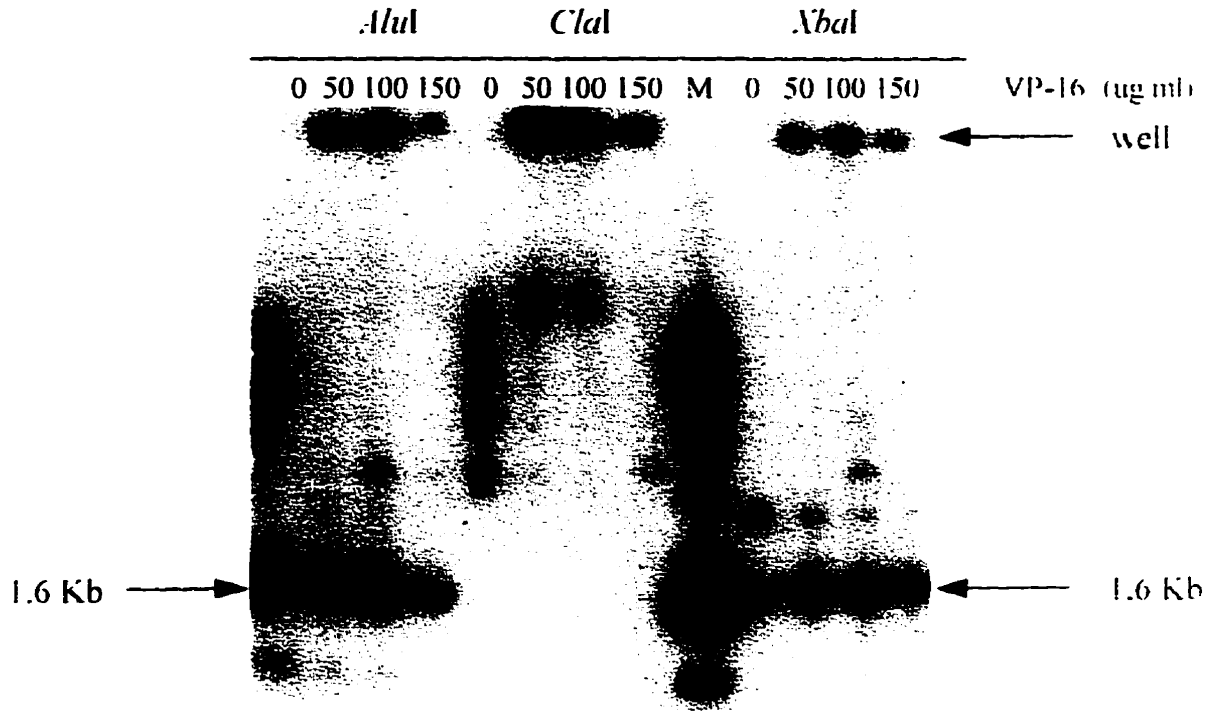

Figure 17. Selective etoposide-induced amplification of a 1.6-Kb fragment containing the NR2/tandem repeat sequence. Monolayers of BSC40 cells were infected with WR29 at 37°C in the presence of 0, 50, 100 or 150 ug/ml etoposide. Cells were harvested 24 hours postinfection to make plugs. Half a plug was digested with *Alu*I, *Cla*I or *Xba*I respectively and the fragments were separated on 0.7% agarose gel. DNA was transferred to a nylon filter and hybridized with the NR2 probe (p1322). The amplified 1.6-Kb fragment is indicated. This amplification could not be detected by *Cla*I digestion.

Replication of the recombinant plasmid containing the 1.6-Kb fragment (NR2/tandem repeat of WR29) in WR6-infected SIRC cells.

We have demonstrated that the near-terminal DNA structure of WR29 is different from that of WR6. We have also demonstrated that with the inhibition of the WR29 genomic replication, a 1.6-Kb fragment containing NR2 and tandem repeat sequences was amplified in the presence of etoposide. This amplified band could be detected by cleavage with restriction enzymes with a recognition site in NR2. Previous studies have shown that plasmid DNA can replicate in vaccinia virus infected mammalian cells to form head-to-tail high molecular weight concatemers (DeLange, et al., 1986; Merchlinsky and Moss, 1986). We were interested to determine whether pGEM-1.6 is capable of replication in mammalian cells infected with the standard vaccinia virus strain (WR6) without being affected by etoposide. For this purpose, we transfected pGEM-1.6 into BSC40 and in rabbit cornea (SIRC) cells which had been previously infected with WR6. Our initial data using BSC40 cells for transfection gave very weak signals, consistent with previous observations suggesting that replication of exogenously added plasmid DNA in virus-infected BSC40 cells is very inefficient. We then chose to work with SIRC cells because plasmid replication has been demonstrated to be highly efficient in these cells (DeLange and McFadden, 1986).

The pGEM-1.6 plasmid was used to transfect WR6-infected SIRC cells, and 24 hours post infection, cellular DNA was harvested and analyzed by restriction enzyme

digestion and Southern blotting. The DNA was digested with *Xho*I plus *Dpn*I. *X*hoI can cut the plasmid once and *Dpn*I was chosen because this enzyme cleaves input methylated plasmid DNA but not the newly replicated, unmethylated plasmid DNA (Peden et al., 1980). This procedure allowed us to distinguish input DNA from newly replicated DNA. After digestion, separation in a 0.7% agarose gel and hybridization with tandem repeat probe (p1369), two major bands were detected: a 6-Kb fragment which is the terminal hairpin fragment derived from the viral genome and a 4.6-Kb fragment consistent with these of a replicated plasmid (Fig. 18; the *Dpn*I fragments produced by cleavage of the input DNA could be seen but are not shown in this figure). This interpretation was confirmed by hybridization of the same filter with pGEM-7Z probe (Fig. 19). Our results with SIRC cells have bearing on our previous data showing selective amplification of the 1.6-Kb fragment in WR29 infected cells. It suggests that a 1.6-Kb plasmid generated by homologous recombination is replicated normally even in the presence of high concentrations of etoposide that inhibit viral DNA replication. This interpretation will be further considered in the "Discussion" section. In order to further prove that this plasmid replication is not due to the 1.6-Kb viral insertion, the bacterial vector pGEM-7Z, lacking the 1.6-Kb insert, was used for the transfection. The same results were obtained: pGEM-7Z plasmid replication in the SIRC cells infected with WR6 was not affected by etoposide (Fig. 20).

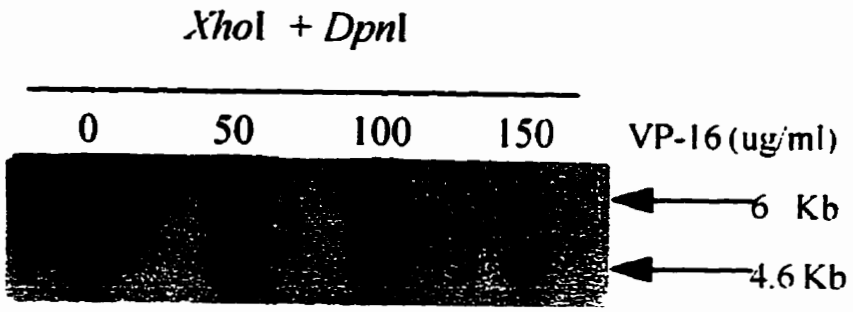

Figure 18. Replication of plasmid pGEM-1.6 in the vaccinia virus WR6 infected SIRC cells. Monolayers of SIRC cells were infected with WR6 and transfected with 1 ug of plasmid DNA. Infected cells were incubated at 37°C in the presence of 0, 50, 100, or 150 ug/ml etoposide. DNA was isolated after 24 hours of infection to make plugs. Half of each plug was digested with *Xho*I plus *Dpn*I for 2 hours and DNA samples were separated by electrophoresis in a 0.7% agarose gel, transferred to nylon filter and hybridized with p1369 (tandem repeat) probe. The 6-Kb genome fragment and the 4.6-Kb plasmid band are indicated by the arrows.

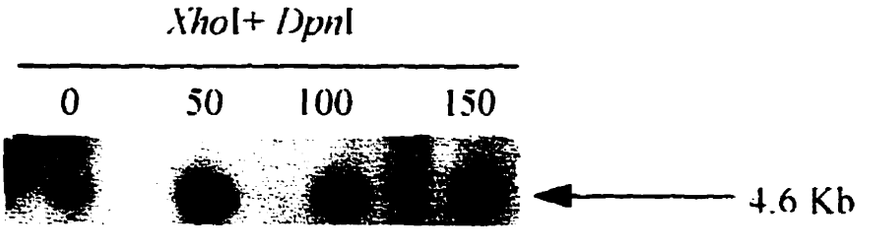

Figure 19. Plasmid replication in the SIRC cells infected with vaccinia virus strain WR6. The membrane from Figure 18 was reprobed with pGEM-7Z probe. The pGEM-7Z is the vector that was used to clone the amplified 1.6-Kb fragment. The 4.6-Kb digested fragment is indicated by the arrow.

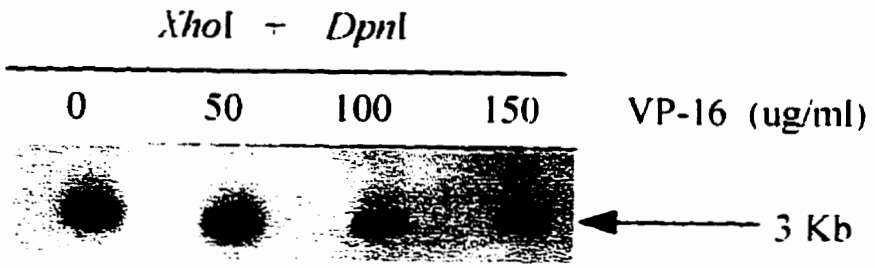

Figure 20. pGEM-7Z plasmid replication in vaccinia virus WR6 infected SIRC cells. Monolayers of SIRC cells were infected with WR6 and transfected with 1 ug of plasmid DNA. Infected/transfected SIRC cells were incubated at 37°C in the presence of 0, 50, 100 or 150 ug/ml etoposide. DNA was isolated 24 hours later and fragments were separated by 0.7% agarose gel electrophoresis, transferred to nylon filter and hybridized with pGEM-7Z vector. The 3-Kb linear vector DNA fragment is indicated.

DNA sequencing of three etoposide-resistant mutants

Previous studies indicated that etoposide could inhibit vaccinia virus telomere resolution and drug-resistant mutants were isolated and mapped to the DNA ligase gene (DeLange et al., 1995). This is somewhat unexpected because it had been reported that the viral topoisomerase is inhibited by this drug. Totally, eleven independent drug-resistant mutants were isolated and eight of these mutants have an identical G→A mutation resulting in a cysteine to tyrosine change at amino acid 11 of the ligase gene (DeLange et al., 1995). The C-termini of the family of DNA ligases are highly conserved and they contain the lysine residue to form the covalent link with AMP. The N-termini of DNA ligases are not conserved and the function(s) are unknown. The N-terminus of poxviral DNA ligase may have roles in etoposide resistance and telomere resolution. For example, disulfide bond formation could affect conformation of the ligase protein and thereby change one of its functions, causing drug resistance. To determine whether the other three VP-16 resistant mutants (VP81, VP85 and VP75) carry a mutation in the DNA ligase gene, we sequenced plasmids (pDLL15, pDLL23 and pDLL31 respectively) that carry inserts from these etoposide-resistant viruses.

As a control, I sequenced the ligase gene of plasmid pDLL17. This plasmid was obtained from virus VP82 which was one of the eight mutants with a G→A mutation mentioned above. A single point mutation in vaccinia virus DNA ligase gene was found in each of the three remaining mutants. The base and amino acid changes are 1) G→A transition,changing cysteine to tyrosine at amino acid position 313 in pDLL15; 2) G→A

transition,changing alanine to threonine at amino acid position 271 in pDLL23; and 3) C→T transition,changing alanine to valine at amino acid position 419 in pDLL31 (Fig. 21).

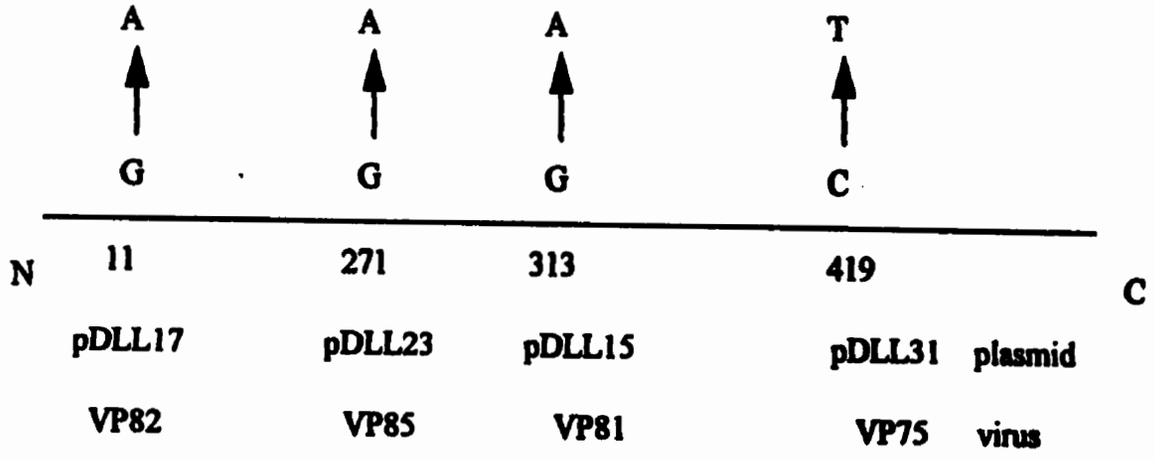

Figure 21. Point mutations in the vaccinia DNA ligase gene of four drug-resistant mutants. The base pair changes are indicated. The numbers show the amino acid positions (diagram not to scale).

Discussion (I)

In this study, we have demonstrated that vaccinia virus variant strain WR29, a plaque purified isolate from the standard wild-type strain (WR6), has specific features at the genome level, particularly at the end of the genome. WR29 consists of a heterogeneous virus population, containing variable sets of NR2/repeat sequences at the ends of the viral genome. One extra NR2/repeat sequence is approximately 1.6 Kb in length. The model illustrated in Figure 22 explains how this variant virus genome may have been generated. The model postulates homologous recombination between the first set of tandem repeats of one genome and the second set of tandem repeats of another viral genome. This should be a rare event because tandem repeats in vaccinia virus are normally quite stable. The recombination event would create a new viral genome which has three sets of tandem repeats and two sets of NR2. NR2 is a unique region and would therefore be expected to recombine at a high frequency characteristic of unique DNA (Fenner, 1969). Our model proposes that the newly generated virus recombines with itself through two NR2 regions to generate extra copies of the NR2 region (Figure 22; step II). This process could continue several times to produce a heterogeneous virus population with a variable length of terminal repeats. Instability and high frequency of recombination at the termini of some strains of vaccinia virus have been reported previously (Moss et al., 1981). Other mechanisms to produce the variant WR29 genome can not be ruled out. For example, the variant genome could have been produced by a recombination-deficient mutation. In this scenario, the frequency of the first step, shown in Figure 22, would be much increased. Subsequently, multiple copies of

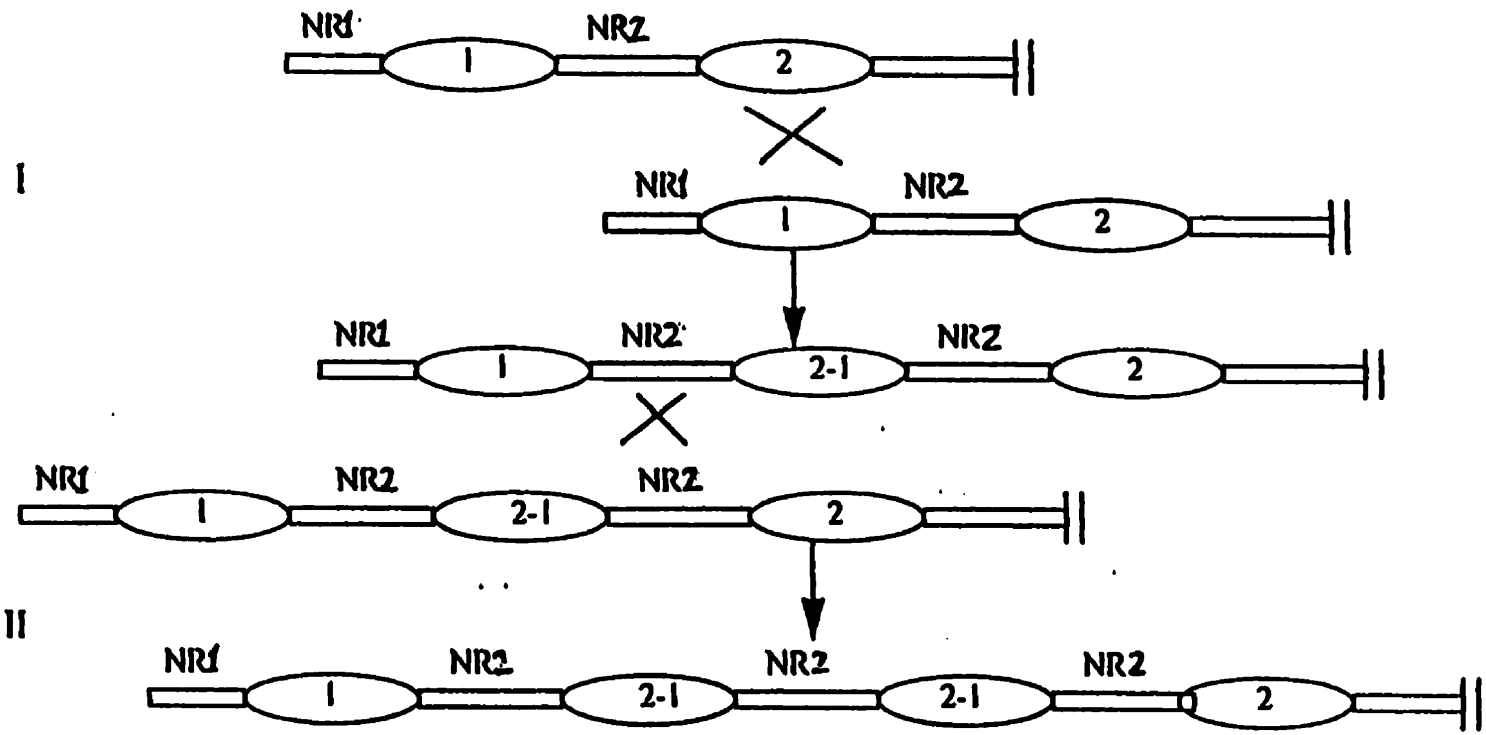

Figure 22. Recombination model to explain the generation of the variant vaccinia virus strain WR29. 1 and 2 indicate the two sets of tandem repeats. In step I, through a rare homologous recombination of tandem repeat set #1 from one genome and tandem repeat set #2 from the other, a variant strain which has two NR2 elements and three sets of tandem repeats would be produced. In step II, the new viral genome could continue homologous recombination with itself through the NR2 region to produce more copies of NR2. This process could be continued several times.

NR2/repeat would be generated by the high frequency of homologous recombination between copies of NR2, as postulated in step II of Figure 22. To distinguish between these two versions of the model, we would have to obtain plaque isolates from strains WR29 and determine whether a reasonable percentage would have "reverted" to the normal stable form as in WR6. It is relevant to note that previous studies with a similar unstable variant virus indicated that roughly 20% of plaque isolates revert back to the stable form after only one round of plaque purification (Moss et al., 1981).

It is not known whether the same number of copies of the NR2/repeat element is present at each end of the viral genome. Previous studies have shown that small deletions in this region are rapidly copied to the other side of the genome (McFadden and Dales, 1979). It is therefore probable that each viral genome contains an identical number of tandem repeats at its end.

In this study, we have again demonstrated that etoposide treatment strongly inhibits telomere resolution of our standard wild-type vaccinia virus strain WR6. Our data suggest that the rate of viral DNA replication of this strain may be somewhat affected by etoposide and that the inhibitory effect of this drug is more noticeable in the interior of the genome than at the ends. We also noticed that etoposide has an effect on DNA replication which is reflected in much reduced amount of large restriction fragments and only a minor effect on small restriction fragments. This suggests that etoposide may cause fragmentation of DNA molecules besides the inhibition of DNA replication. The

mechanism of this damage is unknown. Etoposide could inhibit the cellular topoisomerase II activity by stabilizing the enzyme-DNA complex after DNA cleavage (reviewed in Liu, 1989). It could also inhibit the vaccinia virus-encoded type I topoisomerase (Traktman, 1990). The stabilized enzyme-DNA complex could be the reason for the observed DNA fragmentation (Fig. 5).

Etoposide was found to have a much more extreme effect on the DNA replication of WR29. It strongly inhibited replication of the viral genome except for the NR2/tandem repeat region. Etoposide also inhibited telomere resolution in WR29 as it did in WR6. At the same time, the replicated NR2/tandem repeat sequence had an abnormal conformation such that the DNA could not move into the agarose gel. DeLange (1989) showed that high molecular weight replicative intermediates similarly fail to migrate out of the well during pulsed-field gel electrophoresis (PFGE). Only restriction enzymes which cleaved NR2 allowed release of the 1.6-Kb etoposide-induced fragment from the well. The NR2/tandem repeat region is located at the end of the viral genome and it is believed that this non-transcribed region might perform some functions in promoting DNA replication and recombination (Moss et al., 1981). Promoter activities were found not only in NR2 but also in the terminal non-repeated region NR1, which harbors the TRT (Parsons and Pickup, 1990; Hu and Pickup, 1991). The transcripts do not encode any protein but one or both might function during vaccinia virus telomere resolution. I will discuss this possibility in some more detail in Part II of this thesis.

It is known that plasmid DNA replicates efficiently in the cytoplasm of poxvirus infected mammalian cells in a sequence non-specific manner (DeLange and McFadden, 1986). As demonstrated in this study, when a plasmid containing the 1.6-Kb sequence was transfected into WR6 infected SIRC cells, etoposide did not inhibit plasmid replication. That the etoposide-resistant plasmid replication was not due to the viral insert was demonstrated by the observation that replication of the bacterial vector pGEM-7Z was also unaffected by etoposide. We now propose a model explaining how vaccinia virus strain WR29 may have preferentially replicated a 1.6-Kb viral DNA fragment in the presence of etoposide (Figure 23). We propose that homologous recombination between NR2 elements on the same DNA molecule normally generates a low amount of the 1.6-Kb circular plasmid, and that replication of circular plasmids is either stimulated or at least not inhibited in the presence of etoposide. In contrast, etoposide strongly inhibits replication of the linear viral genome of WR29. It is not known why etoposide inhibits genome replication of strain WR29 but has little effect on WR6. One possibility is that the many copies of the 1.6-Kb fragment are generated rapidly and that this replication itself or sequences present on this endogenous plasmid are the cause of such inhibition.

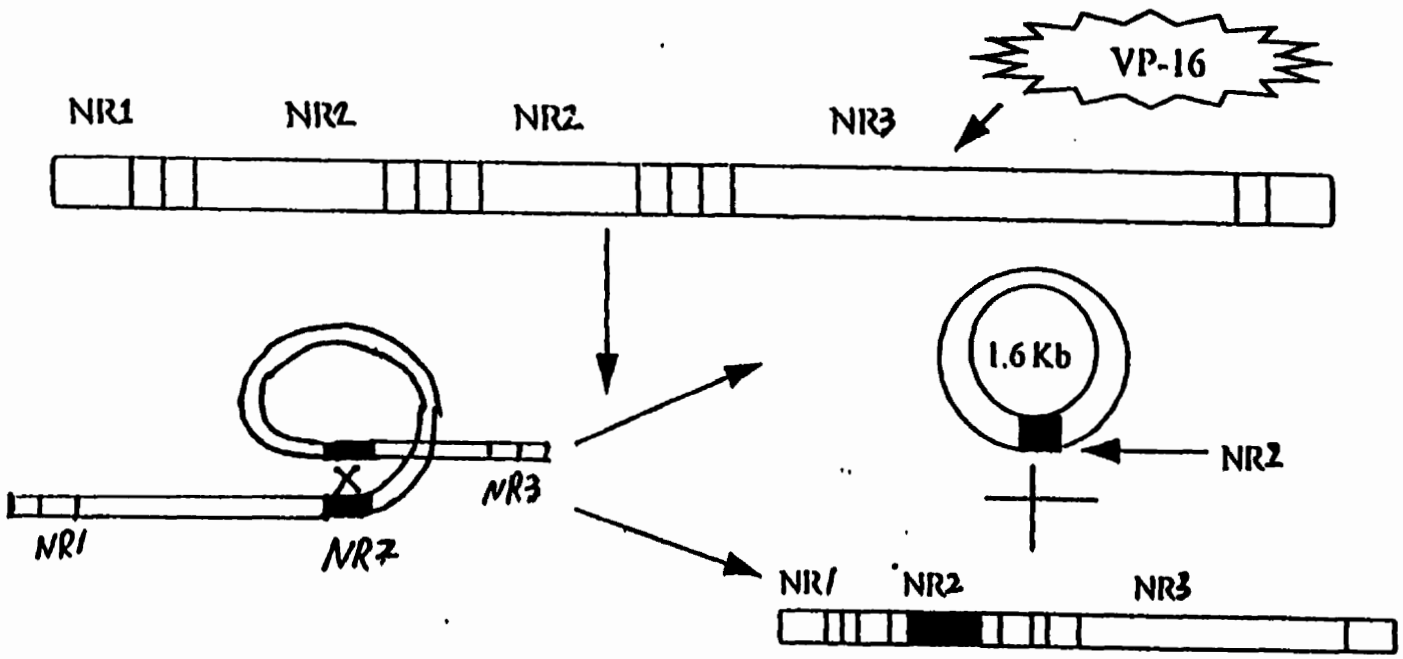

Figure 23. A model to explain selective amplification of a 1.6-Kb DNA fragment derived from vaccinia virus strain WR29. Pairing and homologous recombination between two successive NR2 elements would account for generation of a 1.6-Kb circular plasmid containing NR2 and a set of 70-bp tandem repeats. The circular plasmid is then replicated even in the presence of high concentrations of etoposide which inhibit replication of the viral genome (see text for a detailed description of the model).

The DNA ligase gene of the eleven independently isolated etoposide-resistant mutants have now been sequenced and the lesions determined. Each had a single point mutation in the viral DNA ligase gene. This is somehow unexpected since it is known that the target of etoposide is cellular topoisomerase II (reviewed in Liu, 1989). Eight of these mutants were previously shown to carry an identical point mutation near the 5' end of the virus-encoded DNA ligase gene. I have sequenced the remaining three mutants and shown that they are similarly located in the viral DNA ligase gene, though not at the 5' end.

The C-termini of the family of DNA ligases are highly conserved and it includes the active lysine residue. The N-termini are not conserved and the function(s) of this region is unknown. This could be the ligase domain with which etoposide interacts and causes its lethal effect. A point mutation in this region would then prevent drug interaction and therefore cause drug resistant phenotype. However, this interpretation seems unlikely because vaccinia-encoded DNA ligase in not inhibited by etoposide (Shuman, 1995).Interestingly, two of the mutations, one in the conserved and one in the nonconserved region, cause a change from cysteine to tyrosine. This brings up the possibility that there is a disulfide bond formation between these two cysteines. The other two mutations are in the conserved region of ligase. Could the ligase perform ligation function only during telomere resolution? If that were the case, we would expect nicked hairpin ends rather than unresolved concatemer junctions. The other mechanism of drug resistance could be that DNA ligase forms a component of a DNA repair complex. The other component of this complex could be viral topoisomerase I or cellular topoisomerase

II. Etoposide would then target the topoisomerase in the multi-protein complex.

As further research directions, we could test whether there is a disulfide bond formation by diagonal paper electrophoresis (Brown and Hartley, 1966; Creighton, 1984) We could also detect protein-protein interaction by the yeast two-hybrid system. We could test whether the mutant ligase has lost or gained a function by cloning the ligase into a gene expression vector and purifying the protein after expression.

At this point in time, the mechanism of the effects of etoposide on DNA replication and telomere resolution of vaccinia virus are not fully understood. Further study should include genetic and biochemical approaches to gain a better understanding of the mechanism of DNA synthesis and recombination in this eukaryotic virus model. These studies may also give more detailed information on the action of an antitumour drug such as etoposide.

PART II

TRANSCRIPTIONAL CONTROL OF TELOMERE RESOLUTION BY THE VACCINIA VIRUS ENCODED mRNA CAPPING ENZYME

Materials and Methods

The source of materials was the same as described in Part I.

1. Cells and virus

The media and reagents for the cells were the same as described in PartI. Confluent Africa green monkey kidney cells (BSC40) were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Rabbit cornea cells (SIRC) were maintained in DMEM with 10% FBS and 1x non-essential amino acids. Wild-type strain vaccinia virus WR was obtained from Dr. Richard Condit (University of Florida, Gainesville, Florida), and wild-type strain IHD-W was donated by Dr. McFadden (University of Alberta, Edmonton, Alberta). Temperature-sensitive mutant ts9383 and ts793 were kindly provided by Dr. S. Dales (University of Western Ontario, London, Ontario) and Dr. Richard Condit respectively. Crude virus preparation and virus titration were the same as described before. The permissive temperature for mutants ts9383 and ts793 was 37^{o}C and the non-permissive temperature for the two mutants was 40^{o}C.

2. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE, Southern blotting, DNA probe labeling using the random primer method, and hybridization were performed as described in PartI.

3. Restriction enzyme digestion

Restriction enzyme digestion of virus DNA in plugs, normal agarose gel electrophoresis was the same was described before.

4. RNA isolation

RNA was isolated by TRIzol reagent from Gibco/BRL. Briefly, 6-well dishes (35 mm each) with confluent BSC40 cell monolayers were infected with IHD-W, ts9383 or ts793 at a multiplicity of 20 pfu/ml at 37°C (330 ul/well). After a 30 minute adsorption period, the virus was removed and replaced with 2 ml pre-warmed medium. Infection continued for 10 or 16 hours. At each given time after infection, plates were taken out from the incubators and the medium was aspirated. One ml of TRIzol reagent was added to each 35 mm diameter dish. The reagent was trituated completely to lyse the cells. After setting at room temperature for 5 minutes, the liquid in the well was transferred to a clean 1.5 ml microcentrifuge tube and 0.2 ml chloroform per 1 ml TRIzol was added. The tube

was shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. After incubation, the tube was centrifuged at 12,000 x g at 4°C for 15 minutes. The colorless upper phase was transferred to a new eppendorf tube and 0.5 ml isopropanol was added. The sample was mixed well, incubated at room temperature for 10 minutes, and centrifuged at 12,000 x g at 4°C for 10 minutes. The supernatant was removed and the RNA pellet was rinsed once with 1 ml of 75% ethanol. The pellet was then air-dried and dissolved in an appropriate volume of diethylpyrocarbonate (DEPC)-treated water and stored at -80°C. The concentration of each RNA sample was determined by using a Gilson Spectrophotometer. RNA samples were diluted (usually 1 in 150 to 1 in 300) and placed in quartz cuvettes. Absorbency values were determined at 260 nm and 280 nm. One A_{260} unit was considered to be equivalent to 37 ug of single-stranded RNA. The purity of the RNA samples was indicated by the ratio of $A_{268/280}$. A value between 1.8 and 2.0 was considered to be free of contamination of protein.

5. Northern and slot blotting

10-20 ug of RNA in 12 ul DEPC-treated water was used for each sample. The RNA was mixed with 10 ul of formamide, 2.75 ul of 5 x Gel Running Buffer [0.2 M MOPS (3-(N-morpholino)propanesulfonic acid, pH 7.0, 50 mM sodium acetate, 5 mM EDTA pH 8.0), and 4. 73 ul of formaldehyde]. The above mixture was placed in a 65°C

waterbath for 15 minutes and then put on ice for 5 minutes. The RNA samples were loaded in the gel following the addition of 3 ul of gel loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol). The RNA was electrophoresed at 26 Volts in a 2.2 M formaldehyde/1% agarose gel (Sambrook et al., 1989) with circulation of the running buffer for 16 to 20 hours. The gel was then stained with 0.5 ug/ml ethidium bromide. The success of electrophoresis was determined by visualization of distinct 28S and 18S ribosomal RNA bands with short-wavelength UV. RNA was transfered to Zetaprobe membrane according to the manufacturer's instruction (BioRad). Briefly, after staining and photography, the gel was rinsed twice with sterile water and gentle shaking. The nylon membrane was then soaked in 10 x SSC for 10 minutes. The RNA was then transferred onto the nylon membrane for 16-20 hours in 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0). Following transfer of RNA, the nylon membrane was rinsed in 2 x SSC for 5 minutes and air-dried for 10 minutes. The filter was baked at 80^{o}C in a vacuum oven for 2 hours and stored in a plastic bag at 4^{o}C. At this time, it was ready for Northern hybridization.

Slot blots were prepared as follows. Ten ug of RNA was used to make 1 in 2 serial dilutions. After an appropriate dilution, RNA was mixed with 3 volumes of denaturing solution (500 ul formamide, 162 ul formaldehyde, 100 ul MOPS buffer) and placed in a 65^{o}C waterbath for 15 minutes, and then placed on ice. The manifold for the slot blot was rinsed once with 0.1 M NaCl and once with DEPC-treated water. The nylon membrane was soaked in 10 x SSC for 10 minutes and assembled with the manifold connected to the

vacuum. After assembly, each slot was filled with 10 x SSC to eliminate air bubbles. The vacuum was turned on to pass the 10 x SSC. The speed of the suction was set such that 500 ul solution was passed in 5 minutes. The RNA samples were loaded to each slot. Each slot was then washed with 1 ml 10 x SSC and the apparatus was dismantled. The membrane was air-dried by placing it on a piece of Whatman 3 MM paper. The filter was baked at 80°C in a vacuum oven for 2 hours, and stored in a plastic bag at 4°C. At this point, it was ready for hybridization. Hybridization signals were scanned by using the computer programs "Desk Scan" and "Photofinish".

6. Riboprobe synthesis and Northern hybridization

Single-stranded RNA probes were synthesized by using the riboprobe kit from Promega. First, template plasmid p1369 DNA was extracted and purified by the alkali lysis method of Birnboim and Doly (1979) described in Part I. *Hin*dIII or *Eco*RI was used to linearize the plasmid. After restriction enzyme digestion, DNA was extracted with phenol/chloroform and chloroform. The DNA was then precipitated by the addition of one tenth volume of 3 M sodium acetate and two volumes of absolute ethanol, followed by incubation at -80°C for 15 minutes. The pellets were collected by centrifugation at 4°C for 15 minutes and washing once with 70% ethanol. The pellets were vacuum-dried and dissolved in a small amount of DEPC-treated water for a final concentration of 1 ug/ml. DNA was stored at 4°C. Probe labeling was performed as follows. One ug of the

linearized template DNA was incubated at 37°C for 1 hour in a 20 ul reaction containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 20 units of RNAsin, 2.5 mM ATP, 2.5 mM GTP, and 2.5 mM UTP, 100 uCi (400 Ci/mmol) α^{32}P-CTP, and 10 units of either SP6 or T7 RNA polymerase. The reaction was stopped by the addition of 1 ul of 0.5 M EDTA and incubation at 65°C for 10 minutes. The DNA template was removed by RNase-free DNase (2U per reaction) digestion at 37°C for 15 minutes. Unincorporated nucleotides were removed by chromatography on the Sephadex G-50 Nick column (Pharmacia). The efficiency of riboprobe synthesis was determined by the Beckman LS 1801 liquid scintillation system. The labeled probe was stored at -20°C for no more than 3 days.

Pre-hybridization was performed in buffer B (50% deionized formamide, 6x SSPE, 5x Denhardt's solution, 0.5% SDS and 100 ug/ml sonicated salmon sperm DNA) (20x SSPE is 3 M NaCl, 0.2 M $Na_2HPO_4.H_2O$, 0.2 mM EDTA pH 7.4) at 55-60°C for 15 minutes. The solution was replaced by fresh buffer B (150 ul/cm^2) containing the labeled probe (1x 10^6 cpm/ml). Hybridization was at 55-60°C for 12 to 19 hours. The membrane was then washed with constant shaking in 2x SSC/0.1% SDS at room temperature for 30 minutes and 0.1x SSC/0.1% SDS at 65°C for 20 minutes. The washing time was variable. The filter was air-dried and placed into a fresh plastic bag. Exposure was at -80°C from 30 minutes to 24 hours. After the film was developed, the membrane was stripped of label in a boiling solution of 0.1x SSC/0.1% SDS and allowed to cool to room temperature.

The filter was then reprobed with another riboprobe using the same method as described above.

Results

Previous studies have shown that the telomere resolution defective phenotype of mutant ts9383 is due to a mutation in the small subunit (D12) of the virus-encoded mRNA capping enzyme. Since late gene expression is normal in ts9383, the mRNA capping enzyme may have a direct role in telomere resolution (Carpenter and DeLange, 1991). Immunoprecipitation indicated that D12 is stably produced in the ts mutant either at the permissive temperature (37°C) or at the non-permissive (40°C) but that the physical association of this small subunit with the large subunit (D1) is dramatically reduced at the non-permissive temperature (Carpenter and DeLange, in preparation). The association of these two subunits has been shown to be essential for the 5' mRNA cap methyltransferase activity (Cong and Shuman, 1992; Higman et al., 1992). The tandem repeat region at the end of the viral genome has been previously used as a DNA probe to show that the amount of telomeric RNA from ts9383 and wild-type virus infected cells are roughly identical, but that the migration pattern of ts9383 RNA is slightly faster from that of wild-type (Carpenter, Ph.D. thesis, 1994).

Here I describe further characterization of telomeric RNA in ts9383. I also describe analysis of another mutant, ts793, which harbours a mutation in the D1/D12 subunit interaction domain of the D1 subunit of the capping enzyme.

1. Ts793 is defective in telomere solution

In order to test whether there was plaque formation of ts9383 and ts793 at the non-permissive temperature of 40°C, serial dilution of each ts mutant and of the two wild-type viruses from which they were derived, IHD-W and WR respectively, were made. BSC40 cells were infected at either 37°C or 40°C with the four different vaccinia virus strains. The wild-type vaccinia virus strains IHD-W and WR were used as controls. After 48 hours of infection, infected monolayers were fixed and stained with 0.1% crystal violet (Fig. 24). At 37°C, both wild-type and ts mutants could form normal size plaques. At 40°C, there was plaque formation in IHD-W and WR infected cells, although the size was smaller compared to that observed at 37°C. Plaque formation was negligible for ts9383 and ts793 at 40°C, even at high virus titres (low dilution).

To determine whether ts793 is defective in telomere resolution, we examined viral DNA replication at the permissive temperature and non-permissive temperature. PFGE allowed us to distinguish this class of ts mutants which is defective in the conversion from high molecular weight replicative intermediates to mature monomers (DeLange, 1989). Using this technique, we obtained quantitative and qualitative data of DNA replication.

We routinely infected BSC40 cells with ts793, ts9383 and WR at a multiplicity of 10 pfu/cell. WR and ts9383 were used as controls. The permissive temperature for ts9383 and ts793 was 37°C and the non-permissive temperature was 40°C. DNA was harvested

harvested 24 hours post infection and used to make plugs for PFGE. Viral DNA was identified with the terminal probe p1369 (tandem repeat probe) (Fig. 25). The patterns of DNA replication exhibited by WR at either 37°C or 40°C, and ts9383 and ts793 at 37°C were identical. A single band approximately 185 Kb in length was detected. However, both ts mutants showed a dramatic difference at 40°C. Besides the mature monomer, a ladder of oligomers was also detected. Such oligomeric ladders are typical of a defect in telomere resolution of vaccinia virus (DeLange, 1989; Merchlinsky and Moss, 1989a).

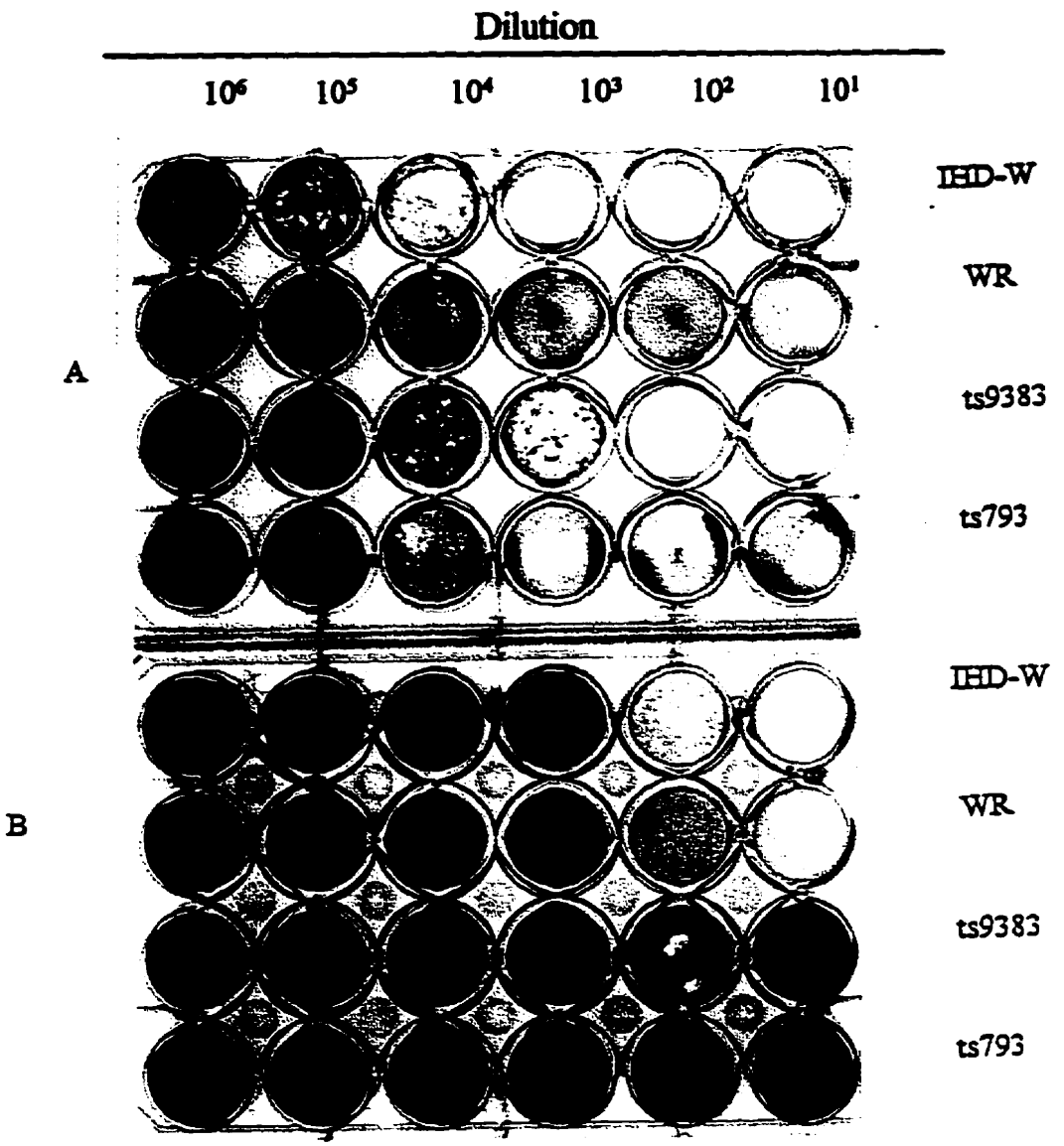

Figure 24. Phenotype testing of two wild-type strains and two temperature-sensitive mutants of vaccinia virus at the permissive and at the non-permissive temperature. Serial dilution (1 in 10) of vaccinia virus strains IHD-W, WR, ts9383 and ts793 were used to infect monolayers of BSC40 cells. After 30 minutes of adsorption at 37°C, virus was removed, fresh medium was added and cells were incubated at either 37°C (A) or 40°C (B) for 48 hours. Plaques were visualized by staining with 0.1% crystal violet.

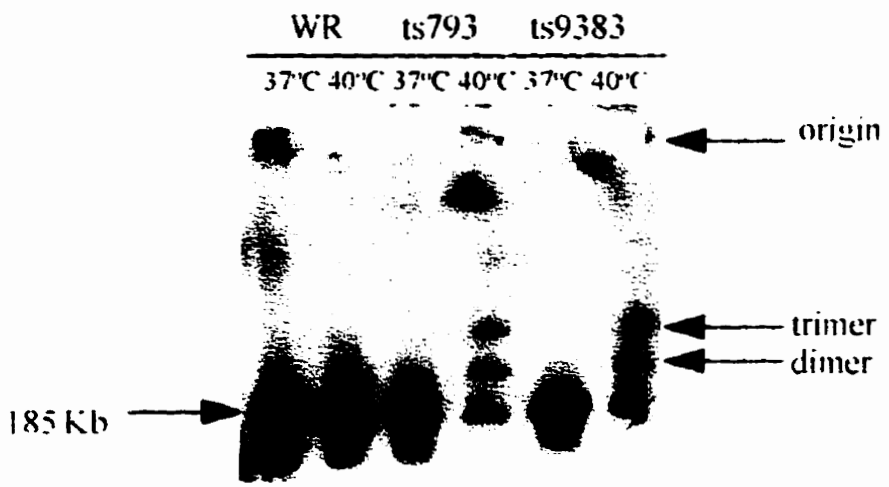

Figure 25. Pulsed-field gel electrophoresis (PFGE) of wild-type vaccinia virus strains WR, ts9383 and ts793. Confluent monolayers of BSC40 cells were infected either with WR, ts9383 or ts793 at 37°C or 40°C at a multiplicity of 10 pfu/cell. 24 hours post infection, monolayers were harvested, lysed in agarose plugs, and the DNA subjected to PFGE. The DNA was transferred to nylon membrane and hybridized with ^{32}P-labeled terminal probe p1369 (tandem repeat probe). The mature monomer (185 Kb), dimer, trimer and the gel origin are indicated.

To confirm the telomere resolution defective phenotype, the viral DNA was digested with *Xho*I and separated by agarose gel electrophoresis. The terminal probe (p1369) was used to detect the concatemer junction fragment. For WR, either at 37°C or 40°C, only the 6-Kb hairpin fragment was observed. At the nonpermissive temperature, both ts9383 and ts793 showed not only the resolved terminal hairpin fragment but also the 12-Kb unsolved telomeric fusion fragment (Fig. 26). This observation confirmed that both ts9383 and ts793 were defective in telomere resolution at 40°C.

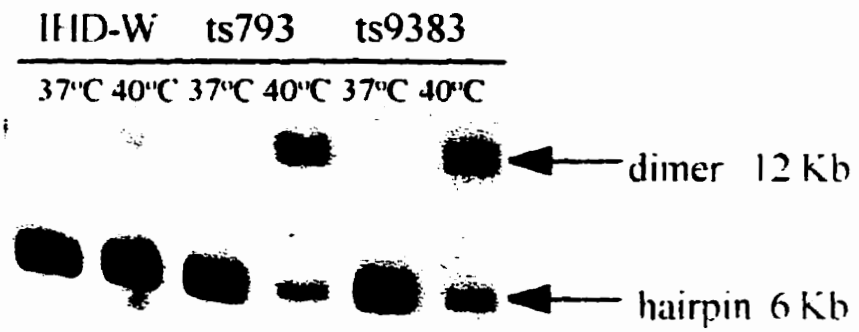

Figure 26. Both ts9383 and ts793 were defective in telomere resolution at 40°C. Monolayers of BSC40 cells were infected at multiplicity of 10 pfu/cell with IHD-W, ts9383 or ts793 either at 37°C or 40°C. After 24 hours of infection, DNA was embedded in agarose plugs and digested in situ with *Xho*I at 40°C for 2 hours. DNA samples were separated in a 0.7% agarose gel, transferred to a nylon filter and hybridized with terminal probe p1369 (tandem repeat probe). The 6-Kb hairpin indicates the resolved terminal fragment and the 12-Kb dimer represents the unresolved inverted repeat fragment.

2. Telomeric transcription of ts9383 and ts793

Previous studies indicated that mutants ts9383 and ts793 had a single point mutation in the D12 and D1 subunit, respectively, of the vaccinia virus encoded mRNA capping enzyme. Yet, in each case, late protein synthesis was normal (Carpenter and DeLange, 1991; Condit, personal communication). The role(s) of this protein in the process of telomere resolution remains unclear. Does it have a direct role in telomere resolution? In order to answer the above question, we decided to analyze the telomeric transcripts of both ts mutants since there were studies which showed that the telomere resolution target (TRT) could act as a late promoter (Stuart et al., 1991) and at least one of the late transcripts started from the TRT region (Hu and Pickup, 1991). We wanted to know whether the promoter located in the TRT region was used the same in the wild-type and ts mutant viruses.

BSC40 cells were infected by wild-type vaccinia virus (IHD-W) and ts9383 at a multiplicity of 20 pfu/cell at 37°C or 40°C. After 10 or 16 hours of infection, total RNA was isolated. The RNA was loaded on a slot blot apparatus and hybridized to single-stranded RNA probe from the tandem repeat region (Fig. 27A). The T7 probe which allows detection of transcripts in the NR2 to NR1 direction (reading towards the hairpin ends), failed to detect a significant difference between IHD-W and ts9383, either at 37°C or at 40°C. Using the SP6 probe, which recognizes transcripts in the NR1 to NR2 direction, the wild-type and ts9383 behaved essentially the same at 37°C. However, we

could detect a significant difference between IHD-W and ts9383 at 40°C. Wild-type IHD-W produced a normal amount of this late telomeric transcripts at 40°C, but ts9383 produced a much reduced amount at 40°C either 10 or 16 hours post infection (Fig. 27B).

The same approach was used to determined the presence of telomeric transcripts of ts793 and the same observation was obtained (Fig. 27C). There was no significant difference of late transcription when the T7 probe was used. However, the SP6 probe demonstrated a sharp decrease in the amount of telomeric transcripts of ts793 when compared to IHD-W at the non-permissive temperature. The above data was confirmed by Northern hybridization analysis (Fig. 27D). To obtain quantitative data of late transcription of wild type and ts mutants, we scanned the slot blots and the results are shown in Fig. 28. The scanning data allowed us to estimate that at the non-permissive temperature, the amount of NR1 to NR2 late transcript of ts9383 was 17-18% compared to wild-type virus (Fig. 28-IB). For ts793, this late transcript was only 8-12% of the IHD-W equivalent (Fig. 28-IIB). We also noticed that at the permissive temperature, the transcription from NR2 to NR1 of ts793 is slightly more efficient than that of IHD-W (Fig. 28-IIA). The reason for this is not clear.

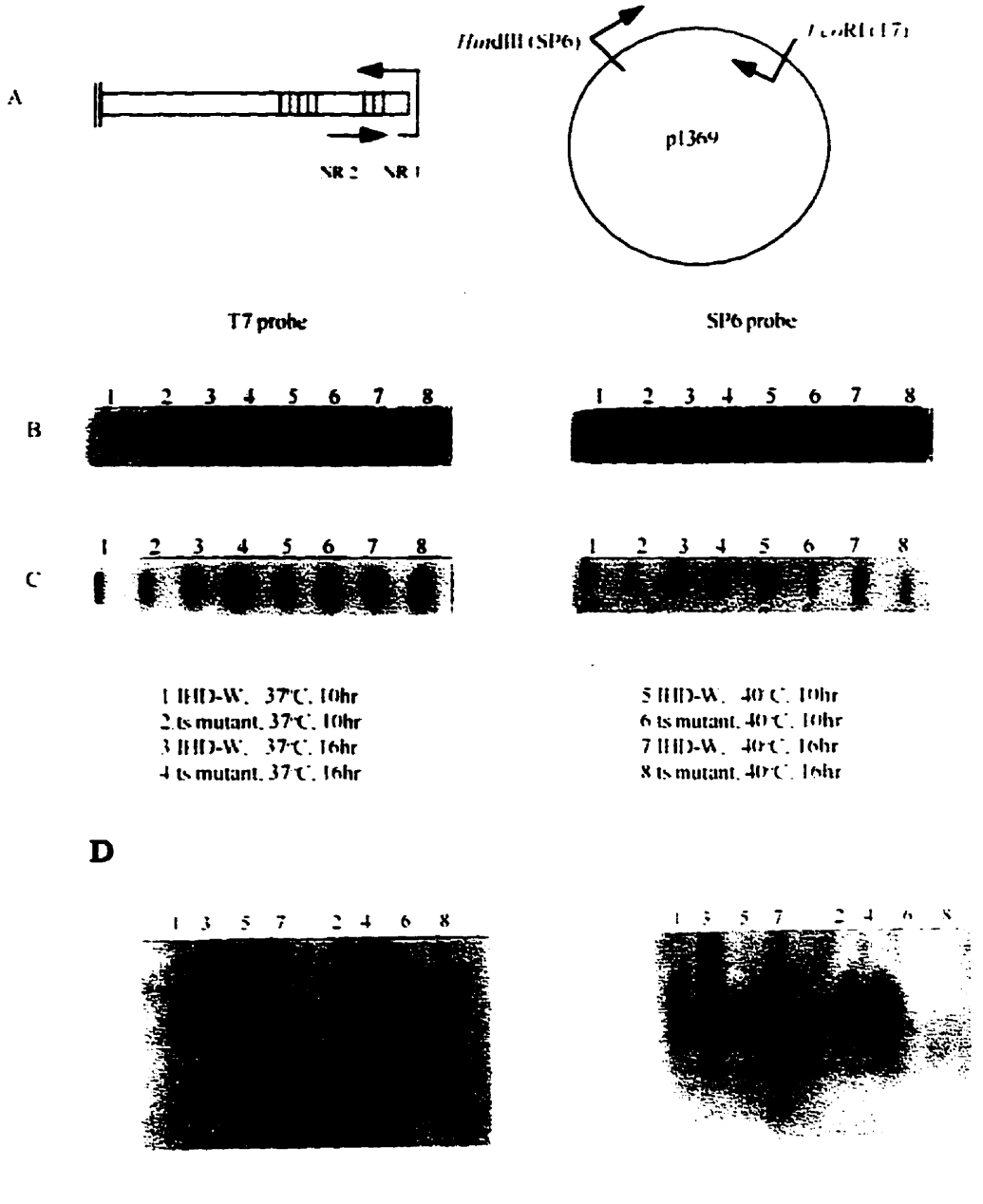

Figure 27. Slot blots and Northern hybridization analysis of two late transcripts of IHD-W and ts mutants. 'A' indicates the positions of two late promoters at the ends of the viral genome, and the SP6 and T7 promoters on plasmid p1369. BSC40 monolayers were infected at a multiplicity of 20 pfu/cell with IHD-W, ts9383 or ts793 at 37°C or 40°C for 10 and 16 hours. RNA was isolated as described in the Materials and Methods section. Five ug of total RNA was subjected to slot blot hybridization analysis and ten ug of total RNA was used in the Northern blots. The RNA on the membrane was hybridized with T7 and SP6 probes respectively. 'B' shows the slot blot results from ts9383 and 'C' represents the slot blot data from ts793. 'D' shows the Northern hybridization data from ts793 (Northern data from ts9383 are not shown).

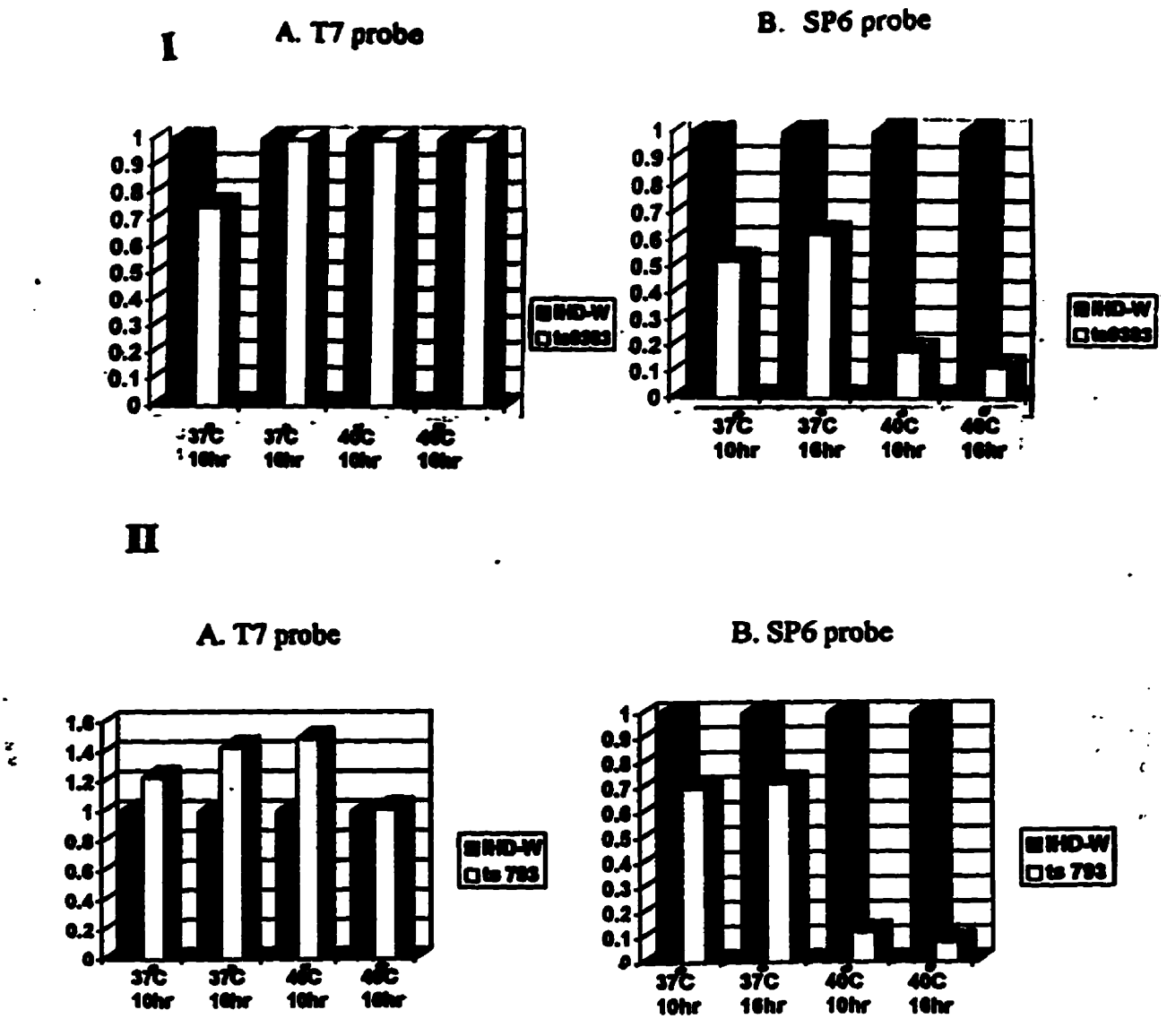

Figure 28. Quantitation of telomeric transcripts in BSC40 cells infected with wild-type strain IHD-W or two ts mutants. Total RNA was isolated after 10 and 16 hours of infection and subjected to slot blot analysis. Two radiolabelled single-stranded RNA probes were used for Northern hybridization is as described in the Materials and Methods section. Telomeric transcription was quantitated by scanning of the slot blot. Values were normalized to 100% of each IHD-W sample. Incubation temperature and infection time are as indicated. 'A' T7 probe; 'B' SP6 probe. I is ts9383 and II is ts793.

Discussion (II)

In Part II we have used two ts mutants to study the function(s) of the vaccinia virus encoded mRNA capping enzyme in telomere resolution. The first mutant is ts9383 which has a single point mutation in the small subunit of the mRNA capping enzyme (DeLange and Carpenter, 1991). The second mutant is ts793 which has one single point mutation in the large subunit of the mRNA capping enzyme (Condit, personal communication). The common feature of these two mutants is that, although telomere resolution is defective, late gene expression is normal. This suggests that the mRNA capping enzyme might have a direct role in the process of telomere resolution.

The mRNA capping enzyme is a heterodimeric enzyme which is composed of two subunits. The large subunit is 96 kDa and the small one is 33 kDa. They are encoded by the D1 and D12 genes, respectively. This enzyme is responsible for the first three enzymatic steps of mRNA cap function, namely polynucleotide 5' triphosphatase, mRNA guanylyltransferase and mRNA (guanine-7) methyltransferase (Martin et al., 1975; Venkatesan et al., 1980).

We have demonstrated that ts793 is defective in telomere resolution at 40°C as is ts9383. At 40°C, there is no plaque formation of both ts mutants but there is plaque formation of wild-type vaccinia virus. PFGE showed that at the non-permissive temperature, both ts mutants have high molecular weight concatemers in addition to

mature monomers.

We also studied transcript formation in the terminal region of both wild-type virus and ts mutants since previous studies indicated that the noncoding regions at the ends of the vaccinia virus genome and some other *Orthopoxviruses* can act as templates for the synthesis of late RNAs, and there are two promoters located in NR1 and NR2 region respectively (Parsons and Pickup, 1990; Hu and Pickup, 1991). Since telomere resolution is a post-replicative process which takes place when these late transcripts are produced, it is possible that one or more of these late transcripts are involved in the process of telomere resolution.

Previous studies (Carpenter and DeLange, in preparation) have shown that telomeric transcript formation was not affected significantly at either the permissive or non-permissive temperature when a terminal double-stranded DNA probe was used. With the double-stranded probe, we could not tell the difference of promoter activity which locates in NR1 and NR2 regions. In order to distinguish the difference between these two promoter activities during late time of infection, especially for the production of late transcripts at the ends of the genome, single-stranded RNA probes were used. Our results indicated that only the NR1 promoter is poorly used by the two ts mutants at the non-permissive temperature. Our observation supports the concept that telomere resolution is correlated with NR1 promoter function (Stuart et al., 1991). It also suggests that mRNA capping enzyme might have a direct role in the control of both NR1 promoter function and

telomere resolution. However, the relationship between telomere resolution and TRT transcription is not known. One possibility is that the mRNA capping enzyme forms a protein complex with RNA polymerase (Shuman et al., 1987). When transcription takes places, this protein complex proceeds through the double-stranded DNA molecule, relaxing the helical structure of double-stranded DNA to allow the formation of a cruciform (Fig. 10) The cruciform would then be cleaved at its base to form mature monomers. The mutation in the mRNA capping enzyme could change the conformation of the protein complex and inhibit transcription and therefore cruciform formation and telomere resolution. It is also possible that transcription from NR1 region is not required for telomere resolution to take place. It may be that the enzyme binds at NR1 and in doing so regulates telomere resolution by inducing a conformational change in the DNA.

The fact that a point mutation in both the small and large subunits of the vaccinia virus encoded mRNA capping enzyme results in identical defects during telomere resolution, and that both mutants are simultaneously defective in telomeric transcription provides strong evidence for a role of the mRNA capping enzyme in telomere resolution, possibly through the control of telomere resolution by late transcription. The mRNA capping enzyme could be one component of a protein complex which controls telomere resolution. Further studies need to be done to identify this protein complex.

General Discussion

Vaccinia virus is an excellent model for studying the mechanism of DNA replication, repair and recombination in eukaryotes. Telomere resolution is the last step of viral DNA replication and includes the conversion of the inverted repeat conformation into daughter hairpin termini (DeLange and McFadden, 1990). This process has important similarity to site-specific recombination events. Therefore, understanding the mechanism of this event might shed light on the biochemistry of site-specific recombination as well (DeLange and McFadden, 1990).

This thesis deals with several aspects of vaccinia virus DNA replication and recombination. Part I emphasizes the effect of etoposide on DNA replication of two different virus strains and the sequence of etoposide-resistant mutants. Recent studies have shown that etoposide, the topoisomerase II inhibitor, can inhibit telomere resolution of vaccinia virus (DeLange et al., 1995). Etoposide resistant mutants were isolated and 8 of the 11 mutants were mapped to the same point mutation at the N-terminus of the virus encoded DNA ligase gene (DeLange et al., 1995). The first part of this thesis further analyzes the remaining three drug-resistant mutants, the role of ligase in telomere resolution and the effect of etoposide on viral DNA replication.

In part II of this thesis, two temperature-sensitive mutants (ts9383 and ts793) were used to identify *trans*-acting factor involved in the process of telomere resolution.

Resolvase proteins are defined as those proteins which are required in the process of telomere resolution. Identification of resolvase protein(s) can be greatly facilitated by availability of res- mutants that are not also defective in late gene expression. Both ts9383 and ts793 fit this criterion. They are defective in telomere resolution without a concomitant defect in gene expression. Interestingly, the mutation in ts9383 was mapped to the small subunit of mRNA capping enzyme (D12L) (Carpenter and DeLange, 1991) and the mutation in ts793 was mapped to the large subunit of the same enzyme (D1R) (Condit, personal communication). It has been shown that D12 in ts9383 could not be co-immunoprecipiated with D1 at the non-permissive temperature (Carpenter, Ph.D. thesis, 1994) and the lesion in ts793 is located in the C-terminal half of D1, which is the domain involved in subunit association (Condit, personal communication). The above findings suggest that this enzyme might play a direct role in telomere resolution. So the question is how the mRNA capping enzyme is involved in telomere resolution?

There are two promoters in the terminal region of the viral genome. One is located in NR1 and the other is in NR2 (Parsons and Pickup, 1990; Hu and Pickup, 1991). Our data suggest that the promoter which is in the NR1 region is poorly used by both of the ts mutants. This brings up the correlation between telomeric transcription, telomere resolution and the role of the mRNA capping enzyme. This hypothesis is reasonable considering the various functions of the capping enzyme in transcription, and the fact that this enzyme is packaged in virions and exists as part of a multiprotein complex that includes the virus-encoded multi-subunit RNA polymerase (Zhang et al., 1994). Several

pieces of evidence have already suggested that telomeric transcription and resolution might be directly linked. First, the resolution sequence, which is highly conserved among the poxvirus family, could act as a strong late promoter (Stuart et al., 1991). Second, late transcription of the telomere region in *Orthopoxviruses* is a conserved feature of these viruses although it doesn't encode any known proteins. It is possible that the mRNA capping enzyme is involved in a protein complex (including RNA polymerase) which can transcribe from NR1 and induce site-specific recombination leading to resolution of the concatemer junctions to monomers. This could be achieved by localized changes in superhelical density produced by movement of RNA polymerase along the helical DNA. This twin-supercoiled-domain model (Liu and Wang, 1987) could explain the cruciform formation, which is the intermediate structure for site-specific recombination (DeLange and McFadden, 1990). However, the finding that promoter activity is not always strictly correlated with resolution efficiency suggests that transcription from telomere region may not be the only factor for resolution (Merchlinsky, 1990; Stuart et al., 1991). The resolution event could also be driven by changing DNA conformation, such as DNA bending. In fact, bending of the phage attachment site attP is a prerequisite to the formation of the active nucleosome assembly unit which promotes site-specific recombination of lambda phage (reviewed in Nash, 1990). This DNA bending is induced by binding of a host protein to the attP site (reviewed in Nash, 1990). Similarly transcription complex binding to NR1 could also induce DNA bending, followed by strand-exchange and branch migration. Although the mRNA capping enzyme doesn't bind DNA itself, RNA polymerase does bind to DNA (Luo and Shuman, 1993; Higman et al.,

1994). It is known that the mRNA capping enzyme forms a complex with RNA polymerase (Zhang et al., 1994). This gives further evidence that the capping enzyme is one of the components of a transcription complex that induces a change in DNA conformation as the first step in telomere resolution.

It should be noted that there are three telomeric transcripts that have been described previously (Parsons and Pickup, 1990; Hu and Pickup, 1991). Two of these were described in my thesis (see part II). The third one, which starts and finishes both at NR2, is twice the size of the other two. We failed to detect such telomeric transcripts. The reason for this is unknown. It could be the difference in cell lines and /or growth conditions. It is also possible that the larger transcript is not palindromic as suggested by Parson and Pickup, but that it is a transcript starting outside the region of tandem repeats. Our data do suggest such an interpretation. We also see larger RNA molecules, but they are strand-specific. Such strand-specificity would not be expected for a palindromic transcript that starts and stops at NR2.

Considering the multifunction nature of the mRNA capping enzyme, why couldn't we detect the defect in general gene expression at the same time as the defect in telomere resolution? There are two possibilities. First, the virion enzyme which is packaged with the core of the viruses provides enough functional enzyme to allow functions required for gene expression, especially at the high multiplicity of infection that was used in my experiments. There are two kinds of temperature-sensitive mutants called thermolabile

(TL) and temperature-sensitive synthesis (TSS) (Gordon and King, 1993). Ts9383 may fall into the latter group and once normal protein is synthesized and assembled at a low temperature, it will still function even after a shift to the non-permissive temperature This hypothesis could be tested by temperature-shift experiments. Second, normal gene expression in these capping enzyme mutants might be explained if a cellular or viral complementing factor exists. For example, the methyltransferase activity of the viral capping enzyme might be supplemented or complemented with a cellular homology.

The poxviral ligase is non-essential in cell culture (Colinas et al., 1990; Kerr and Smith, 1991 and Parks et al., 1994) and it is thought to be primarily involved in DNA repair (Kerr and Smith; 1991; Odell et al., 1996). This enzyme, like the mRNA capping enzyme, could also be a candidate as a trans-acting factor for telomere resolution. All of the 11 drug-resistant mutants are mapped to this gene which gives firm evidence that DNA ligase is crucial in telomere resolution. The next question is concerned with how DNA ligase is involved in this process? Does this enzyme act at the telomere region? According to the similar functions of ligase and topoisomerase II, etoposide could act on ligase in the same manner as topo II. Recent studies have shown that etoposide does not inhibit ligase activity by itself *in vitro* (Shuman, 1995). It is possible that ligase is one of the components of the resolvase complex and the function(s) of this complex is/are inhibited by etoposide. Previous studies show that viral type I topoisomerase (topo I) is inhibited by etoposide (Traktman, 1990). It is possible that ligase and topo I form a protein complex that acts at the telomere region of vaccinia virus, and that drug-resistant

mutants are defective in complex formation.

When we investigated the effect of etoposide on DNA replication of two virus strains, a surprising discovery was made. Etoposide inhibits viral genome replication and at the same time, a plasmid which contains NR2/repeat sequence replicates effectively. It is possible that the replication of this tandem-repeat-containing plasmid is the reason of inhibition of genome replication. Could this plasmid DNA have titrated out some essential replication factor? Further studies would help us to get a better understanding of the mechanism of poxvirus DNA replication.

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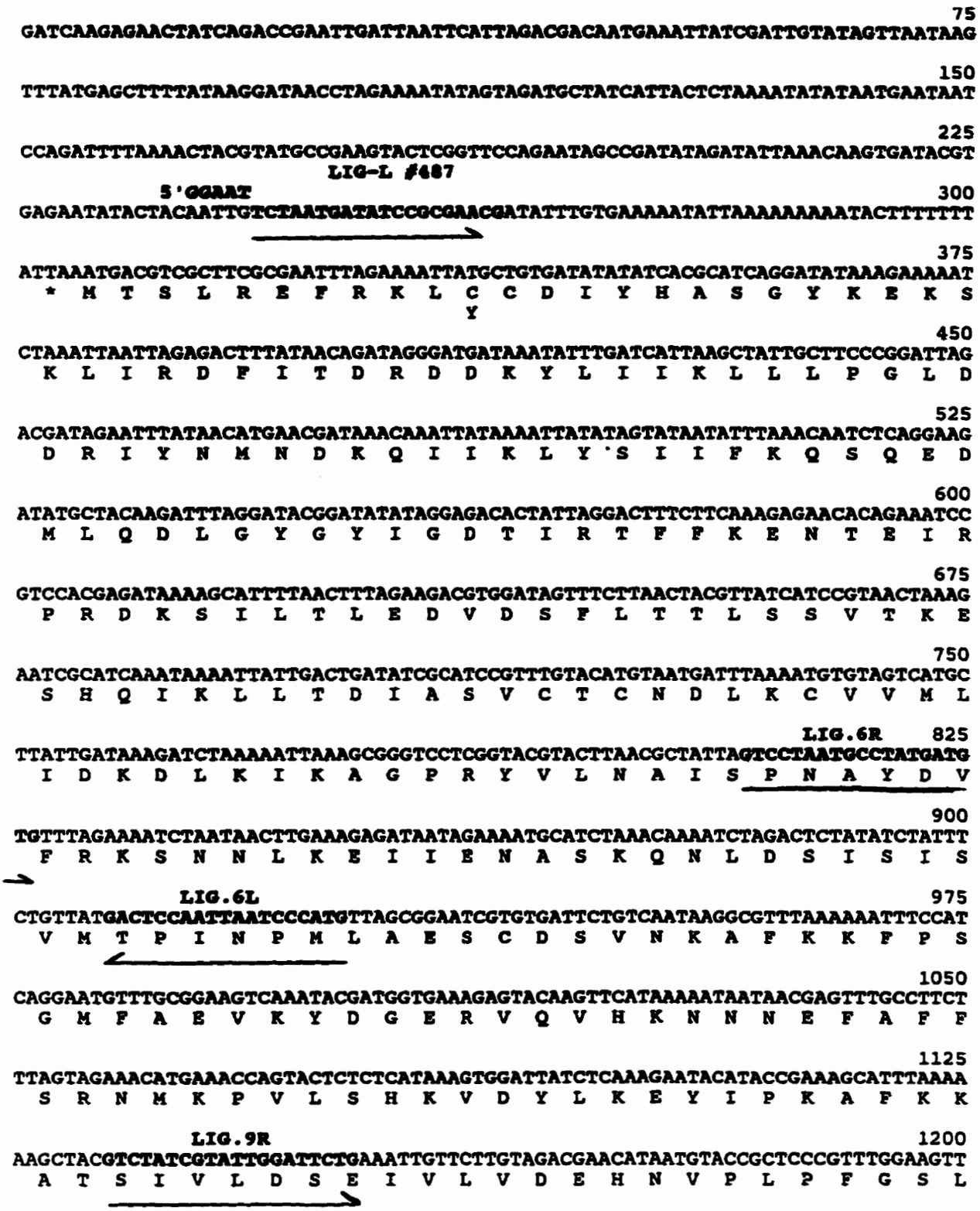
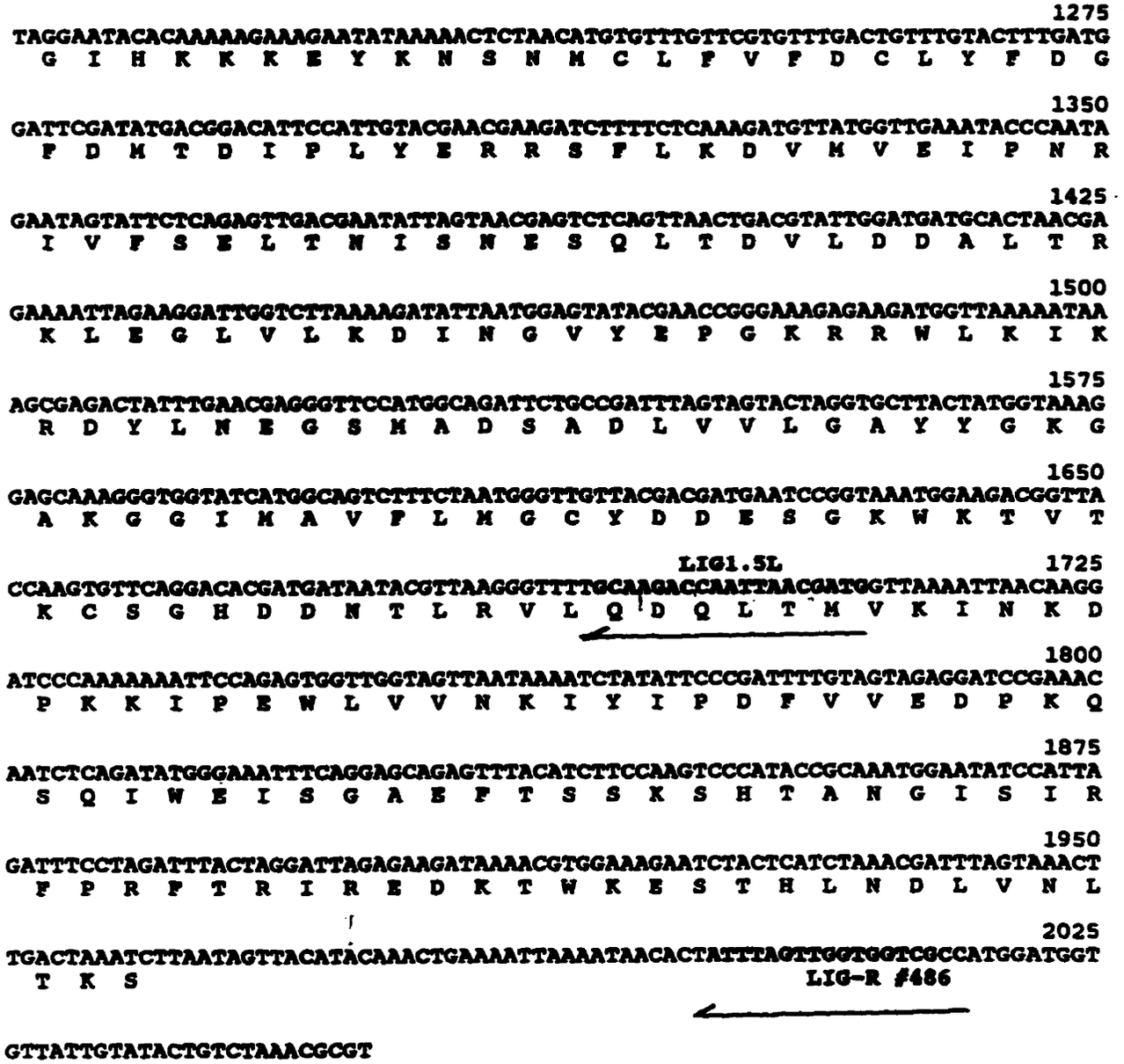
Appendix





Vaccinia WR DNA ligase gene----sequencing strategy. The primers are indicated by the arrows.