MOLECULAR GENETIC ANALYSIS OF CONDITIONAL LETHAL VACCINIA VIRUS MUTANTS DEFECTIVE IN TELOMERE RESOLUTION

A thesis submitted to the
Faculty of Graduate studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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Department of Human Genetics
University of Manitoba
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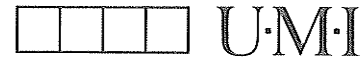

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BY

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For Sandra

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Abbreviations

I. Units of Measure:

bp	base pairs
cm	centimeter
cpm	counts per minute
Da	daltons
h	hour
kb	kilobase
K or kDa	kilodalton
min	minute
nt	nucleotides
rpm	revolutions per minute
sec	second
µCi	microcurie
v/v	volume per volume
w/v	weight per volume
x g	gravitational force (where g =9.81 metres/second^2)

II. Reagents

ATP	adenosine-5'-triphosphate
UTP	uridine-5'-triphosphate
GTP	guanosine-5'-triphosphate
CTP	cytidine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dTTP	2'-deoxthymidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
BSA	bovine serum albumin
$CHCl_3$	chloroform
CsCl	cesium chloride
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ddH_2O	distilled deionized water
EtBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
IPTG	isopropylthiogalactoside
K-MES	2[N-morpholino]ethone sulphonic acid
MOPS	morpholinopropane sulfonic acid

PBS	phosphate-buffered saline
PIPES	1,4 piperazinediethanesulfonic acid
SDOC	sodium deoxycholate
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TAE	Tris acetate/EDTA buffer
TBE	Tris borate/EDTA buffer
TE	Tris EDTA
TEMED	N,N,N',N'- tetramethylethylenediamine
Tris-HCl	tris (hydroxymethyl) aminoethane hydrochloride
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

III. Enzymes

RNase	ribonuclease
DNase	deoxyribonuclease
β-gal	β-galactosidase

IV. Other Abbreviations

A_{260}	optical absorbance at 260 nm wavelength
ATI	A-type inclusion body
CPV	cowpox virus
DME	Dulbecco's Modified Eagle medium
ds	double stranded
ss	single stranded
m.o.i	multiplicity of infection
mRNA	messenger RNA
M.W.	molecular weight (expressed in Daltons)
ORF	open reading frame
p.i.	postinfection
PFGE	pulsed-field gel electrophoresis
PFU	plaque forming unit
PVDF	polyvinylidene difluoride
RCN	racoonpox virus
res^-	telomere resolution defective
rpo	RNA polymerase
SFV	Shope fibroma virus
tRNA	transfer RNA
TRT	telomere resolution target
ts	temperature sensitive
UV	ultraviolet

V	volt
VETF	vaccinia early transcription factor
VITF	vaccinia intermediate transcription factor
VLTF	vaccinia late transcription factor

Abstract

Vaccinia virus, a DNA virus of vertebrate hosts, is characterized by its complex morphology, cytoplasmic site of replication and large genome size. The approximately 185-kb linear, double-stranded DNA genome is bounded by incompletely base-paired, A-T rich, terminal hairpins. During DNA replication, these genomes are converted into high molecular weight concatemers with unit length genomes joined together by the replicated inverted repeat configuration of the terminal hairpin. Regeneration of the monomeric, mature genomes from these replicative intermediates can be viewed as a site-specific recombination event and is referred to as telomere resolution. Although the cis-acting elements required for this process are well defined, no specific trans-acting factors have been identified to date. A detailed study of two temperature-sensitive (ts) mutants defective in telomere resolution (res^- mutants tsC63 and ts9383) was undertaken in an attempt to identify elements involved in the resolution process.

Mutant tsC63 was previously characterized as a res^- mutant defective in the synthesis of some but not all postreplicative (i.e. post-onset of DNA synthesis) proteins at the nonpermissive temperature (40°C). Using a combination of marker rescue and DNA sequencing techniques, the defect was localized to open reading frame (ORF) A1. S1 nuclease analysis demonstrated that at the nonpermissive temperature, ORF A1 was transcribed normally whereas other prototype late genes were not. This observation supports previous evidence for the existence of two classes of postreplicative genes in vaccinia, now termed intermediate and late based upon their temporal appearance. Furthermore, it demonstrates that ORF A1 is a required factor in the production of stable late mRNAs in an *in vivo* situation. Whereas previous studies had demonstrated the need for postreplicative gene expression in telomere resolution, this mutant has shown that the intermediate class is insufficient for the resolution process. Therefore, the res^- phenotype is likely a consequence of the defect in late gene expression.

Mutant ts9383 also exhibits a res^- phenotype, and is of particular interest because all levels of gene expression appear normal. The defect in this mutant was determined to reside in ORF D12, a gene previously shown to encode the small subunit of the viral heterodimeric mRNA capping enzyme. Intuitively, one might have expected a mutant like this to have a defect early in the infection cycle and to affect gene expression. An examination of RNA and protein production in this mutant suggests that this is not so. Both the small and large subunit of the mutant virus were found to be stable during infections. However, as determined by co-immunoprecipitation of D12 with a D1 antibody, the mutant D12 protein failed to interact with the D1 subunit at the nonpermissive temperature. This suggests that a physically normal subunit association of the capping enzyme is essential for telomere resolution and the final stages of virion morphogenesis but not for transcription and translation during virus infections.

CHAPTER I: GENERAL INTRODUCTION

The problems with linear DNA

Notwithstanding the normal insults to the genome by spontaneous and induced mutagenesis, the faithful replication and reproducible transmission of the genetic material to progeny is a critical facet of life for any organism. Perhaps most critical to this statement is that genetic information not be lost during each subsequent replication phase.

Linear genomes pose special problems for this process. First, all known DNA polymerases require a 3' hydroxyl primer from which to initiate synthesis (Watson, 1972). Often this function is performed by an RNA primer which is ultimately excised at the end of the replication phase. Therefore, each time a linear DNA molecule is replicated, gaps are generated at the 5' ends of the *de novo* synthesized strands resulting in the production of progressively shorter daughter molecules. Second, free DNA ends have a tendency to fuse or recombine (McClintock, 1941; 1942) and therefore, structures must be in place to suppress inappropriate interactions at the ends of the DNA molecule. Finally, without appropriate protection, free ends may be susceptible to attack by exonucleases. The ends of linear DNA are formally known as telomeres, and the mechanisms employed to replicate and segregate these structures are complex and varied.

Mechanisms to replicate and segregate linear DNA genomes

There are several basic mechanisms by which linear, genomic molecules are reproduced. Bacteriophage lambda eliminates the problem of replicating a linear DNA

genome altogether by eliminating ends during its replication phase. Because its 12 base *cos* ends have complementary sequences, they can base pair to form a circle (Yarmolinsky, 1971). Bacteriophages T2 and T7 utilize a second method. Each 3' end of the linear DNA molecule is a duplicate sequence of the 5' end. During DNA replication, small 5' end-gaps are formed in the replicated genomes, but as the sequences at the ends of the molecule are identical, the 3' ends of the chromosomes can join end-to-end to form dimers (or larger concatemers). The missing genomic segments at the 5' end are then filled in by a DNA polymerase activity and the resulting structures ligated. Subsequently, site-specific cleavage regenerates monomeric daughter genomes (Stratling et al., 1973). The mammalian adenovirus and bacteriophage ϕ29 utilize a protein to which a deoxyribonucleotide is covalently bound. When the protein binds at the extreme end of the chromosome, the attached nucleotide serves as a primer and thus, this mechanism ensures that no information is lost from the 5' ends (Rekosh et al., 1977; Lichy et al., 1981; Wimmer, 1982).

In a number of eukaryotic cells, the telomere replication problem is solved by adding a non-coding reiterated DNA sequence to the ends of the genome (Blackburn and Szostack, 1984; Zakian, 1989). In eukaryotes from yeast to mammals, the mechanism appears to operate in the same manner; an enzyme termed the telomerase adds repeats to the ends of linear DNAs in a non-specific manner. In many respects the process is akin to a complex terminal transferase activity (Greider and Blackburn, 1985). In the yeast *S. cerevisiae*, the approximately 300-bp telomere consists of repeats of 5' $C_{2\text{-}3}A(CA)_{1\text{-}3}$ (Shampay et al., 1984).

Another method of dealing with the telomere replication problem is to maintain the ends of the molecule in a hairpin structure. Hairpin ends have been observed in creatures as

diverse as *Paramecium* (mitochondrial DNA; Pritchard and Cummings, 1981), yeast (plasmids; Kikuchi et al., 1985), bacteria (plasmids of *B. burgdorferi*; Barbour and Garon, 1987), *Tetrahymena* (ribosomal DNA; Blackburn and Gall, 1978), parvoviruses (Berns, 1990) and poxviruses (Baroudy et al., 1982). Replication problems are obviated by the ability to perform synthesis through the hairpins and thereby replicate the linear DNA molecule in a dimeric, double-stranded circular form. Another mechanism is to nick one strand of the molecule and use the ability of the hairpins to fold back on one another (and thereby act as primers). In many cases, this latter process can lead to the generation of multimeric replication intermediates. Once the DNA is faithfully replicated, proper segregation of the genetic material to progeny must be ensured by resolution of the multimers..

Poxviruses, of which vaccinia is the prototypal member, are eukaryotic viruses which represent excellent model systems for studying the nuances of replication and segregation of hairpin terminated DNA chromosomes. In these viruses, DNA replication generates long concatemeric structures and the process of converting these replication intermediates into progeny daughter molecules is known as telomere resolution. While concatemerization is generally accepted as the method by which vaccinia DNA replicates, the process of telomere resolution is not well understood. An understanding of this process is further complicated by the fact that there is an obvious and dramatic interplay of DNA replication and telomere resolution with gene expression and possibly with virion morphogenesis. To fully comprehend the process of telomere resolution in vaccinia, it is necessary to have a thorough understanding of how telomere resolution fits into the virus life cycle.

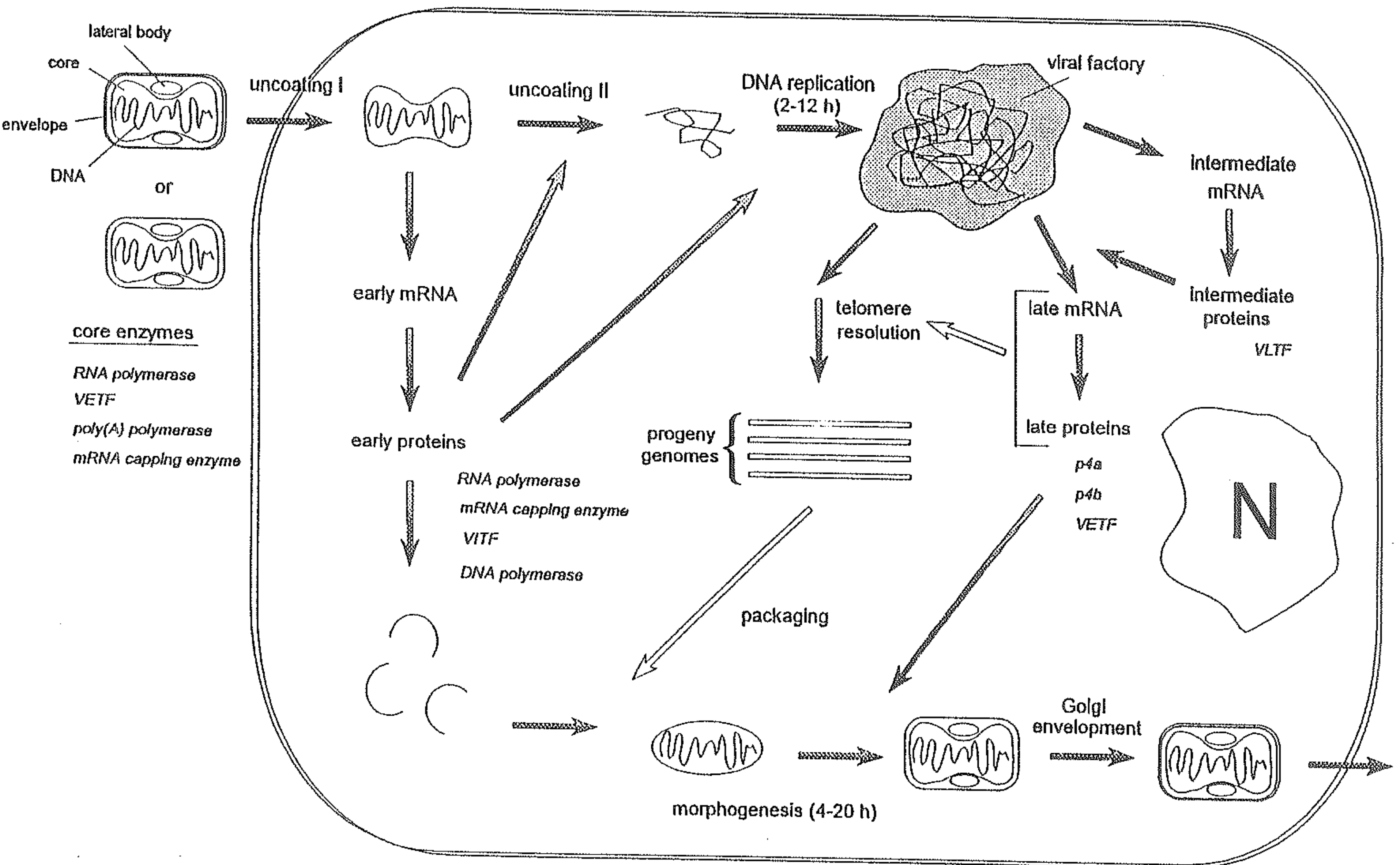
Vaccinia virus as a model system

Poxviruses comprise a family of eukaryotic viruses characterized by their large size, complex morphology, and cytoplasmic site of replication (Moss, 1990a). Members of this family have been identified which are capable of infecting insects (Entemopoxviridae) as well as birds and mammals (Chordopoxviridae). Vaccinia virus, the prototypal member of the *Orthopoxvirus* genus, maintains its genetic information in the form of an approximately 185-kb linear, double-stranded DNA molecule. It has proven to be an exceptionally intriguing model for the study of DNA replication, recombination and transcription for a number of reasons. First, because of its cytoplasmic site of replication, factors normally provided by the host nucleus must now be synthesized by the virus. For example, vaccinia encodes its own DNA and RNA polymerases. Second, the virus can be grown to a relatively high titre in tissue culture thus simplifying biochemical analysis of the encoded polypeptides. Furthermore, the virus rapidly shuts-down host metabolism, a feature which greatly facilitates study of viral functions. Finally, methods for the genetic manipulation of the virus are available such that essentially any gene (virus-derived or not) may be placed downstream of an inducible promoter and expressed to high levels. The life cycle for vaccinia as it is currently understood appears in Fig. 1 and is described in the following sections.

Vaccinia gene expression

Vaccinia exhibits a surprisingly complex cascade of gene expression (Moss, 1990b). The cytoplasmic replication site, while freeing the virus from host cell-cycle progression, forces it to encode its own transcriptional machinery thereby allowing the production of 5'

Figure 1. Vaccinia virus life cycle. Shaded arrows indicate general temporal order of progression, open arrows indicate poorly understood aspects of the life cycle. Time periods post infection in hours are indicated for DNA replication and virion morphogenesis. Abbreviations: VETF, VITF, VLTF, vaccinia early, intermediate and late transcription factors; p4a, p4b, precursor polypeptides 4a and 4b; N, cell nucleus. Elements not drawn to scale. (Modified from Moss, 1990a).


lateral body
core
envelope
DNA
or
core enzymes
RNA polymerase
VETF
poly(A) polymerase
mRNA capping enzyme
uncoating I
uncoating II
DNA replication
(2-12 h)
viral factory
Intermediate
mRNA
Intermediate
proteins
VLTF
early mRNA
early proteins
RNA polymerase
mRNA capping enzyme
VITF
DNA polymerase
telomere
resolution
late mRNA
late proteins
p4a
p4b
VETF
progeny
genomes
N
packaging
Golgi
envelopment
morphogenesis (4-20 h)

capped and 3' polyadenylated mRNA. The temporal regulation of vaccinia gene expression has, until recently, been subdivided into two phases: expression which occurs prior to the onset of DNA replication (early) and expression which occurs following the onset of DNA replication (post-replicative). The latter class has traditionally been termed late. However, the recent finding of an intermediate class of transcription has necessitated a reclassification (Vos and Stunnenberg, 1988). A current model for the temporal regulation of vaccinia gene expression is outlined in Fig. 2. In this model, each class of expressed genes is responsible for the activation of a subsequent class of genes. Viral genes have a high packaging density made so, in part, by the absence of introns. Separation of only a few nucleotides between transcription initiation and termination sites of different genes is not uncommon, nor is it uncommon to have regions with small overlaps. Because of this genome organization, the virus is capable of expressing an estimated 150-200 genes. In the case of vaccinia virus strain Copenhagen which has been sequenced in its entirety, 198 major and 65 minor overlapping open reading frames have been identified (Goebel et al., 1990; Johnson et al., 1993).

i) virus encoded transcriptional machinery

Vaccinia's genome encodes a complex multi-subunit, DNA-dependent RNA polymerase with a molecular mass of approximately 500-kDa (Baroudy and Moss, 1980). Currently, 8 polypeptides of 147, 132, 35, 30, 22, 19, 18 and 7-kDa are believed to comprise the enzyme, and the genes which encode these polypeptides have been identified (see Moss, 1990b; Moss et al., 1991; Johnson et al., 1993). The two largest proteins contain similarities to both prokaryotic and eukaryotic RNA polymerase subunits. The enzyme is present in

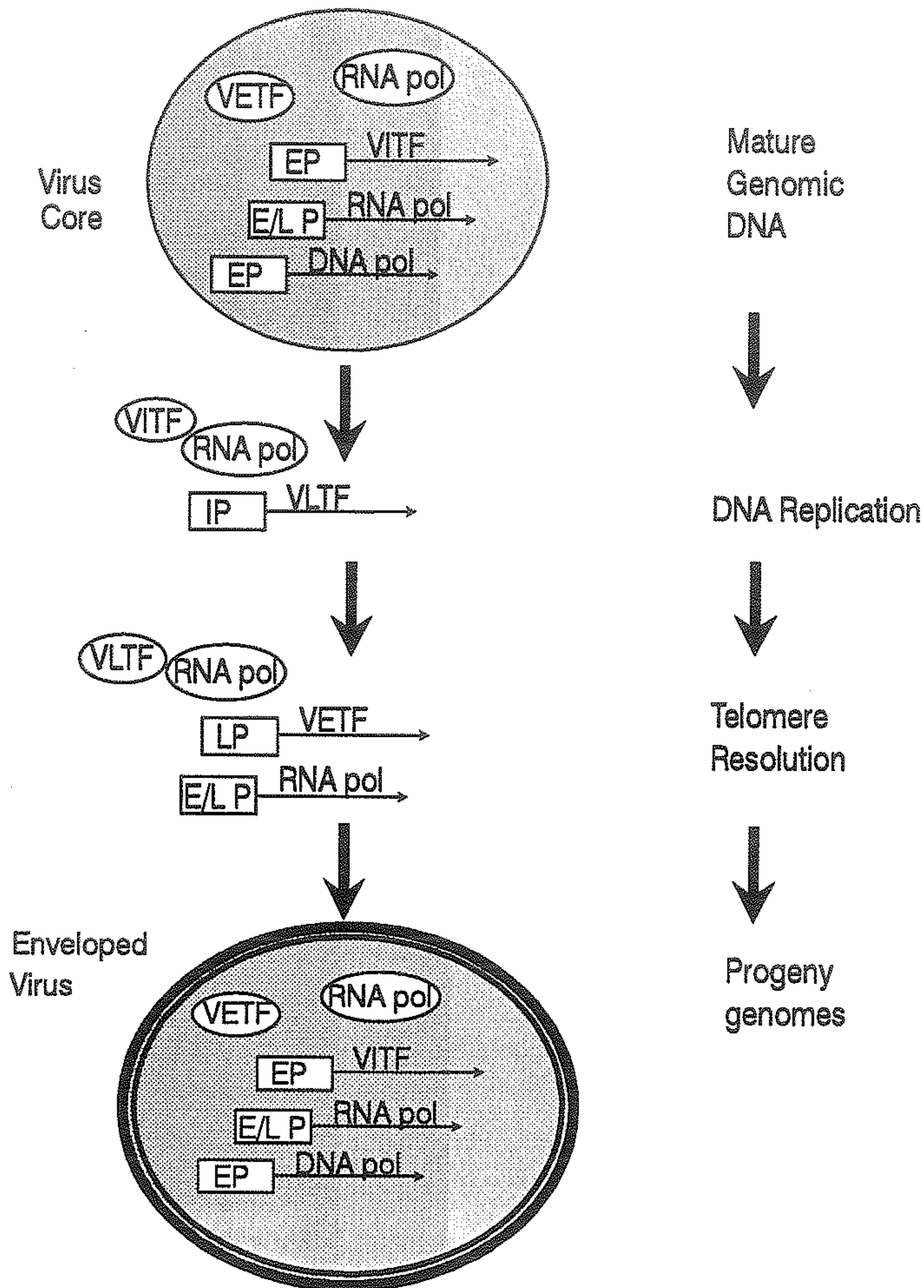


Figure 2. Vaccinia virus gene expression cycle. Thin arrows indicate open reading frames, thick arrows indicate order of progression, ovals indicate proteins. Abbreviations: EP, IP, LP, early, intermediate and late promoters; E/LP, early/late tandem promoter; VETF, VITF, VLTF vaccinia early, intermediate and late transcription factors; RNA pol, RNA polymerase; DNA pol, DNA polymerase. (Modified from Moss, 1990b).

purified virions and the promoters for the polymerase subunits are of the early type, although some have a compound early/late promoter (Fig. 2).

In addition to the RNA polymerase, the vaccinia genome also encodes a number of other enzymes involved in mRNA synthesis. These include: a 127-kDa multifunctional mRNA capping enzyme composed of 95 and 31-kDa subunits (Martin et al., 1975), a 38-kDa RNA(nucleoside-2)methyltransferase (Barbosa and Moss, 1978a,b), an 80-kDa heterodimeric poly(A) polymerase (Moss et al., 1975), two nucleoside triphosphate phosphohydrolases; one of which likely has a role in postreplicative gene expression (Kunzi and Traktman, 1989) and a type I DNA topoisomerase (Shaffer and Traktman, 1987; Shuman and Moss, 1987). Transcription factors critical for the activation of the different gene classes will be discussed in the relevant sections below.

ii) early gene transcription

Early gene expression occurs immediately after virus entry into the host cell taking place concomitantly with, or immediately following, the first virus uncoating stage. The synthesis of virus early transcripts is dependent on the viral RNA polymerase and a heterodimeric early transcription factor (VETF) which are packaged into the virion during the previous replication cycle (reviewed in Moss, 1990b).

RNA-DNA hybridization studies have suggested that approximately one-half of the genome is transcribed during the early gene expression phase (Paoletti and Grady, 1977; Boone and Moss, 1978) and the polypeptides encoded by the early gene class include elements involved in DNA replication (e.g. DNA polymerase) and RNA transcription (e.g.

RNA polymerase, mRNA capping enzyme, etc.). Transcription factors required for the intermediate phase of gene expression are also thought to be produced at this time.

The early promoter is found approximately 30-bp upstream of the transcription initiation site (Weir and Moss, 1987). Detailed mutational analysis of one early gene (encoding a 7.5-kDa polypeptide) identified a region at -13 to -28 relative to the mRNA start site (+1) which was critical for function (Davison and Moss, 1989a). The sequence of the 7.5-kDa polypeptide gene promoter is indicated below with the critical region underlined:

5' TAAAAGTAGAAAATATATTCTAATTTATTG (+1)

Most of the adenosine residues within the critical region are of significant importance for activity as virtually any nucleotide substitution in this region decreases expression of a reporter gene. Interestingly, the 7.5-kDa polypeptide gene promoter sequence detailed above is not optimal for promoter strength as substitution of an adenylate with a guanylate residue at -22 results in a 3-fold increase in promoter strength. Variations within the critical region are likely responsible, in part, for the significant variation seen in the quantities of different early transcripts (Kaverin et al., 1975; Paoletti and Grady, 1977; Boone and Moss, 1978). Purified vaccinia RNA polymerase is incapable of transcribing a double-stranded DNA template unless supplemented with vaccinia early transcription factor (VETF). VETF is a heterodimeric protein (Broyles et al., 1988) whose subunits are expressed late in infection and then packaged into virions for use during the subsequent infection cycle (Gershon and Moss, 1990). The two components of VETF are 82 and 77-kDa in size with the 77-kDa subunit containing an ATP binding/ATPase motif. This observation is consistent with the fact that ATP hydrolysis is a requirement for early gene transcription (Broyles and Moss, 1988).

All virus early mRNAs examined to date have a discrete length and the sequence TTTTTNT (N any nucleotide), which appears approximately 20-50 nucleotides upstream of the poly(A) addition site, is essential for efficient termination (Yuen and Moss, 1987). Interestingly, it is the nascently produced RNA sequence U_5NU and not the DNA template sequence that is recognized by the transcription termination activity (Rohrmann et al., 1986; Shuman and Moss, 1988). Even more intriguing is the fact that the termination activity, as measured in an *in vitro* complementation assay, is conferred by the viral mRNA capping enzyme (Shuman et al., 1987). It appears that termination is a separate activity of the viral capping enzyme as *in vitro* produced nascent transcripts need not be capped for termination to take place (Shuman et al., 1987; Luo et al., 1991).

iii) intermediate gene transcription

Normally, when vaccinia infections are performed in the presence of a DNA replication inhibitor such as hydroxyurea, postreplicative gene expression is suppressed (Cochran et al., 1985). It is for this reason that DNA replication has been considered the demarcation point between early and postreplicative classes of gene expression. The existence of two different classes of postreplicative genes was suggested at an early stage in virus research. This suggestion was based on the temporal appearance of polypeptides during the later stages of the virus infection cycle (Moss and Salzman, 1968; Pennington, 1974; Opperman and Koch, 1976). Vos and Stunnenberg (1988), used a novel plasmid transfection assay to provide definitive evidence for the existence of these two postreplicative classes termed intermediate and late.

Vos and Stunnenberg (1988) assumed that factors required for intermediate gene expression would belong to the early gene class and would therefore be synthesized prior to DNA replication (see Fig. 2). For their experiment, they examined four genes whose promoters were initially classified as post-replicative: 11K, 7.5K, I8 and I3. Of these, both the 11K and 7.5K genes contained the typical late promoter consensus sequence TAAAT, but neither the I3 nor I8 genes had such a sequence present at the transcription initiation site (Schmitt and Stunnenberg, 1988; Vos and Stunnenberg, 1988). Infections performed in the presence of the DNA replication inhibitor hydroxyurea did not express any of the four transcripts. In contrast, when plasmids containing the I8, I3, 11K or 7.5K genes were transfected into vaccinia-infected, hydroxyurea-blocked cells, the plasmid-borne I8 and I3 genes were expressed whereas the 11K and 7.5K genes were not. This demonstrated that: i) the factors essential for transcription from I8 and I3 promoters were present prior to DNA replication, ii) a naked DNA template was essential for expression since transfected but not genomic DNA was transcribed, and iii) the I3 and I8 genes belonged to a different regulatory class than the 11K and 7.5K genes. Subsequent to Vos and Stunnenberg identifying I3 and I8 as intermediate genes, Keck et al. (1990) identified three other genes belonging to this intermediate class: A1, A2 and GK1/G8.

Determination of a consensus promoter sequence for intermediate genes has remained somewhat tenuous considering that only five intermediate genes have been identified to date. Three of these promoters, A1, A2 and G8 have a long polypyrimidine tract adjacent to a TNAAAT sequence (where N is any nucleotide). This TNAAAT sequence bears similarity to the late promoter sequence TAAAT (Davison and Moss, 1989b) and has served to confuse

the issue of intermediate/late promoter structures. Two other intermediate genes, I3 and I8 do not have the TNAAAT sequence further bringing into question the nature of the recognition mechanism. Linker-scanning mutagenesis of the I3 promoter identified two critical regions in intermediate promoters at positions -20 to -9 and +1 to +9 relative to the transcription initiation site (Hirschmann et al., 1990). More specific single-base mutational of intermediate promoters A1, A2 and G8 defined an AT-rich element at -26 to -13 and a TAAA sequence at -1 to +3 on the non-template strand (relative to the mRNA start site) which were critical for expression (Baldick et al., 1992).

The intermediate gene transcription factor is composed of at least two elements, one of which is required for promoter melting (Vos and Stunnenberg, 1991). The second element is the viral mRNA capping enzyme (Vos et al., 1991). Capping activity is not a prerequisite for intermediate transcription factor activity since *in vitro*, intermediate transcripts are not capped until reaching >15 nucleotides in length (Harris et al., 1993). This observation suggests that the transcription factor activity represents yet another function of the capping enzyme. At this point, the actual number and functions of intermediate genes is unclear but a recent estimate from one dimensional polyacrylamide gel electrophoresis places the number at likely greater than thirteen (Zhang et al., 1992). The function of the intermediate class of genes is currently not understood. At least some of the factors are responsible for activating the late class of genes, while other intermediate gene products such as I8 appear to have an involvement in virion assembly (Fathi and Condit, 1991).

iv) late gene transcription

Late genes expressed by the virus likely encode elements involved in virion assembly and in the production of factors involved in the transcription of early genes during the next infection cycle. The promoter sequence for late genes has been well characterized due to a comprehensive mutational analysis of one late gene (28K) promoter (Davison and Moss, 1989b). There is a conserved TAAAT motif present downstream of a run of A or T residues in the non-template strand. In many cases, a G immediately follows the TAAAT sequence and dictates the translation start site. The 28K late gene promoter upon which the single site mutagenesis was performed is A_6N_6TAAAT. One construct containing a run of T residues ($T_{20}N_6$TAAAT) was over 100-times stronger for expression than the 28-kDa promoter (Davison and Moss, 1989). In fact, T-runs, in the non-template DNA strand, generally produce higher expression than A-runs suggesting that these sequences contribute to more than just a region with a low melting temperature.

Keck et al. (1990), using a similar transfection assay to that described by Vos and Stunnenberg (1988), identified three factors necessary and sufficient to activate transcription from a reporter gene containing a late promoter. The results matched previously obtained biochemical observations suggesting the need for three distinct intermediate factors in late gene transcription transactivation (Wright and Moss, 1989; Wright et al., 1991). The three elements identified were the virus-encoded proteins A1, A2 and G8 (GK1); a full understanding of their role in transactivation remains to be elucidated.

There are two significant structural alterations present in late and intermediate transcripts. First, most late transcripts are not specifically terminated; rather, heterogeneous

length mRNAs are produced. Second, each transcript contains a characteristic 5' capped poly(A) tract of approximately 30-40 nt (Bertholet et al., 1987; Baldick and Moss, 1993). This poly(A) head is not encoded by the genome and is believed to arise through a slippage mechanism at the TAAAT (ATTTA in the template strand) sequence within the promoter (Schwer et al., 1987).

Recently it has become clear that not all postreplicative transcripts have heterogeneous 3' ends. Several genes have been characterized as containing discrete 3' termini. These include the cowpox and vaccinia virus equivalent A-type inclusion (ATI) body protein (Patel and Pickup, 1987; Patel et al., 1988; Amegadzie et al., 1990) and non-coding transcripts present at the telomere (Parsons and Pickup, 1990). At least in the case of the cowpox ATI protein, the discrete termini appear to be produced through the actions of an endoribonuclease. This enzyme cleaves primary RNAs near a cis-sequence designated the AX element (which includes the sequence 5'-UUUUAU-3'). The newly derived 3' end is then polyadenylated (Antczak et al., 1992). Whether the telomeric transcripts are processed in the same fashion is unknown.

Vaccinia DNA structure

The complex nature of gene expression discussed above is reflected in the size of the genome and the fact that the virus is responsible for its own transcription and DNA replication. The mature vaccinia chromosome size is 185-200-kb (1-2 x 10^5 kDa). The Copenhagen strain, recently sequenced in its entirety, has a size of 192-kb (Goebel et al., 1990). The genome is a double-stranded, covalently-closed, continuous DNA structure with

the telomeres containing hairpin ends. There is a relatively high AT richness associated with the genome (64%), although this value varies greatly among other genera of the poxvirus family (Moss, 1990). In general, the physical/genetic structure of the viral DNA can be broken down into three components: a conserved central region, an inverted terminal repeat region, and the telomeric sequences which include the hairpin termini (Fig. 3).

i) central region

Although no clear demarcation point exists, the central portion of the vaccinia genome maintains a high level of similarity with many other poxvirus genomes examined to date, and it appears that the majority of genes essential for virus propagation in tissue culture are located in this region (Mackett and Archard, 1979; Esposito and Knight, 1985; Earl and Moss, 1989). Many of the genes located closer to the telomeres are believed to play roles in host-range/tropism/species-specificity determination (see Turner and Moyer, 1990).

ii) inverted terminal repeats

The inverted terminal repeats are identical but oppositely oriented sequences which occur at the telomeric ends of the poxvirus genome (Garon et al., 1978; Wittek et al., 1978) (Fig. 3). In vaccinia, the repeat is usually 10-12-kb in length and itself has some interesting properties; in particular, a series of tandem repeats found within the most extreme 3-4-kb of the molecule. There are two sets of repeats separated by a unique 325-bp region (Wittek and Moss, 1980). Analysis of one plaque purified isolate showed a series of 13 x 70-bp repeats separated by the aforementioned 325-bp fragment and followed by another 18 x 70-bp repeat

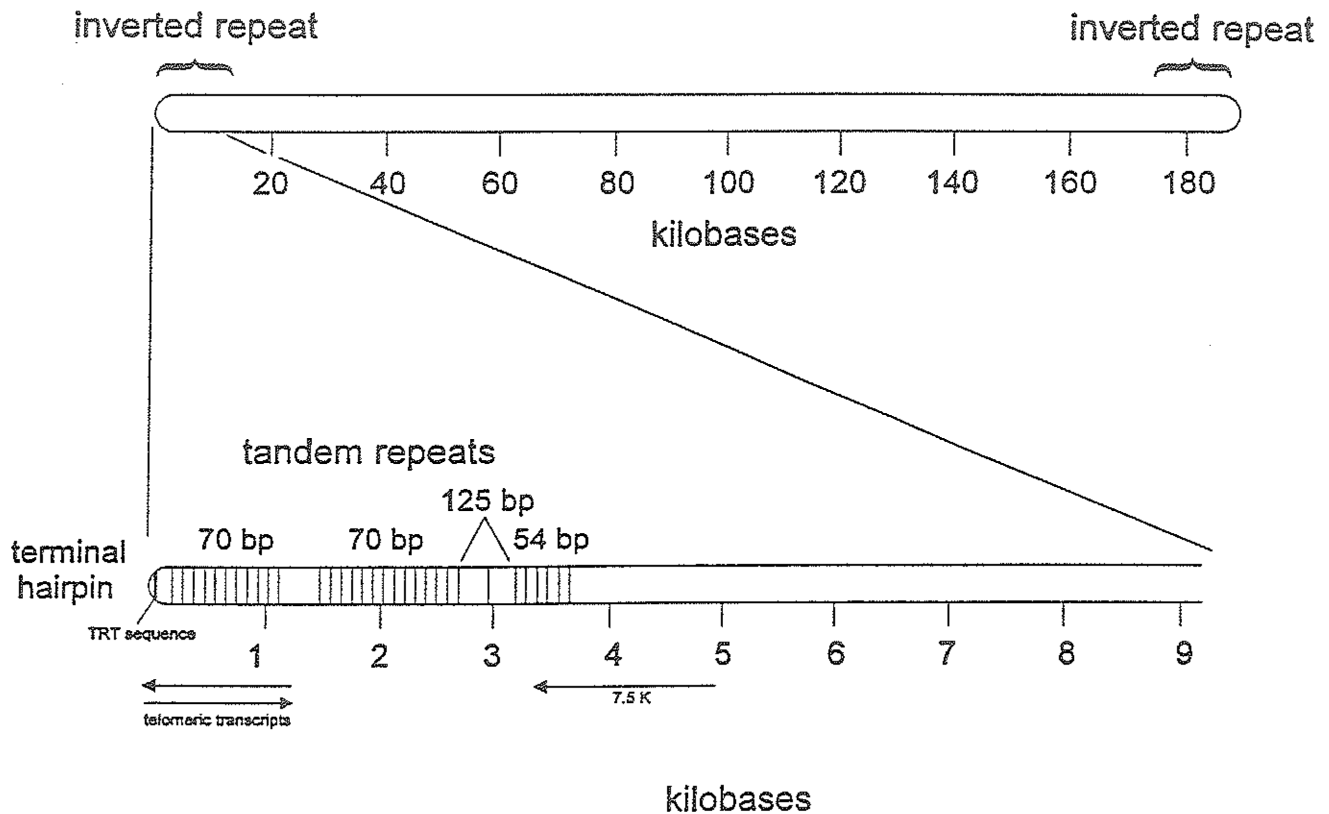


Figure 3. Vaccinia virus DNA structure. Upper portion of figure shows the approximately 185-kb hairpin-terminated mature DNA chromosome. Lower portion of figure shows an enlargement of the 9 kilobases closest to the hairpin terminus which includes the inverted repeat region. Blocks of tandem repeats are indicated as are the locations and names of transcripts which have been detected near the terminal regions. The location of the telomere resolution target (TRT) sequence which is required in telomere resolution is also indicated. (Modified from Moss, 1990a and Parsons and Pickup, 1990).

set, 2 x 125-bp repeats and finally a series of 8 x 54-bp repeats (Fig. 3) (Baroudy and Moss, 1982b; Baroudy et al., 1982). Based upon sequence information, it appears that the 125 and 70-bp repeats evolved from the 54-bp repeats (Baroudy et al., 1982a,b; Baroudy and Moss, 1982). Even though the significance of the inverted terminal repeat is not yet clear, findings by Esposito and Knight (1985) that 37 of 38 *Orthopoxvirus* strains show cross-hybridization with the vaccinia 70-bp repeat, suggest a conserved and significant role for this region. Only raccoonpox did not show a hybridization signal, although subsequent analysis of this region identified other repeat units whose general conceptual make-up is similar to that of the vaccinia repeat. Interestingly, the inverted terminal repeat of variola virus, the causative agent of smallpox, is very short and may not contain any tandem repeats at its end (Archard and Mackett, 1979; Esposito and Knight, 1985).

The number of repeats which exist in a given vaccinia purified plaque isolate varies dramatically. It is presumed that the variation occurs through incomplete crossing-over as the virus is known to exhibit significant levels of intra- and intergenic recombination (see Condit and Niles, 1990). Intriguingly, Shope Fibroma virus, a member of the *Leporipoxvirus* genus, shows little length heterogeneity in its repeat region possibly because its genome contains only a low number of related repeat units; i.e. each repetition unit is a unique (but related) imperfect palindrome (Upton et al., 1987).

A number of postreplicative mRNA species are produced within the repetitive region although their functions remain unknown (Wittek et al., 1980; Wittek et al., 1981; Cooper et al., 1981) (e.g. 7.5K transcript in Fig. 3). It also appears that non-coding transcripts are produced in this region. Parsons and Pickup (1990) have noted several non-coding telomeric

transcripts, one of which originates from a region between the tandem repeats and another from a sequence known as the telomere resolution target sequence (TRT) adjacent to the hairpin terminus (Hu and Pickup, 1991) (Fig. 3).

iii) the vaccinia telomere

Vaccinia telomeres contain an uninterrupted covalently-closed linear hairpin structure. That the DNA strands at the telomeres are terminally cross-linked was demonstrated at an early stage both by denaturation of the DNA chromosome followed by neutral gradient sedimentation (Szybalski et al., 1963; Junwirth and Dawid, 1967) and by a failure of vaccinia DNA strands to denature during alkaline gradient sedimentation (Berns and Silverman, 1970). Definitive evidence for the existence of cross-linked ends was provided by electron microscopy of denatured and partially denatured viral DNA and the site of this cross-linking was localized to the ends of the DNA molecule (Geshelin and Berns, 1974). Terminal cross-links have since been seen in every poxvirus examined to date (see DeLange and McFadden, 1990 for a review).

The telomeric nucleotide sequence of vaccinia virus strain WR DNA was determined by Baroudy et al. (1982) who showed conclusively that the cross-linked structures corresponded to hairpin termini (Fig. 4). The hairpin is 104-bp in vaccinia and exhibits three significant features: i) a high AT richness of the region (92%); ii) the presence of a number of incompletely base-paired nucleotides; and iii) the fact that the hairpins from either end exist in two conformations which are inverted and complementary to one another and are often referred to as "flip" and "flop" configurations (Fig. 4). A similar structure exists for Shope

```
                                                                                                     C A              10
A-A-G-T-T-A-G-T-A-A-A-T-T-A-T-A-T-A-T-A-T-A-A-T-T-T-T-A-T-A-A-T-T-A-A-T-T-A-A-T-T-T-A-A-T-T-T-T-A T A-T-T-T-T|A-T-T-T-A-G-T-G-T-3'
|  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . |. . . . . . . . .
A-T-C-A-A-T-C A-T-T-T-A-A T-A T-A-T-A-T-A-T-T A-A-A-A-T A-T T-A-A-T-T-A A-A-T T-A-A-A-A-T A-T-A-A-A-A|T-A-A-A-T-C-A-C-A-5'
             T            T   T             C         C   G           T     A           T-T

             A           A   A             G         G   C           A     T           A-A                10
T-A-G-T-T-A-G T-A-A-A-T-T A-T A-T-A-T-A-T-A-A T-T-T-T-A T-A A-T-T-A-A-T T-T-A A-T-T-T-T-A T-A-T-T-T-T|A-T-T-T-A-G-T-G-T-3'
|  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . |. . . . . . . . .
T-T-C-A-A-T-C-A-T-T-T-A-A-T-A-T-A-T-A-T-A-T-T-A-A-A-A-T-A-T-T-A-A-T-T-A-A-A-T-T-A-A-A-A-T A T-A-A-A-A|T-A-A-A-T-C-A-C-A-5'
                                                                                           G T
```

Figure 4. Nucleotide sequence of the hairpin loops of vaccinia virus DNA telomeres. The sequences represent the two alternate inverted and complementary forms of the hairpin termini known as flip and flop. The terminal loops are shown to maximize base-pairing. Italicized letters indicate a portion of the contiguous identical sequence. The most proximal portion of the telomere resolution target region (TRT) is boxed in. (Modified from Baroudy et al., 1982a).

fibroma virus (DeLange et al., 1986). Any model for replication must be able to accommodate these features.

Cloning of the poxvirus telomeric region was a difficult process since it is in the form of a hairpin structure. Three distinct strategies have been employed to clone the virus telomere. DeLange et al. (1984) used the fact that linear yeast plasmids can be propagated when the plasmid telomere sequences are replaced with telomeres from another source (Szostak and Blackburn, 1982). Gel-purified telomeric fragments from Shope Fibroma virus (SFV) and vaccinia were ligated to yeast linear plasmids and the constructs used to transform yeast. Interestingly, the replicated form of the plasmid was not linear, but circular in nature such that synthesis through the telomere resulted in an inverted repeat (telomere fusion) configuration of the hairpin sequence (Fig. 5). The viral DNA termini in the double-stranded inverted repeat form were subsequently sub-cloned into recombination-deficient bacteria (DeLange et al., 1986). A second strategy was used by Winters et al. (1985). This group generated a fusion element by annealing the complementary strands of denatured hairpins and then cloning these artificially-derived telomere fusions into bacterial plasmids. A third strategy made use of the observation that restriction enzyme analysis of replicating poxvirus DNA showed palindromic forms of the terminal sequence (i.e. a fusion element). Merchlinsky and Moss (1986) isolated this restriction fragment directly from replicating DNA and inserted the sequence into bacterial vectors. The sequence of the vaccinia telomere fusion/concatemer junction clone is seen in Fig. 5.

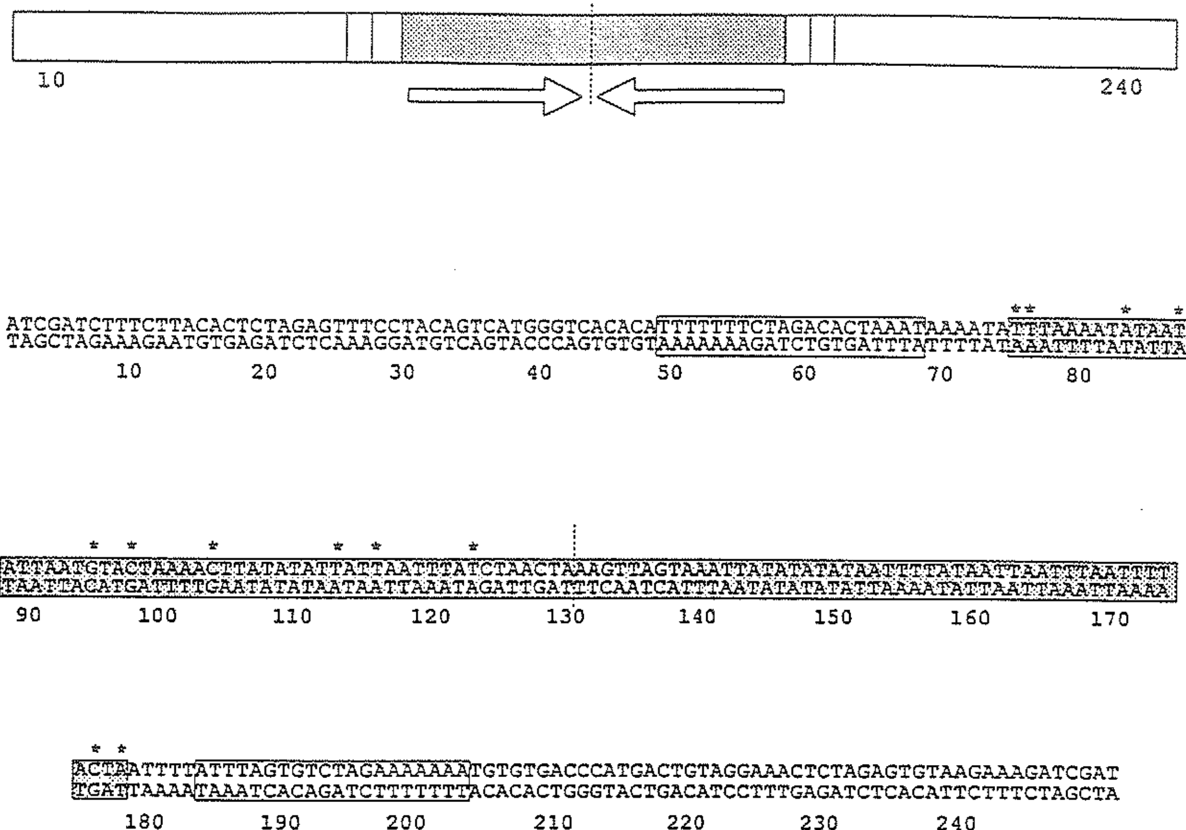


Figure 5. Schematic representation and nucleotide sequence of the vaccinia virus telomere fusion (concatemer junction). The upper schematic depicts the structure of the telomere fusion after DNA synthesis has proceeded through the terminal hairpin. The lower portion depicts the nucleotide sequence. Nucleotides within the shaded box correspond to the double-stranded form of the terminal hairpin loop. This sequence forms an imperfect palindrome with the axis of symmetry indicated by a vertical dashed line. Nucleotides which contribute to the imperfect palindrome and which are normally not base-paired in the mature hairpin termini are indicated with an asterisk (*). Arrows in the upper schematic represent the palindrome formed by the telomere fusion. The minimal telomere resolution target (TRT) sequence required for telomere resolution is bounded by an unshaded box. (Modified from Merchlinsky and Moss, 1989b).

Vaccinia DNA replication

DNA replication marks a significant point in the virus life cycle. Not only does it represent a commitment by the virus to the production of progeny genomes, but it is also essential for the transition from early to postreplicative gene expression. Furthermore, it is generally accepted that eukaryotic DNA viruses tend to utilize elements of the host nucleus when replicating their genomes. In contrast, the cytoplasmic site of replication seen in vaccinia infections is significant since the virus must either encode the elements required for synthesis of its own genome or it must actively recruit these factors from the host nucleus to the cytoplasm. There is some debate regarding the extent to which the latter process is employed (see Dales, 1990), however it is generally accepted that most of the proteins required for viral replication are, in fact, virus-encoded. One consequence of the fact that vaccinia encodes its own replication machinery is that it allows the virus to replicate independent of the host S-phase. Further proof of vaccinia's autonomy from the host cell nucleus comes from the observation that vaccinia can undergo DNA replication in mouse L-cells enucleated with the drug cytochalasin B (Prescott et al., 1971; Pennington and Follet, 1974). Intriguingly, while DNA replication and all levels of gene expression appear unaffected, mature infectious progeny are not produced (Pennington and Follet, 1974). However, an α-amanitin resistant mutant has been identified which can replicate in enucleated BSC40 cells and produce infectious virus particles (Villarreal et al., 1984).

Vaccinia DNA replication occurs in specific sites in the cytoplasm known as viral factories or virosomes (Cairns, 1960; Dales and Siminovitch, 1961). In a synchronous (i.e. high multiplicity) infection, DNA replication can take place as early as 2-3 h postinfection

(p.i.) and increase in intracellular viral progeny DNA continues at a rapid rate until approximately 12 h p.i. At this point, viral DNA synthesis slows and ultimately plateaus. Estimates have placed productive infections at generating about 10,000 genome copies, although only about one-third of these sequences are packaged (Salzman, 1960; Joklik and Becker, 1964).

A number of different models have been proposed to explain the mechanism by which poxviruses, including vaccinia, replicate their DNA. One current model based on an hypothesis proposed by Cavalier-Smith (1974) and Bateman (1975) explains essentially all of the current data (Moyer and Graves, 1981; McFadden and Dales, 1982). This model, the self-priming replication model, is illustrated in Fig. 6. In order to examine the process in greater detail, each aspect of DNA replication will be covered in a separate section below.

i) initiation of DNA replication

Based on the altered sedimentation properties of viral DNA (Pogo, 1977), it was postulated that DNA replication is initiated by the introduction of a site-specific nick at, or near, one of the terminal hairpins (Esteban and Holowczak, 1977a,b; Pogo et al., 1981; 1984). The nick would then allow access by a DNA polymerase to a 3' OH end, which could be used as a primer for DNA synthesis (self-priming). That replication begins at the ends of the genome seems likely based on the fact that pulse-labelling with [^{3}H] labelled precursors results in incorporation at the ends of the genome first (Pogo et al., 1984).

The sequence specificity of this hypothesized near-terminal nicking is currently unknown. Moreover, an actual origin of replication as seen in virtually all other prokaryotic,

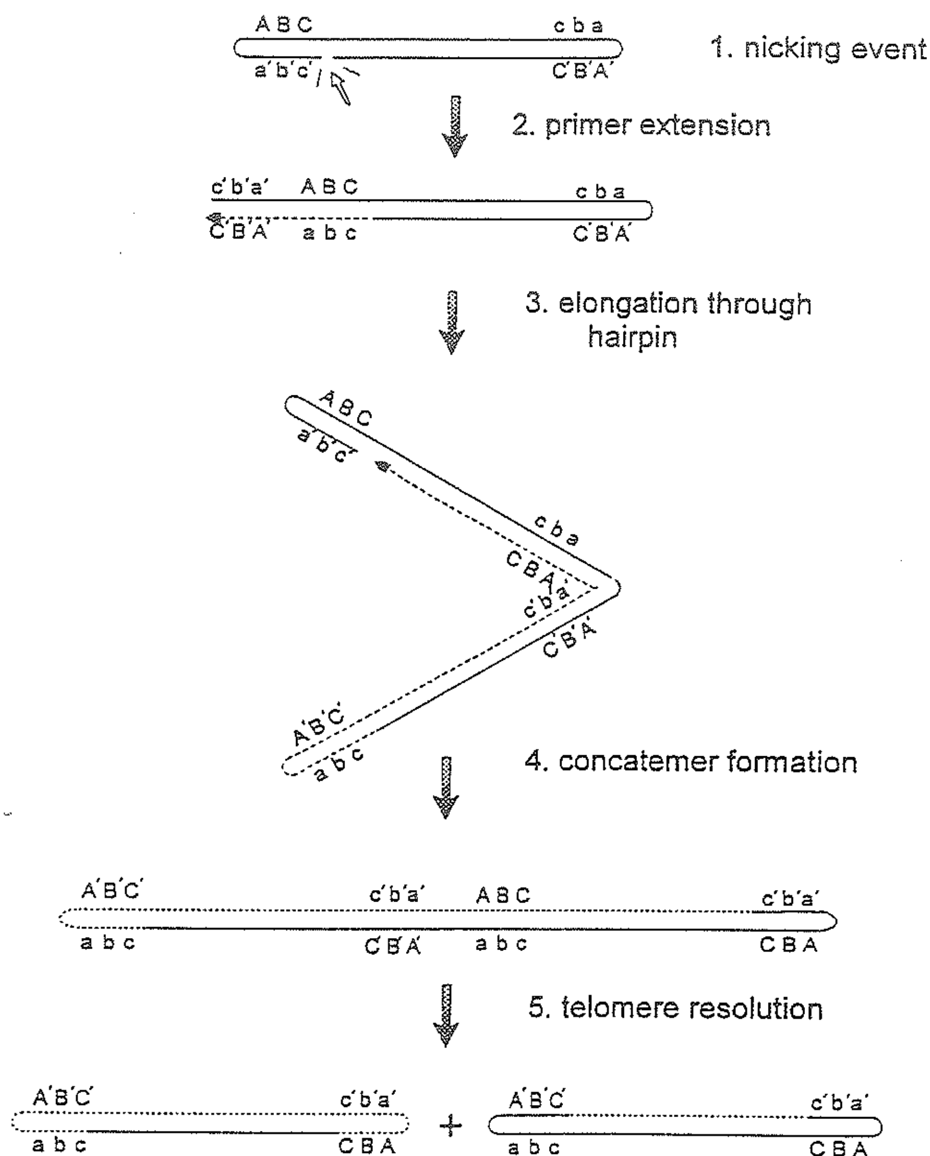


Figure 6. Postulated self-priming model for poxvirus DNA replication. Letters A, B, C (a, b, c for complementary strands) represent sequences located near the hairpin termini and within the approximately 10 kilobase-pair inverted repeat region. As the termini are not identical, the apostrophe (') symbols are used to indicate extrahelical bases in the flip and flop isomers (see Fig. 3 this work). Dotted lines indicate newly synthesized DNA. For simplicity, step 4 only shows a dimer formation. However, larger concatemers are known to be generated. (Modified from Traktman, 1990).

eukaryotic and viral genomes has thus far eluded identification. Interestingly, any circular plasmid, irrespective of sequence, when transfected into vaccinia or SFV-infected cells, is replicated into high molecular weight complexes (DeLange and McFadden, 1986; Merchlinsky and Moss, 1988)

ii) elongation

The first step in elongation is to synthesize a small stretch of DNA corresponding to the hairpin region (see Fig. 6, step 2) by displacing the complementary strand. Because the newly synthesized region contains a self-complementary inverted repeat, the 3' end can fold back upon itself and again allow priming (Fig. 6, step 3). Synthesis continues past the opposite hairpin thereby producing a dimeric concatemer in a head-to-head conformation. Likewise, further DNA synthesis can generate large, multimeric structures (Moyer and Graves, 1981, DeLange, 1989; Merchlinsky and Moss, 1989a). Because concatemers are seen, it suggests that during the initiation of DNA replication, nicks are not always introduced at both ends of the genome. If nicks were introduced at both termini, only dimeric replication intermediates would be produced. Importantly, the self-priming model can account for the regeneration of incompletely base-paired loops and the retention of the flip/flop sequences present at either end of the DNA molecule (see section on telomere resolution).

Other models have been proposed to explain vaccinia DNA replication including the *de novo* synthesis model, which suggests that replication begins with the synthesis of an internal RNA primer (Esteban et al., 1977; Pogo and O'Shea, 1978; Baroudy et al., 1982b;

Moss et al., 1983), and another model which invokes lagging strand synthesis (Esteban et al., 1977). However, such models have not received support recently.

iii) concatemer formation

The predominant form of vaccinia/poxvirus DNA replication involves the production of concatemeric structures (DeLange et al., 1986; DeLange, 1989; Merchlinsky and Moss, 1986; Merchlinsky et al., 1988; 1989a). Early in the study of poxvirus DNA replication, it was determined that rapidly sedimenting forms of intracellular concatemeric DNA existed (Moyer and Graves, 1981). Later, actual visualization of these DNA species was achieved by using pulsed-field gel electrophoresis (Merchlinsky and Moss, 1989a; DeLange, 1989).

Concatemers obtained from sucrose gradient fractionation of replicating rabbitpox DNA contained head-to-head and tail-to-tail arrays of genomes (Moyer and Graves, 1981). However, more recent evidence derived from an examination of resolution defective mutants suggests the presence of head-to-tail fusions as well (DeLange, 1989; Merchlinsky and Moss, 1989a). These forms likely arise as a result of recombination.

iv) vaccinia genes with roles in DNA replication

Genetic analysis has identified four conditional-lethal DNA$^-$ mutants (for review see Traktman, 1990; Millns et al, in press). These include mutants with defects in: the viral DNA polymerase (Traktman et al., 1989), the gene product of ORF D5 (Roseman and Hruby,

1987; Evans and Traktman, 1987), a gene product encoded by B1R (a known serine-threonine protein kinase) (Rempel et al., 1990; Rempel and Traktman, 1992) and the gene product of ORF D4 which has uracil DNA glycosylase activity (Millns et al., in press; Stuart et al., 1993; Upton et al., 1993). An attempt to insertionally inactivate a type I DNA topoisomerase was unsuccessful suggesting an essential role for this protein, possibly in DNA replication (Shuman et al., 1989).

The DNA replication mechanism is also postulated to require a nuclease which initiates DNA replication via a nicking activity, an activity which separates concatemeric intermediates into monomeric forms, and a ligase to seal the products of this reaction. Interestingly, Kerr and Smith (1989) have demonstrated that a virus-encoded DNA ligase is not essential for virus viability in BSC40 cell cultures. Whether there is another ligase activity, perhaps host-provided, or whether cleavage and ligase activity are both components of a multi-functional enzyme (such as a DNA topoisomerase), awaits further study. Moreover, the recent observation that a ts mutant defective in DNA replication has a lesion in a gene encoding a uracil DNA glycosylase suggests that a role for DNA repair enzymes coupled to the replication process is plausible (Millns et al., in press).

Telomere resolution

The process of converting viral concatemeric structures to monomeric, mature, hairpin-terminated genomes is referred to as telomere resolution and the mechanism by which this process occurs has been one of the most elusive goals of recent poxvirus studies. The tools of genetics, biochemistry and molecular biology have all combined to provide a basic

framework for understanding this process. In general, the essential cis-acting sequences are already known; the challenge remains to identify the trans-acting factors and their roles in the process.

i) in vivo resolution of cloned poxvirus telomere fusions

The observation by DeLange and McFadden (1986) that transfected plasmid DNA could be replicated by the viral replication machinery, but in a sequence non-specific manner, was an important step in the development of a telomere resolution assay. In this assay, circular plasmids when transfected into virus-infected cells are replicated into high molecular weight concatemeric structures. Plasmids which contain the cloned viral telomere fusion element are also replicated but are then resolved into linear hairpin-terminated minichromosomes (DeLange et al., 1986; Merchlinsky and Moss, 1986) (Fig. 7). Uninfected cells are neither able to replicate or resolve plasmids transfected into the cytoplasm and therefore it is likely that the factors responsible for this are virus-encoded. Importantly, this resolution machinery must be highly conserved among the poxviruses since the telomere resolution fusion element from SFV can be resolved by vaccinia-infected cells; likewise, the cloned vaccinia fusion element can be resolved in SFV-infected cells (DeLange et al., 1986; Merchlinsky, 1990b)

ii) identification of the telomere resolution target (TRT) region

The assay described above has allowed a means of determining the sequence and positional organization of the cis-elements necessary for telomere resolution. In an attempt

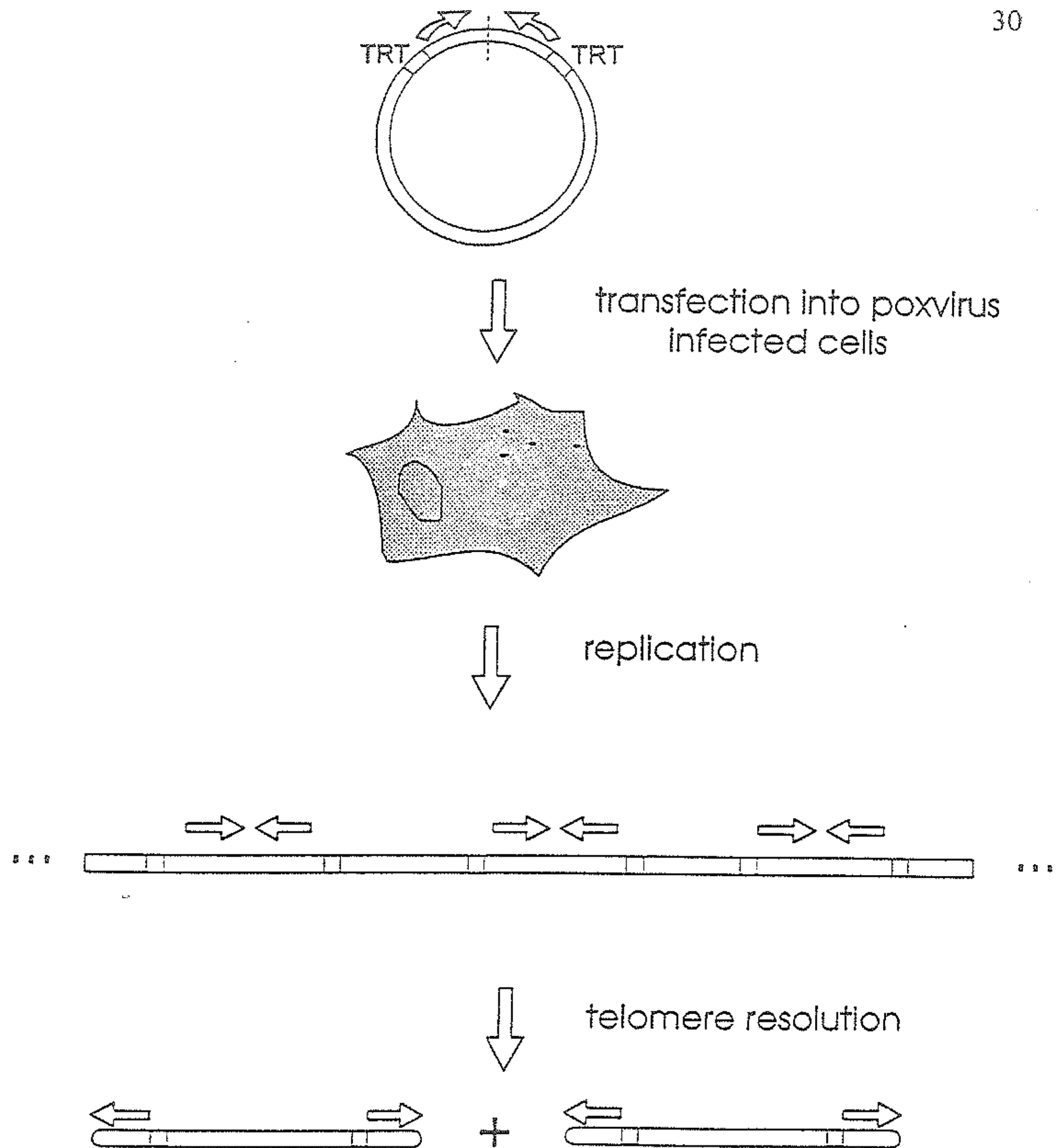


Figure 7. Plasmid transfection assay for telomere resolution. Bacterial plasmids containing the telomere resolution target sequence, when transfected into poxvirus-infected cells, are replicated into high molecular weight concatemers and subsequently resolved into hairpin-terminated minichromosomes. The TRT sequence is indicated, as is the obligatory palindromic repeat (indicated by inverted arrows).

to delineate the sequence required, plasmids containing telomere fusion elements from SFV and vaccinia were subjected to unidirectional deletions at both ends of the inverted repeat, as well as bidirectional deletions from the AflII site at the axis of symmetry (DeLange et al., 1986; DeLange et al., 1987). The resulting staggered-deletion constructs were transfected into virus-infected cells and the conversion to monomeric hairpins monitored by harvesting the DNA and examining its structure following agarose gel electrophoresis. In the SFV construct, a complete domain of 58-76-bp (corresponding to region I/IA, II, III) present just downstream from the non-palindromic nucleotides in the viral hairpin was found to be essential for optimal resolution (Fig. 8). While a 20-bp sequence (regions I/IA) is sufficient to direct some telomere resolution, the efficiency of the process is increased by the adjacent regions II and III. It was also found that the resolution sequence had to be present as two copies on either side of the axis of symmetry in an inverted repeat configuration (e.g. Fig. 5) as deletion of any part of either of the sequences resulted in a decrease or abrogation of resolution. Likewise in vaccinia, copies of a 20-bp sequence proximal to the hairpin loop must be present in an inverted repeat configuration for resolution (Merchlinsky and Moss, 1989b; Merchlinsky, 1990a). The minimal <u>core</u> and <u>complete</u> cis-acting sequence have been designated TRT (telomere resolution target) and correspond to regions I/IA and I/IA-III, respectively, in Fig. 8 (DeLange et al., 1986; Delange and McFadden, 1987; Merchlinsky and Moss, 1989b; Merchlinsky, 1990a). For now, the requirement for regions II and III of the vaccinia TRT in the resolution process remains an unresolved issue.

The copies of the TRT in an inverted repeat configuration are subject to three other constraints: i) the TRTs must be within 200-nucleotides of each other in the case of vaccinia

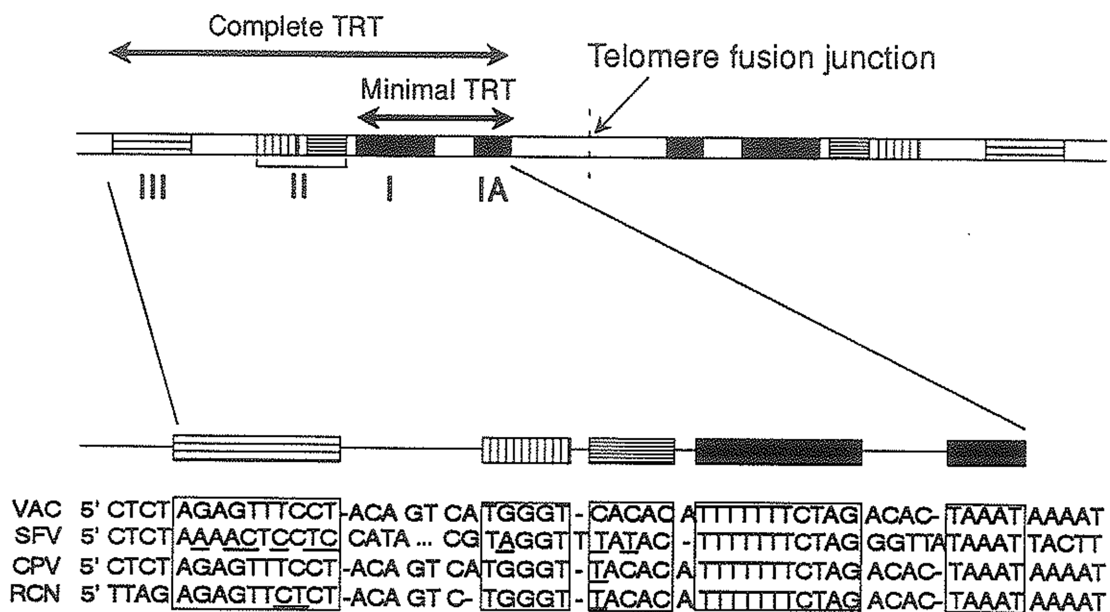


Figure 8. Organization of the telomere resolution target (TRT) region. Upper portion of figure represents the configuration of the vaccinia virus telomere fusion following DNA replication through the hairpin. The axis of symmetry is indicated by a dashed vertical line. The regions making up the complete and minimal telomere resolution sequences are indicated by shaded boxes (regions I/IA, II, III). Lower portion of figure shows the nucleotide sequences of the telomere resolution domain from a number of poxviruses including vaccinia (VAC), Shope Fibroma (SFV), cowpox (CPV) and raccoonpox (RCN). The boxed in regions indicate areas of sequence conservation. Nucleotides which do not match the vaccinia sequence within these conserved regions are underlined. (Modified from DeLange and McFadden, 1990).

and within approximately 100-nucleotides in SFV, as constructs with larger separations are not resolved (McFadden et al., 1988; Merchlinsky and Moss, 1989b); ii) the TRTs are resolved more efficiently when the two TRTs are similar in sequence and the distance towards the axis from each TRT is similar (Merchlinsky and Moss, 1989b); and iii) the TRTs must lie on either side of a palindromic sequence, as attempts to insert non-palindromic DNA, while having no effect on DNA replication, completely eliminate resolution. Interestingly, insertion of any palindromic sequence between the TRTs can be resolved (McFadden et al., 1988).

The fact that SFV-infected cells are able to resolve vaccinia containing telomere fusions and vice versa, coupled with the high degree of sequence similarity in the TRT, suggested that the resolution process is conserved. In fact, representatives from many of the poxvirus genera share a sequence similar in composition to the vaccinia TRT. Plasmids containing the cloned telomere fusion from cowpoxvirus and fowlpoxvirus, as well as SFV, are resolved in vaccinia-infected cells (Merchlinsky, 1990b). However, in keeping with constraint (ii) above, constructs containing heterologous TRTs (e.g. one from SFV and one from vaccinia in the same construct) are not resolved efficiently. Apparently, one cannot mix and match sequences which would otherwise work well in the resolution assay (Merchlinsky, 1990a).

A mutational analysis of the core vaccinia TRT (region I/IA) determined that the sequence ATTTAGTGTCTAGA$_7$ could only be altered marginally without affecting resolution. The critical consensus sequence is (A/T)TTT(A/G)N$_{7\text{-}9}$A$_7$, where N is any nucleotide (Merchlinsky, 1990b). Furthermore, the evidence by Merchlinsky (1990a) demonstrating that the resolution sequences must be essentially identical for resolution to

occur, suggests that processing of each resolution site does not occur in an autonomous manner, rather it suggests conservative strand exchange/processing is involved.

The TRT contains a motif which bears similarity to a vaccinia postreplicative promoter (Davison and Moss, 1989b). In fact, the core sequence of the TRT corresponds to a very active postreplicative promoter (Stuart et al., 1991). However, the correlation between resolution and promoter activity does not appear to be absolute. Merchlinsky (1990b) suggested that many sequences which serve as late promoters are incapable of telomere resolution. Using constructs generated by Davison and Moss (1989b) to test late gene promoter strength, Merchlinsky identified one sequence which was a strong late promoter but possessed weak resolution, and one weak promoter which exhibited excellent resolution (Merchlinsky, 1990b).

iii) mutants defective in telomere resolution (res$^-$)

In an attempt to identify trans-acting factors which function in telomere resolution, screening of temperature-sensitive (ts) mutants was undertaken. DeLange (1989), using pulsed-field gel electrophoresis (PFGE) as an assay, and Merchlinsky and Moss (1989a) using predominantly the *in vivo* plasmid transfection/resolution assay, identified a total of six ts res$^-$ mutants. Of these, five (tsC7, tsC21, tsC22, tsC53 and tsC63) were obtained from the collection of Dr. R. Condit (Thompson and Condit, 1986) while another (ts9383) was obtained from the collection of Dr. S. Dales (Dales et al., 1978).

From the results of this screen, DeLange (1989) proposed three groups based on the protein synthesis patterns:

1) tsC7, tsC21 and tsC53 were all defective in the production of intermediate and late proteins. Subsequent analysis has determined that each of these mutants has a lesion in a subunit of the multi-protein viral RNA polymerase complex. Mutant tsC7 has a defect in the rpo22-kDa subunit, mutant tsC21 a defect in the rpo21-kDa subunit and tsC53 a lesion in the rpo147-kDa subunit (Hooda-Dhingra et al., 1989; Thompson et al., 1989).

2) Mutant tsC22 had a normal switch from early to postreplicative gene expression. However, at later times in infection, late gene expression abruptly ceased. This "abortive late" phenotype is due to the rapid degradation of all RNA species at late times during the virus infection (Pacha and Condit, 1985), and appears to be due to activation of the 2-5A pathway (Cohrs et al., 1989). The mutant gene has been identified as ORF A18 (Pacha et al., 1990). Furthermore, it has now been shown that activation of the 2-5A degradation pathway was due to an increase in the amount of double-stranded RNA (Bayliss and Condit, 1993).

What all of the res$^-$ mutants in groups 1 and 2 share is the inability to either produce or maintain the production of intermediate or late proteins. In agreement with these observations, addition of the antiviral drug isatin-β-thiosemicarbazone, which blocks intermediate and late protein synthesis (Pennington, 1977), to a wild-type infection also results in the production of concatemeric replication intermediates with no resolution (Merchlinsky and Moss, 1989a). These results strongly implicate one or more postreplicative gene products in telomere resolution (DeLange, 1989; Merchlinsky and Moss, 1989a). Interestingly, treatment with the drug rifampicin, which interferes with morphogenesis at the level of envelope formation (Moss et al., 1969), does not interfere with telomere resolution (Merchlinsky and Moss, 1989a). Although this has been taken to suggest that packaging is

not critical for resolution, virion morphogenesis is complex and the block seen with rifampicin may represent a later stage of the process. It is not clear if a very early stage in packaging/morphogenesis is a prerequisite for telomere resolution.

3) The last category of res⁻ mutants consists of tsC63 and ts9383. These mutants are unique in that they do not show a complete defect in postreplicative gene expression. The functional characterization of these mutants constitutes the work performed in this thesis and therefore, the background information on each of these mutants will be presented in detail below.

iv) mutant tsC63

This mutant which was derived from the WR strain of vaccinia was generated via mutagenesis with nitrosoguanidine (Condit et al., 1983). Temperature-sensitive mutants were then isolated by plaque enlargement. A total of 32 complementation groups were isolated with tsC63 being the only member of complementation group 14 (Condit and Motyczka, 1981; Condit et al., 1983). Very little was known about tsC63 prior to the studies described in this work. It had a relatively high index of leakiness at 6.5% [leakiness was determined by performing a one-step growth experiment at both 40°C (sample 1) and 32°C (sample 2), assessing the titre of both samples at 32°C to determine yield, then dividing the yield for sample 1 by the yield from sample 2] and exhibited a high reversion index (6.1×10^{-3}) [reversion is efficiency of plating at 40°C/efficiency of plating at 32°C]. It was determined to be DNA replication competent, but had a defect in postreplicative gene expression (Condit

et al., 1983). Data regarding the location of the gene responsible for the ts phenotype was not available prior to this work.

v) mutants ts9383

Mutant ts9383 was originally derived by mutagenesis of wild-type strain IHD-W. Dales et al. (1978) arranged 78 ts mutants into 17 groups based on the virus phenotype at the nonpermissive temperature by electron microscopy. Group E mutants, to which ts9383 was assigned, are characterized by the aberrant formation of virus envelopes, specifically the formation of viroplasmic foci (areas of concentrated virus DNA and proteins) which are surrounded by an envelope but which do not have spicules on the outer membrane (spicules may be important in providing structural rigidity and curvature to the envelope). Furthermore, large DNA paracrystals are apparent in the host cytoplasm. Four other ts mutants were also assigned to this group: ts1085, ts7743, ts9203 and ts9251.

Morphologically, the phenotype exhibited by these mutants appear similar to that seen in wild-type infections incubated with the drug rifampicin. In these types of infections, post-translational cleavage of a number of viral polypeptides essential for propagation is inhibited (Moss and Rosenblum, 1973). Stern et al. (1977) showed that ts1085 had a similar type of defect and suggested that group E mutants were defective in proteolytic processing and envelope formation.

A more detailed analysis of the group E mutants was performed by Lake et al. (1979) who found a rather variable phenotype. Post-translational cleavage of the major core polypeptides p94-->p62 and p65-->p60 was affected in all of the mutants except ts9383 and

ts9251. In fact, an examination of the precursor/product relationships of proteins with molecular masses of 94, 65, 62, 60, 23, 18.5 and 18 kDa showed ts9251 to have no defect whereas ts9383 appeared to have a slight decrease only in the 18.5- and 18.0-kDa proteins. All other mutants showed more dramatic defects. It is clear therefore that the group E mutants have a rather heterogeneous phenotype in nonpermissive temperature infections, and that in mutant ts9383, processing of proteins is not affected at a gross level.

Models for telomere resolution

There are essentially three models which could explain the mechanism by which concatemeric replicative intermediates are converted to monomeric genomes containing flip and flop terminated hairpins (Fig. 9).

i) Nicking and refolding

The first model (Fig. 9, left) is a variation of the terminal palindrome model of Cavalier-Smith (1974), as modified for hairpin telomeres by Bateman (1975). Sequence-specific nicks are introduced in opposite strands of the replication intermediate molecule telomeres. Strands are separated, paired with the complementary strand and a DNA ligase reseals the nicks thereby generating the final product. One significant drawback to this model is that it assumes the resolution sites are processed independently of one another. Findings by McFadden et al. (1988) and Merchlinsky (1990) demonstrating that a close similarity between TRTs on the same substrate is important for resolution, have weakened support for

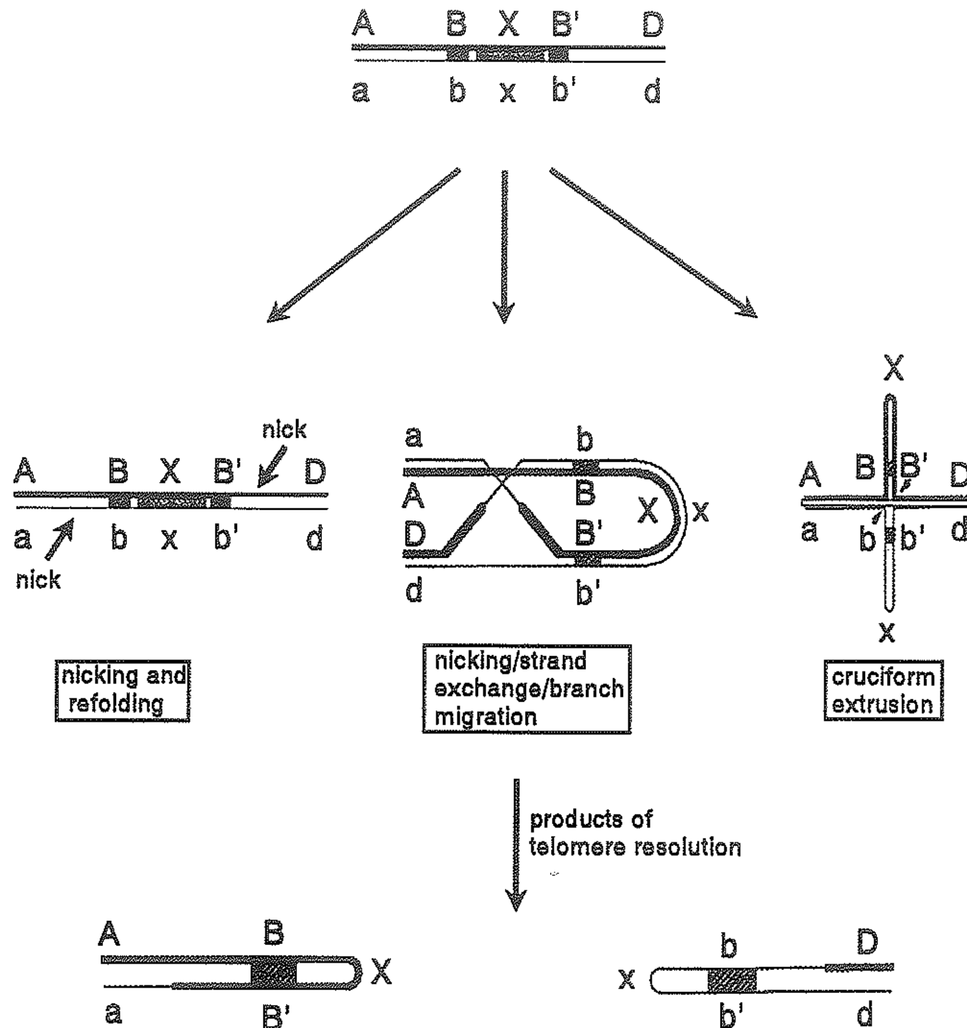


Figure 9. Proposed models for telomere resolution. Upper portion of the figure indicates the telomere fusion/concatemer junction of vaccinia virus DNA following DNA replication. Each of the DNA strands is represented by either a thick or thin strand. Symbols A, B, X, B', D (a,b,x,b',d for the complementary strand) represent sequences near the hairpin terminus. The telomere resolution sequence is indicated by a striped box, the double-stranded copy of the hairpin loop is indicated by a stipple pattern-filled box. The middle portion of the figure indicates three possible mechanisms for telomere resolution (from left to right): 1.nicking and refolding; 2. nicking/strand exchange/branch migration; and 3. cruciform extrusion. Nicking or cleavage sites are indicated with small arrows. The lower portion of the figure indicates the resulting products produced after the resolution event. (Modified from Merchlinsky, 1990a).

this model. Rather, the findings are more compatible with the concept of symmetric strand exchange as proposed in the following two models.

ii) Nicking/strand exchange/branch migration

In the second model (Fig. 9, center), an initial symmetrical nicking event occurs on each strand in the telomere fusion near the axis of symmetry but distal to the flip and flop sequence. It is likely that this event is in some way mediated by the presence of the TRT sequence. Conservative strand exchange is then followed by branch migration to regenerate the two forms of the hairpin termini (McFadden et al., 1988). Two important requirements that arise from this model are: the necessity for topoisomerases to segregate the two strands during branch migration and the requirement for symmetrical nicking near the TRT.

iii) Cruciform extrusion model

This model (Fig. 9, right) requires that initial helix unwinding at the axis of symmetry is followed by extrusion of a cruciform (McFadden and Morgan, 1982; McFadden et al., 1988). This process is made possible by the virtually identical inverted repeat structure of the fusion. The cruciform structure which, at its base, also looks like a Holliday junction, could then be converted to hairpin termini by the action of an enzyme similar to a Holliday junction endonuclease. Such an endonuclease is capable of cleaving the DNA strands then exchanging them and finally re-ligating the substrates. Proteins have been identified from a number of sources which appear to recognize cruciform structures. These sources include mammalian cells (Bianchi, 1988; Elborough and West, 1988; Bianchi et al., 1989), bacteriophage

(Mizuuchi et al., 1982; Kemper et al., 1984; Dickie et al., 1987), yeast (Symington and Kolodner, 1985; Evans and Kolodner, 1988; Parsons and West, 1988) and poxviruses (Stuart et al., 1992).

a) Analysis of cruciform structure in vitro

Because of some of the appealing aspects of the cruciform resolution model, a detailed analysis of the mechanism by which cruciforms arise has been undertaken using the SFV telomere fusion as a representative cruciform-capable structure. Using two dimensional agarose gel electrophoresis, Dickie et al. (1987a) have shown that cruciformation of the SFV fusion requires a relatively high energy of formation (G_f = 44 kcal/mol), provided by the torsional energy of supercoiling, and a low energy of activation. If adequate torsional energy is supplied by negative supercoiling in the individual topoisomers, extrusion occurs relatively easily. In essence, once the cruciform bubble is initiated, extrusion is likely to proceed. Merchlinsky et al. (1988) have examined the kinetics of cruciformation in vaccinia and have found similar results, specifically that the initial event in cruciformation is the rate-limiting step.

While these experiments prove that cruciformation is not an impossible event from either energetic or kinetic standpoints, whether or not cruciformation actually occurs *in vivo* is unknown. However, there is precedence for Holliday junction-like enzymes which act on "recombination intermediates" such as bacteriophage T4 endonuclease VII (Mizuuchi et al., 1982; Kemper et al., 1984; Lilley and Kemper, 1984), bacteriophage T7 endonuclease I (Panayotatos and Wells, 1981; deMassey et al., 1984; Dickie et al., 1987b) and activities from

various mammalian cells (Waldman and Liskay, 1988; Elborough and West, 1990) that can cleave at the base of a cruciform and convert the substrate elements to lineforms.

iv) trans-acting factors required for telomere resolution

As already mentioned, a search for the elements involved in-trans in telomere resolution has already been undertaken from two different approaches. The genetic approach, isolation of mutants with a res$^-$ phenotype under nonpermissive conditions, has already been discussed. A second approach involves the *in vitro* recreation of the resolution event using protein extracts.

Lakritz et al. (1985), using extracts prepared from purified vaccinia virions, identified an activity which was able to perform three sequential reactions on supercoiled target plasmid DNA: introduce nicks, linearize the plasmid, and finally cross-link one of the termini. The reactions occurred in a concerted fashion in that intermediates, such as the linearized DNA form, are not substrates for conversion to a cross-linked hairpin terminated product in the presence of more protein (Lakaritz et al., 1985). The activity had a pH optimum of 6.5 and did not require ATP as an energy cofactor. Sedimentation analysis suggested that the activity sedimented with an apparent molecular mass of 105 kDa (Lakaritz et al., 1985). However, subsequent purification has shown the enzyme to be a 50-kDa species now termed the nicking-joining (N-J) enzyme (Reddy and Bauer, 1989). Whether the 105-kDa protein's activity represents a different (but similar) activity, or whether a homo- or heterodimer involving the 50-kDa protein makes up the 105-kDa form remains unclear.

In the first two stages of the reaction, the substrate plasmid is nicked and subsequently linearized thus generating an intermediate with 3' P and 5' OH ends. In many ways, the enzyme behaves like a type I DNA topoisomerase. Type I topoisomerases form covalent bonds with target sites and cleave the DNA in the absence of ATP also generating 3' P and 5' OH termini before resealing the DNA prior to release. It would not be unlikely, based on the kinetic data and on the concerted nature of the reaction, to assume that the full reaction proceeds via a DNA-protein covalent step.

The N-J activity has low cross-linking activity *in vitro* with incubation conditions typically requiring high temperature and prolonged incubations (up to 12 h) to generate a maximal ratio of 1:5 cross-linked to linear products (Lakaritz et al., 1985). However, generation of cross-linked products is dramatically increased following treatment of the enzyme with trypsin, resulting in the conversion of input plasmid to cross-linked product of 15% at 37°C and 50% at 50°C (compared with a maximum of 15% at 50°C for the non-digested form of the enzyme). This trypsinization corresponds to proteolysis of the N-J enzyme from a 50-kDa to a 44-kDa form. In the 44-kDa form, the salt optimum was shifted from 0 to 50 mM NaCl and the pH optimum was shifted from 6.5 to 7.5. This has raised the possibility that the N-J enzyme is produced in a pronuclear form and that activation requires proteolysis. It has been suggested that other components are likely missing from the purified preparation which would serve to increase efficiency (Reddy and Bauer, 1989).

Interestingly, since the enzyme is packaged into virions, it is present in the initial phase of infection. As telomere resolution is a late event, it is curious that such an enzyme, if involved in telomere resolution, would be packaged. One explanation might be that the

enzyme serves more that one function, for example it may play a role in the initial near-terminal DNA nicking event postulated for the initiation of DNA replication. In this manner, the 50-kDa form of the N-J enzyme would be responsible for stages in the initiation of DNA replication whereas the proteolytically processed form (44-kDa) would be responsible for telomere resolution. That late gene expression is required could reflect a need for production of a protease or regulatory subunit with activity directed towards the 50-kDa N-J enzyme. Of course one would then have to explain how a 50-kDa N-J enzyme rather than a processed form of the N-J enzyme would be packaged into progeny virion. An alternate possibility is that contact with another protein might also increase the efficiency of the cross-linking activity and that the *in vitro* proteolysis merely serves to expose the active site.

The N-J activity is capable of generating cross-linked products in a number of substrate molecules (Lakaritz et al., 1985). In the presence of a plasmid containing the cloned vaccinia telomere fusion, the activity is capable of cleaving within an S1 nuclease sensitive region near the axis of symmetry (Merchlinsky et al., 1988). This region likely corresponds to the apex of an extruded cruciform. For it to be concluded that this enzyme is involved in resolution, either alone or in combination with other factors, a more specific interaction with the TRT and the hairpin structures will need to be shown.

Further attempts to isolate relevant factors for telomere resolution using crude lysates *in vitro* have been hampered by the fact that cleavage and subsequent cross-linking of plasmid substrates occur in a sequence non-specific (i.e. non-TRT dependant) fashion. It appears that the reaction is solely dependent on the capacity of the substrate to form a branched Holliday structure, such as is seen in a cruciform (Stuart et al., 1992).

Goals of research project

The primary focus of this work was aimed at identifying trans-acting elements required in the telomere resolution event. A preliminary identification by DeLange (1989) of two ts mutants which are defective in telomere resolution, but not entirely defective in post-replicative gene expression, allowed a genetic window of opportunity. A thorough analysis of mutants tsC63 and ts9383 was undertaken with the intent of:

1) Characterizing the biological phenotype.

2) Identifying the gene(s) responsible for the ts effect.

3) Characterizing the mutant gene products and determining their role in telomere resolution.

This work attempts to address each of these objectives. The results section of this work is composed of three parts. In the first, telomere resolution defective (res$^-$) mutant ts9383 was characterized. This mutant did not exhibit a typical defect in late gene expression at the nonpermissive temperature; in fact, at a gross level, postreplicative gene expression was present as evidenced by pulse-labelling of proteins. However at the DNA level, concatemeric DNA replication intermediates rapidly built-up. The mutant gene was identified as D12 which encodes the small subunit of the viral heterodimeric mRNA capping enzyme. As no pleiotropic defect in postreplicative gene expression was observed, D12 represents the second candidate protein, along with the N-J enzyme, for a role in the resolution event.

In the second section, mutant tsC63, which is also a res$^-$ mutant, was shown to have a partial defect in postreplicative gene expression; in particular, at the nonpermissive temperature, while intermediate genes were expressed, late genes were not. The defect in

tsC63 was found to contain a mutation in ORF A1 which is now believed to encode a transcription factor responsible for late gene expression. This work, confirms that resolution requires late gene expression and that early and intermediate gene expression alone is insufficient for the process.

In the third section, a more detailed analysis of mutant ts9383 was undertaken. First, using the less invasive method of RNA isolation, it was confirmed that late gene expression at the RNA level was normal in the mutant. Furthermore, one non-coding telomeric transcript was generated in a similar fashion at both permissive and nonpermissive temperatures. At the protein level, newly-synthesized ts9383 D12 and D1 subunits appeared essentially stable. More significantly, co-immunoprecipitation experiments demonstrated that subunit association of the capping enzyme was affected in ts9383 infections at 40°C but not at 32°C. These data are compatible with the notion that the capping enzyme or the small subunit on its own plays a direct role in telomere resolution.

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The contents of chapter 3 have been published as: Carpenter, M.S. and DeLange, A.M. 1992. Identification of a temperature sensitive mutant of vaccinia virus defective in late but not intermediate gene expression. Virology **188**:233-244.

The contents of chapter 4 are being prepared for submission: Carpenter, M.S., E.G. Niles and A.M. DeLange. Altered vaccinia virus mRNA capping enzyme in a telomere resolution defective mutant. (In preparation).

CHAPTER II: A TEMPERATURE-SENSITIVE LESION IN THE SMALL SUBUNIT OF THE VACCINIA VIRUS ENCODED mRNA CAPPING ENZYME CAUSES A DEFECT IN VIRAL TELOMERE RESOLUTION.

Abstract

Using pulsed-field gel electrophoresis, we have demonstrated that the temperature-sensitive (ts) conditional-lethal mutant ts9383 is, at the nonpermissive temperature, defective in the resolution of concatemeric replicative intermediate DNA to linear 185-kilobase (kb) monomeric DNA genomes. The resolution defect was shown to be the result of a partial failure of the mutant virus to convert the replicated form of the viral telomere to hairpin termini. In contrast to other mutants of this phenotype, pulse-labelling of viral proteins at various times postinfection revealed no obvious difference in the quantity or temporal appearance of members of the late class of polypeptides. Using the marker rescue technique, we have localized the ts lesion in ts9383 to an approximately 1-kb region within the HindIII D fragment. Both the ts phenotype and the resolution defect were shown to be caused by a single base C→T point mutation resulting in the conversion of the amino acid proline to serine in the 23rd codon of open reading frame D12. This gene encodes a 33-kDa polypeptide which is known to be the small subunit of the virus-encoded mRNA capping enzyme (E. G. Niles, G. J. Lee-Chen, S. Shuman, B. Moss, and S. S. Broyles, Virology **172**:513-522, 1989). The data are consistent with a role for this capping enzyme subunit during poxviral telomere resolution.

Introduction

The linear DNA genomes of at least two families of eukaryotic DNA viruses, namely parvoviruses and poxviruses, contain characteristic hairpin termini (reviewed in: Berns, 1990; Moss, 1990a). The structures of these genomic termini are apparent adaptations to allow faithful replication of the termini using a DNA polymerase which requires a free 3' hydroxyl end as a primer. Vaccinia virus, the prototypal member of the poxvirus family, is a large and complex virus which replicates entirely within the cytoplasm of infected cells (reviewed in Moss, 1990a). The 185-kilobase (kb) linear double-stranded DNA genome of vaccinia virus is terminally cross-linked by hairpin loops, which are AT-rich, incompletely base-paired and exist in two inverted and mutually complementary configurations (Geshelin and Berns, 1974; Baroudy et al., 1982). These features of the terminal hairpin are characteristic of all poxvirus telomeres whose sequence is known (reviewed in DeLange and McFadden, 1990). During viral DNA replication, the hairpin termini are transiently converted into an inverted repeat configuration. This configuration has been referred to as a "concatemer junction" because at least the great majority of them link genomic-length units into high molecular weight replicative intermediate concatemers (DeLange, 1989; Merchlinsky and Moss, 1989a; Moss et al., 1983; Moyer and Graves, 1981). The term "telomere fusion" has been used to avoid inferring the status of any one particular genomic milieu (DeLange and McFadden, 1990). The hairpin termini are regenerated from these telomere fusion elements in a post-replicative process called telomere resolution. Cloned telomere fusion elements from vaccinia virus or from the leporipoxvirus Shope fibroma virus (SFV) are, after their transfection into poxvirus-

infected cells, resolved to hairpin termini (DeLange and McFadden, 1986; Merchlinsky and Moss, 1986). This *in vivo* transfection assay has allowed the identification of a short, highly conserved DNA sequence, the telomere resolution target or TRT, which is located immediately adjacent to the incompletely base-paired hairpin and is required in cis as two inverted copies to facilitate telomere resolution (DeLange and McFadden, 1987; Merchlinksy, 1990; Merchlinsky and Moss, 1989b).

Both *in vitro* and *in vivo* studies aimed at identifying proteins that act in trans to promote telomere resolution have met with difficulties. In an attempt to reconstruct the resolution event *in vitro*, Reddy and Bauer (1989) demonstrated that a viral 50-kDa nuclease has the ability to cross-link the telomere fusion element, although with low efficiency and under conditions that facilitate melting of the DNA duplex. Significantly, this enzyme cleaves preferentially within an S1-sensitive region of the TRT region (Merchlinsky et al., 1988), and the cross-linking activity was stimulated by partial trypsin treatment (Reddy and Bauer, 1989), suggesting that the nuclease may be part of the putative telomere resolvase complex. The *in vivo* genetic approach involved screening of temperature-sensitive (ts) lethal mutants for a defect in telomere resolution (res$^-$). Using this approach, a total of seven non-complementing res$^-$ mutants have been identified (DeLange, 1989; Merchlinsky and Moss, 1989a). Pulsed-field gel electrophoresis of DNA from res$^-$ mutants showed that the DNA replicated at the nonpermissive temperature existed largely as high molecular weight concatemers rather than as mature hairpin-terminated genomes. It was, however, soon realized that the ts lesions in most of these mutants may not affect the resolution process directly. At the onset of DNA replication in wild type infections, early gene expression is

turned off and two temporal classes of late genes, now known as intermediate and late, are activated in succession (Lake et al., 1979; Vos and Stunnenberg, 1988). The majority of res$^-$ mutants are defective in the switch from early to late gene expression, and hence, only produce at best very small amounts of late proteins. This class includes four mutants that have lesions in various subunits of the virus-encoded RNA polymerase (rpo), namely rpo147, rpo22, rpo18 (Ahn et al., 1990; DeLange, 1989; Hooda-Dhingra et al., 1989; Merchlinsky et al., 1988), and rpo132 (Hooda-Dhingra et al., 1990; DeLange, unpublished results). Another res$^-$ mutant, tsC22, while not defective in the switch from early to late gene expression, displays a defect in the stability of RNA at late times resulting in abortion of late protein synthesis (Pacha and Condit, 1985). The drug isatin-β-thiosemicarbazone (IBT) similarly causes RNA instability, abortion of late protein synthesis and a subsequent defect in telomere resolution. It thus appears that a defect in late gene expression may be responsible for the observed defect in telomere resolution, and hence, efficient telomere resolution may have a requirement for late gene expression (DeLange and McFadden, 1986; Merchlinsky and Moss, 1989a). This requirement for late gene expression may reflect synthesis of one or more late protein(s), that function(s) in trans to promote or regulate telomere resolution. However, late gene expression could in principle also affect the resolution event directly, either through synthesis of noncoding late transcript(s), or through binding of the RNA polymerase and/or other proteins to the telomere fusion element. Such a role of the transcriptional apparatus is given credence by two recent observations: (i) the TRT region can function as an efficient transcriptional promoter (Merchlinsky, 1990b; Stuart et al., 1991); and (ii) noncoding telomeric RNA transcripts are synthesized *in vivo* at late times when telomere resolution takes

place (Parsons and Pickup, 1990).

The present study reports the characterization of a conditional lethal temperature-sensitive res$^-$ mutant, ts9383. The mutation responsible for this ts, res$^-$ phenotype is located in open reading frame (ORF) D12, which encodes the small subunit of the virus-encoded mRNA capping enzyme (Niles et al., 1989). This mutant differs from all other previously analyzed res$^-$ mutants in that it has no apparent defect in the expression of intermediate and late genes. Our findings suggest a more direct role for this enzyme in the telomere resolution event.

Materials and Methods

Cells and Virus. A continuous line of African green monkey kidney cells (BSC40) was grown as a monolayer culture in Dulbecco's Modified Eagle (DME) medium supplemented with 5% fetal calf serum. Wild type strain IHD-W and temperature-sensitive mutant ts9383 were kindly provided by G. McFadden and S. Dales, respectively. Mutant ts9383 was three times plaque-purified in our laboratory before being used in experiments. Crude virus stocks were obtained as described previously (DeLange, 1989). The permissive temperature for mutant ts9383 was 32°C, the nonpermissive temperature was 40°C. A cosmid library of vaccinia strain WR (Thompson and Condit, 1986) was generously donated by R. Condit.

Pulsed-Field Gel Electrophoresis (PFGE). PFGE was performed as described by DeLange (1989). Monolayers of BSC40 cells were infected at a multiplicity of 25 pfu/cell and at various times postinfection, cells were harvested and embedded in 0.7% Incert

agarose (Mandel Scientific Co.) plugs. Plugs were loaded onto 1.5% agarose gels and electrophoresed in 0.1 M TBE in a uniform field of 5 V/cm for 45 minutes and then pulsed for 20 h at 100 sec intervals using a hexagonal attachment.

Southern blotting and detection of viral DNA with ^{32}P-labelled terminal-repeat viral DNA probe pVT-1 were performed as described previously (DeLange, 1989). pVT-1 DNA was labelled with ^{32}P by the random hexanucleotide primer method (Feinberg and Vogelstein, 1983).

Protein Labelling. Pulse-labelling of proteins from infected cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of labelled proteins were performed as described previously (Condit and Motyczka, 1981; DeLange, 1989), except that electrophoresis was done in the Protean-II system from Bio-Rad. Cells were infected at a multiplicity of 25 pfu/cell.

Plasmid Construction and DNA Purification. Viral DNA was purified as described by Esposito et al. (1981). The large scale isolation of cosmid DNA was carried out as described by Thompson and Condit (1986). Cloning of specific restriction fragments involved digestion of viral or plasmid DNA, electrophoresis, electro-elution, and ligation into the multiple cloning site of phagemid vector pTZ18U or pTZ18R (obtained from US Biochemicals) by standard methods (Maniatis et al., 1982). Recombinant plasmids were used to transform bacterial strains DH5α or JM101. Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (1979). Plasmids pC6-1, pC6-3, and pC6-4 were derived

by double digestion of cosmid pWR93-130 (Thompson and Condit, 1986) with either XhoI+KpnI or SacI+KpnI, followed by ligation of the purified fragments into pTZ18R. Plasmid pC6-43 was obtained by digestion of pC6-4 with BamHI followed by ligation; pC6-45 was generated by double digestion of pC6-4 with BamHI, to cleave the multiple cloning site in the vector, and XhoI, followed by treatment with Klenow enzyme and blunt-end ligation. To obtain plasmids pC6-44BX and pC6-44EX, pC6-43 was digested with either BamHI+XhoI (BX) or EcoRI+XhoI (EX), and the 1.8-kb BamHI-XhoI and 1.0-kb EcoRI-XhoI fragments were cloned in pTZ18R digested with BamHI+SalI or EcoRI+SalI. The pC6-44BX-equivalent clones from wild-type IHD-W and mutant ts9383 were obtained by digesting the relevant viral DNA with BamHI+XhoI and ligation of the purified fragments into (BamHI+SalI)-digested pTZ18R. The corresponding EX clones were then constructed by digestion with EcoRI and re-ligation. These clones were designated pCBXN1 and pCEXN1 (the IHD-W equivalents of pC6-44BX and pC6-44EX, respectively), and pCBXD1 and pCEXD1 (the ts9383 equivalents).

Marker Rescue. The marker rescue procedure used in this study was a modification of the method described by DeLange and McFadden (1986). For each rescue, 10 μg of cloned viral insert DNA was released from the plasmid vector by digestion with appropriate restriction enzymes. The digested DNA was extracted once with phenol/chloroform, once with chloroform, and then dialyzed for 30 minutes against an excess of 2 x NNH (50 mM Hepes [pH 7.1], 280 mM NaCl, 1.5 mM Na_2HPO_4). One hour prior to transfection, the DNA in 2xNNH was mixed with agitation into an equal volume of 250 mM $CaCl_2$ and the DNA-

calcium phosphate co-precipitate was allowed to form at room temperature. Confluent monolayers of BSC40 cells in T25 tissue culture flasks (Corning) were infected with ts9383 (in Hanks balanced salt solution + 0.1% bovine serum albumin) at a multiplicity of 0.05 pfu/cell. After a 1.5 h adsorption period at 37°C, the inoculum was aspirated and the calcium phosphate precipitated DNA added to the cells. After another 30 min at room temperature, 10 volumes of fresh medium was added, and the flasks placed in a 40°C incubator. At 5.5 h postinfection, the medium was replaced with fresh DME + 5% fetal calf serum, and the flasks returned to the 40°C incubator. After 72 h incubation, the infected monolayers were fixed with neutral buffered formalin (Sigma) and stained with 0.1% crystal violet. When testing rescue of the resolution-defective phenotype, the monolayers were scraped off, and the resulting virus was either used as an inoculum for PFGE analysis, or it was passaged once in BSC40 cells prior to such analysis.

DNA Sequencing. To facilitate sequencing of both strands of the 1.0-kb insert in plasmids pC6-44EX, pCEXN1, and pCEXD1, we made use of a single functional XbaI site (at position 454-459 in Fig. 13) within this 1.0-kb fragment; XbaI-XhoI and XbaI-EcoRI fragments were subcloned into both pTZ18U and pTZ18R. Strain JM101 was used as the bacterial host for these plasmids, and single-stranded plasmid DNA was produced, packaged, and extruded into the growth medium after infection with helper phage M13KO7 (obtained from US Biochemicals). Single stranded template was prepared according to Dente et al. (1983) and Messing (1983). Sequencing of these single-stranded templates was performed by the Sanger dideoxynucleotide chain termination method (Sanger et al., 1977), using

extension from a 17-mer universal primer or reverse primer with Klenow enzyme, and [^{35}S] deoxyadenosine triphosphate as the labelled nucleotide. The gels were fixed, transferred to blotting paper, dried under vacuum, and exposed to X-Omat AR X-ray film.

Nucleotide Sequence Accession Number. The GenBank accession number for the DNA sequence presented here is M64430.

Results

Ts9383 is defective in concatemer resolution. Mutant ts9383 is a heat-sensitive derivative of vaccinia virus strain IHD-W. When grown at the nonpermissive temperature (39.5°C) in mouse L cells, it incorporates a wild type level of [^{3}H]thymidine into newly replicated DNA and is therefore DNA replication positive (Dales et al., 1978). Electron microscopic observation revealed characteristic DNA paracrystal structures in the cytoplasm of infected cells (Dales et al., 1978; Silver and Dales, 1982). We have adapted the technique of pulsed-field gel electrophoresis as a convenient and rapid method to evaluate not only the quantity but also the quality of replicated DNA. This method has facilitated the identification of a class of mutants that is deficient in the process that converts high molecular weight concatemeric replicative intermediates to the 185-kb monomeric DNA genome (DeLange, 1989; Merchlinsky and Moss, 1989a). These mutants are referred to as resolution-negative or res^{-}. Mutant ts9383 was one of the res^{-} mutants identified in this manner (DeLange, 1989). This study presents a detailed analysis of mutant ts9383 in BSC40 cells. We have routinely used 40°C as the nonpermissive temperature, since it was found that a temperature as high

as 39°C was permissive for this mutant.

To determine whether the resolution defect of ts9383 was a general defect expressed at all times of DNA replication, we infected monolayers of BSC40 cells with wild-type IHD-W or mutant ts9383 at a multiplicity of 25 and, after 0, 2, 4, 6, 8, 12, and 24 h postinfection, analyzed the replicated DNA by PFGE (Fig. 10). The patterns of DNA replication exhibited by ts9383 at the permissive and the nonpermissive temperature during the first three time points (up to 4 h postinfection) were essentially identical, and parallelled the results seen with wild-type at the two temperatures. At 2 h postinfection, a faint band can be seen to migrate in the 185-kb range which corresponds to input viral DNA. At 4 h postinfection, a strong hybridization signal corresponding to replicating viral DNA appeared in the well-DNA (origin) fraction. Therefore, replication at the nonpermissive temperature is not delayed in comparison with replication at the permissive temperature, nor is it delayed in comparison with wild-type DNA replication at either temperature.

Commencing with the appearance of DNA fractions which are capable of entering the agarose gel matrix (here at 6 h postinfection), the mutant showed dramatic differences when comparing replication at the two temperatures. At the permissive temperature, beginning with 6 h, full length 185-kb monomeric genomes were produced with production continuing to a point somewhere between 12 and 24 h postinfection, after which the majority of the well-DNA fraction had entered the gel. In contrast, replication at the nonpermissive temperature was characterized by the appearance of both monomers and oligomers, typical of mutants of the res$^-$ phenotype (DeLange, 1989), even at the earliest time (6 h postinfection) that replicated DNA entered the gel (Fig. 10B; lane 6 at 40°C). To confirm that unresolved end-

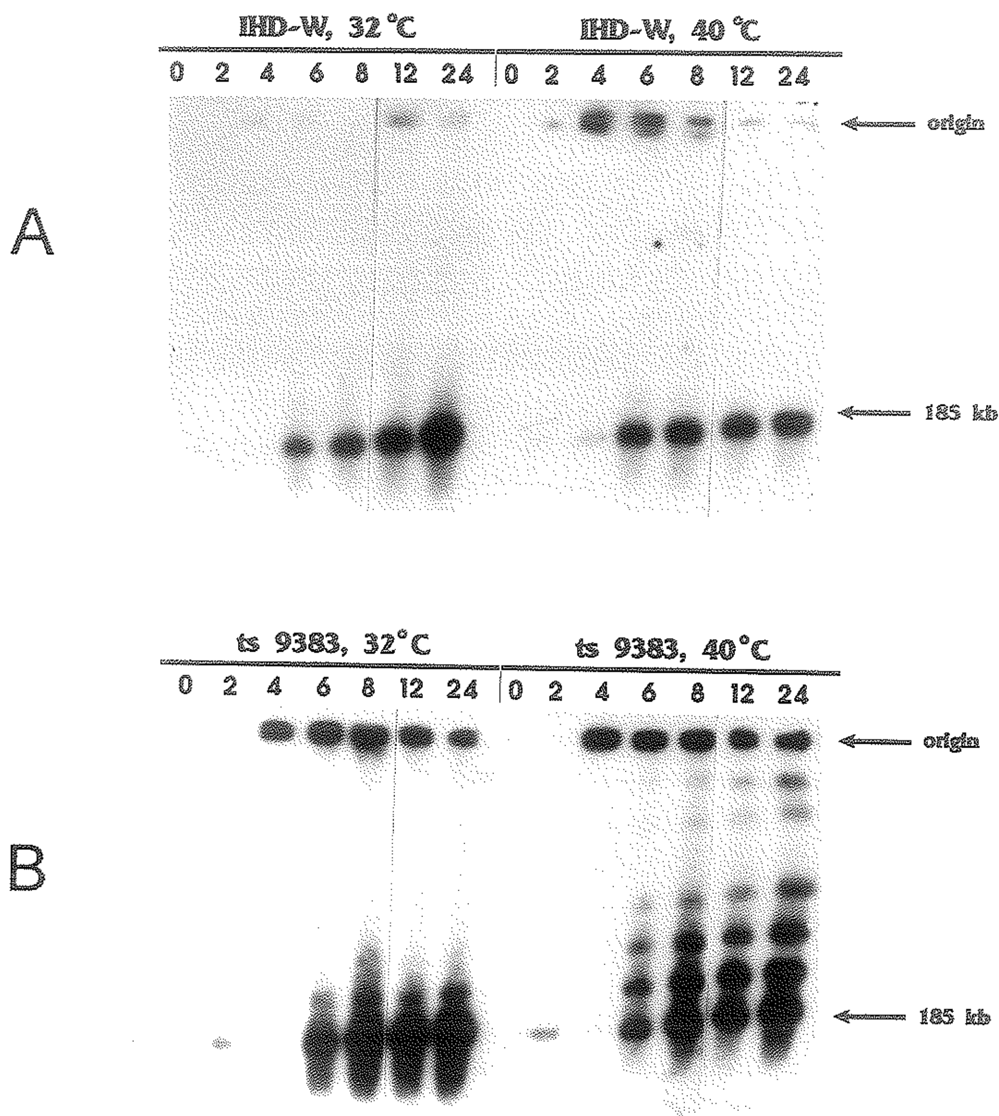

Figure 10. Pulsed-field gel electrophoresis of vaccinia virus DNA from cells infected with wild-type strain IHD-W (A) or the temperature-sensitive mutant ts9383 (B) at the either the permissive (32°C) or the nonpermissive (40°C) temperature. Monolayers of BSC40 cells were infected at a multiplicity of 25 pfu/cell and incubated at 32°C or 40°C. At times 0, 2, 4, 6, 8, 12 and 24 h postinfection, monolayers were harvested and embedded in agarose plugs and treated with sarkosyl and proteinase K to digest protein. The plugs were equilibrated against electrophoresis buffer, inserted into pre-formed wells in a 1.5% agarose gel and subjected to PFGE. The DNA was then transferred to nitrocellulose and hybridized with ^{32}P-labelled terminal probe pVT-1. The migration of the 185-kb monomeric genome size is indicated. To emphasize the concatemers at 6 h postinfection, we show an overexposure of the blot in (B).

to-end telomere fusion was responsible for the presence of the slow-migrating oligomers observed with this mutant, we digested DNA (in plugs) with XhoI and examined the resulting restriction fragments by agarose electrophoresis and Southern blotting. Both the 6.2-kb hairpin fragment and a high proportion of 12.4-kb telomere fusion fragment were observed (not shown), indicating that ts9383 is defective in telomere resolution, and that the resolution defect of this mutant is expressed at all times during the DNA replication cycle.

Protein synthesis in wild-type IHD-W and mutant ts9383. The res$^-$ phenotype has been seen in a number of other mutants, including tsC21, tsC53, tsC63, tsC7, and tsC22, representing at least five complementation groups (DeLange, 1989; Merchlinsky and Moss, 1989a) and ts29, ts32, and ts47 representing a sixth complementation group (Hooda-Dhingra et al., 1990; A.M. DeLange, unpublished data). What all of these mutants share is some defect in late gene expression. To determine whether ts9383 is similarly defective in late gene expression, we infected BSC40 cells with either IHD-W or ts9383, and pulse-labelled the proteins for 15 minutes with [^{35}S] methionine at 0, 2, 4, 6, 8, 10, and 12 h postinfection. The proteins were then analyzed on 12.5% SDS-polyacrylamide gels (Fig. 11). The results of total protein synthesis for IHD-W are similar at both temperatures. At the permissive temperature, ts9383 displayed a pattern of proteins which is essentially indistinguishable from that of wild-type. Interestingly, protein synthesis in ts9383-infected cells grown at the nonpermissive temperature was also indistinguishable from that of wild-type-infected cells. Late polypeptides were produced at the correct times and in comparable quantitative amounts compared with wild-type. Since the analysis of protein synthesis was performed in parallel with the DNA

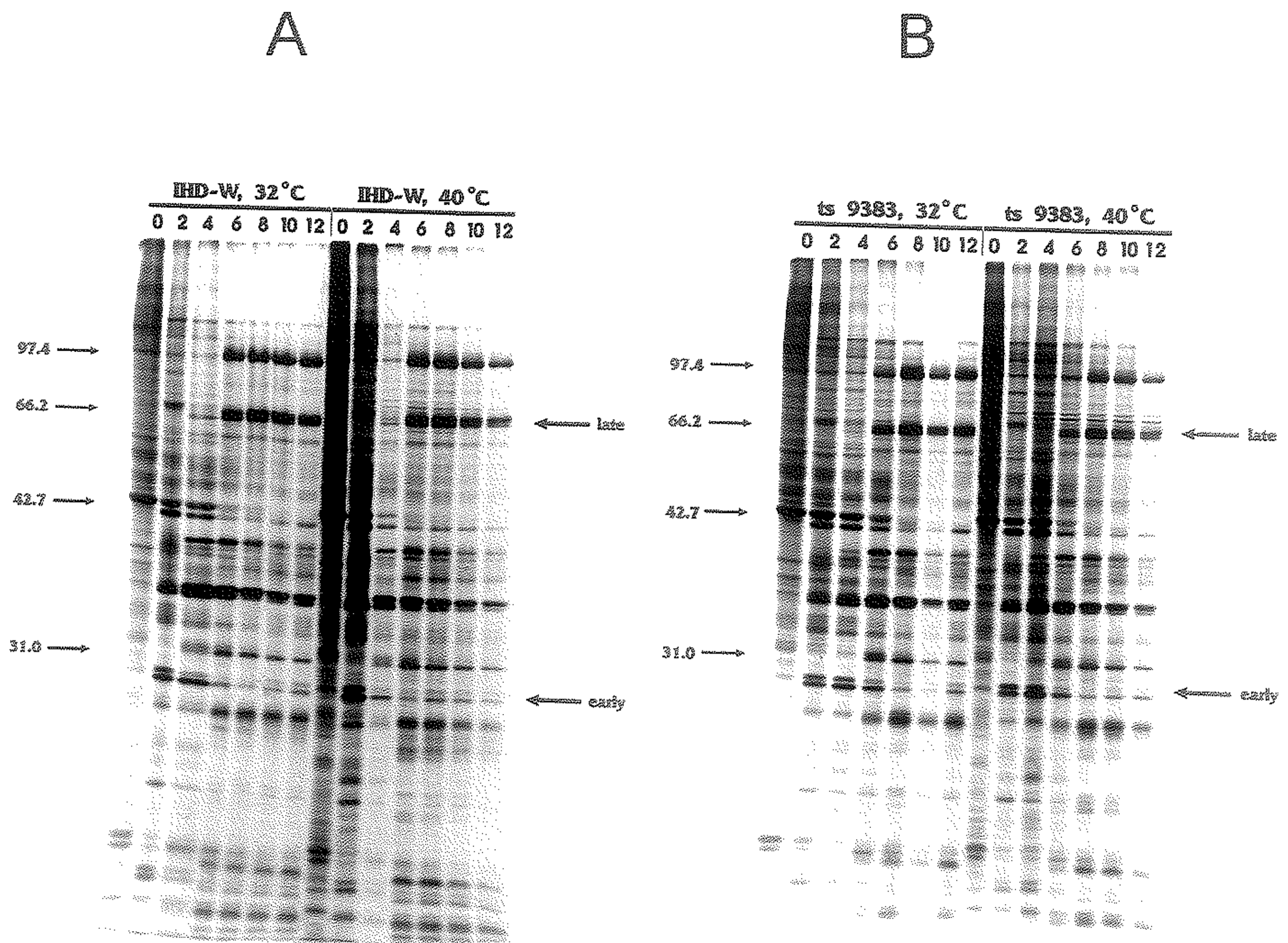

Figure 11. Protein synthesis in wild-type strain IHD-W (A) and mutant ts9383 (B). BSC40 monolayers were infected at a multiplicity of 25 pfu/cell and incubated for 0, 2, 4, 6, 8, 10 and 12 h at 32°C or 40°C, after which total proteins were pulse-labelled with [^{35}S] methionine. Samples were harvested, separated in a 12.5% SDS polyacrylamide gel and exposed to X-ray film. Molecular masses are given in kilodaltons and representative early and late proteins are indicated.

analysis described in the previous section, these data suggest that the defect in telomere resolution in ts9383 is not caused by a general defect in the synthesis of late proteins.

Mapping of the ts9383 lesion by marker rescue. Our next goal was to determine the map position of the mutation in ts9383 on the viral genome, and assign it to a specific open reading frame (ORF). For this purpose, we employed the technique of marker rescue. The HindIII restriction fragment map of vaccinia virus as well as the various cloned fragments are detailed in Fig. 12. The results of the marker rescue experiment are given in Table 1. In an initial attempt to narrow down the region of the genome, we used a set of overlapping cosmids which together contain most of the WR wild-type vaccinia genome (Thompson and Condit, 1986). We were thus able to determine that the lesion lay within an approximately 37-kb fragment of cosmid pWR93-130 which spans the HindIII D/A junction.

This cosmid was used to derive various subclones: pC6-4 contains a 13-kb KpnI-SacI fragment; pC6-1 a 12.5-kb XhoI-KpnI fragment and pC6-3 a 12-kb KpnI-SacI fragment. Of these subclones, only pC6-4 rescued ts9383 (Table 1). By using marker rescue with several subclones of pC6-4, we identified a single 1.0-kb fragment still able to rescue the ts phenotype of ts9383. This clone, pC6-44EX, contains regions of both ORF D12 and ORF D13 (Niles et al., 1986; Weinrich and Hruby, 1986). Further attempts to localize the mutation to a specific open reading frame by marker rescue were unsuccessful. Positive complementation with a mutant (tsC33), known to have a conditional lethal mutation in ORF D13 (Niles et al., 1986), strongly suggested that the ts lesion of mutant ts9383 lay within ORF D12.

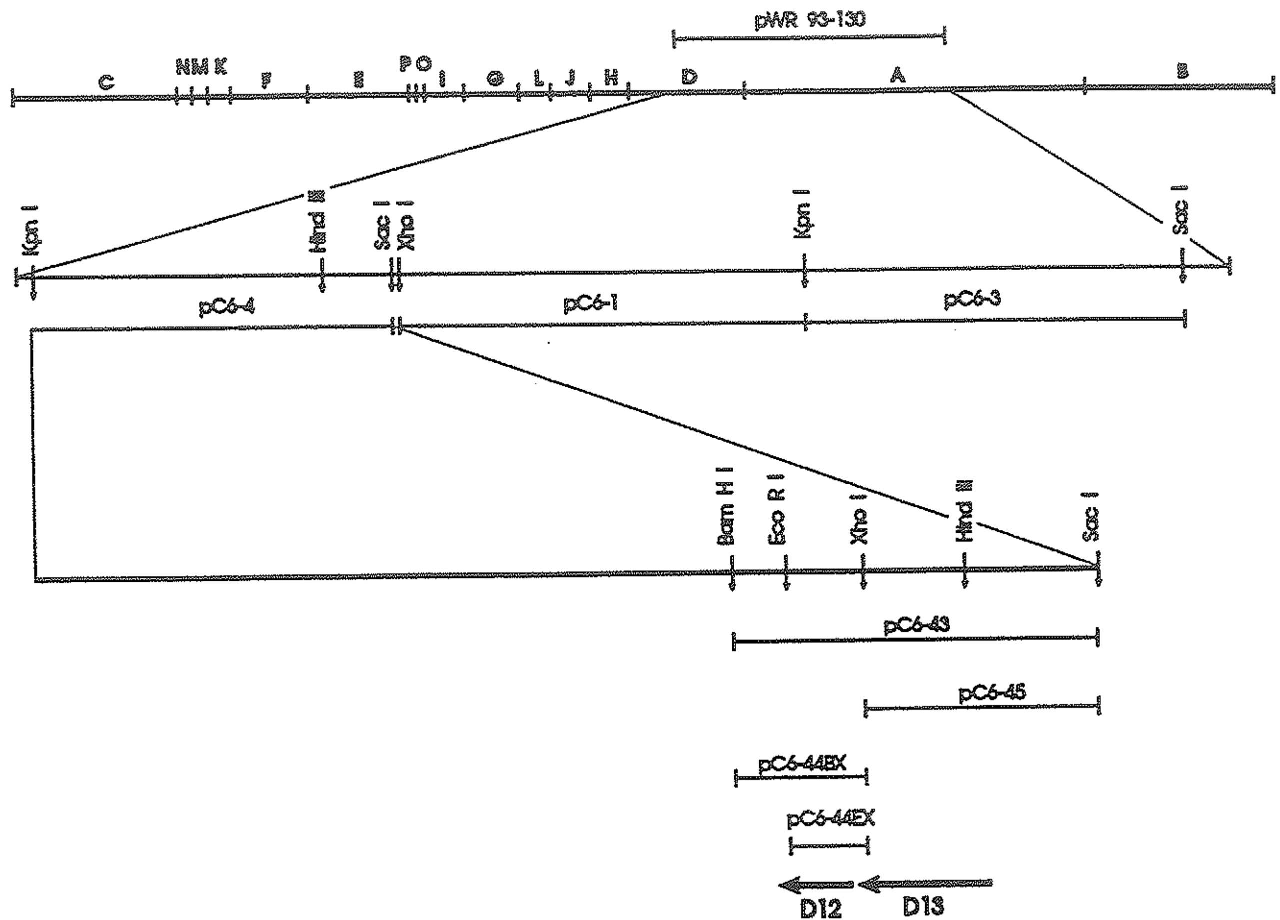

Figure 12. Marker rescue analysis of mutant ts9383. Monolayers of BSC40 cells were infected with ts9383 at a multiplicity of 0.05. For each rescue, 10 μg of linear viral DNA obtained from various subclones of cosmid pWR93-130 were added to the infected monolayers, after which the cells were incubated for 72 h and rescued plaques visualized by staining with crystal violet. The letters A to O represent the HindIII fragments of vaccinia strain WR. The pC6 series of clones were derived from cosmid pWR93-130. Only those restriction sites relevant to the analysis are indicated in the diagram. The location of genes D12 and D13 and the direction of transcription are as indicated. The results of the rescue experiment are given in Table 1.

Table 1. Marker rescue[a] of mutant ts9383.

DNA clone	DNA size (kb)[b]	plaques/10 μg DNA
pC6-4	13.0	> 500
pC6-1	12.5	0
pC6-3	12.0	0
pC6-43	4.9	267
pC6-45	3.1	0
pC6-44BX	1.8	229
pC6-44EX	1.0	49
no DNA	-	0

[a] Marker rescue was performed as described in Materials and Methods and Fig. 12. The locations of the DNA clones on the viral DNA are presented in Fig. 12.

[b] The size of the vaccinia virus DNA insert is indicated in kilobases.

DNA sequencing of the ts9383 mutation. Marker rescue analysis indicated that the ts lesion in mutant ts9383 was contained within a 1.0-kb XhoI-EcoRI fragment in either ORF D12 or ORF D13 near the right hand-side of the HindIII D fragment. To determine the molecular basis for this ts phenotype and assign the mutation to a single gene, we sequenced this 1.0-kb DNA fragment using the Sanger dideoxy chain termination method. Since ts9383 was derived from wild-type strain IHD-W, rather than strain WR from which the DNA used in the above-described marker rescue experiments was derived, we cloned the appropriate restriction fragments from both IHD-W and ts9383 in pTZ vectors, as described in Materials and Methods. The resulting clones were checked in a marker rescue assay to ensure that the correct fragments had been cloned. It was found that pCEXN1 (the IHD-W equivalent of pC6-44EX) was able to rescue the mutant to the same level as was achieved with the corresponding WR-derived clone pC6-44EX. As expected, the ts9383 EcoRI-XhoI fragment (called pCEXD1) was unable to rescue ts9383. The sequence of this fragment is depicted in Figure 13. In the 1035 bases sequenced, only a single alteration was seen in comparing the mutant with the wild-type IHD-W at position 379 in our figure (position 14283 in Niles et al., 1986; 3419 in Weinrich and Hruby, 1986). This alteration is a C→T substitution resulting in the conversion of proline to serine at amino acid position 23 in ORF D12.

We have also determined the DNA sequence of the insert in the WR-derived clone pC6-44EX and found it to be identical to that reported by Niles et al. (1986). In comparing the sequences of this 1035-bp fragment from wild-type strains WR and IHD-W we detected three differences. Two of these differences are due to single base changes that do not alter the corresponding amino acids: a C in strain IHD-W versus a T in strain WR at position 105, and

```
 XhoI

TCGAGAATTTATAATGAACTACTGACAAAAAGCAATAATGGAACAAGAACCCTAACTTTTAACTTTACACCAAAG   75

ATATTCTTTAGGCCGACAACTATTACGGCCAATGTATCTAGGGGGAAAGATAAACTATCTGTTCGAGTAGTTTAT 150

TCCACCATGGATGTCAACCATCCAATCTATTATGTACAAAAACAATTGGTAGTTGTATGTAATGACCTGTATAAG 225

GTATCTTACGATCAAGGGGTAAGTATTACCAAGATTATGGGAGATAATAACTAATAATAATAATGAAAACAAACT 300

ATAGAGTTGTAAATGGATGAAATTGTAAAAAATATCCGGGAGGGAACGCATGTCCTTCTTCCATTTTATGAAACA 375
            MetAspGluIleValLysAsnIleArgGluGlyThrHisValLeuLeuProPheTyrGluThr
   T
   *
TTGCCAGAACTTAATCTGTCTCTAGGTAAAAGCCCATTACCTAGTCTGGAATACGGAGCTAATTACTTTCTTCAG 450
LeuProGluLeuAsnLeuSerLeuGlyLysSerProLeuProSerLeuGluTyrGlyAlaAsnTyrPheLeuGln
   Ser

ATTTCTAGAGTTAATGATCTAAATAGAATGCCGACCGACATGTTAAAACTTTTTACACATGATATCATGTTACCA 525
IleSerArgValAsnAspLeuAsnArgMetProThrAspMetLeuLysLeuPheThrHisAspIleMetLeuPro

GAAAGCGATCTAGATAAAGTCTATGAAATTTTAAAGATTAATAGCGTAAAGTATTATGGGAGGAGTACTAAAGCG 600
GluSerAspLeuAspLysValTyrGluIleLeuLysIleAsnSerValLysTyrTyrGlyArgSerThrLysAla

GACGCCGTAGTTGCCGACCTCAGCGCACGCAATAAACTGTTCAAACGTGAACGAGATGCTATTAAATCTAATAAT 675
AspAlaValValAlaAspLeuSerAlaArgAsnLysLeuPheLysArgGluArgAspAlaIleLysSerAsnAsn

CATCTCACTGAAAACAATCTATACATTAGCGATTATAAGATGTTAACCTTCGACGTGTTTCGACCATTATTTGAT 750
HisLeuThrGluAsnAsnLeuTyrIleSerAspTyrLysMetLeuThrPheAspValPheArgProLeuPheAsp

TTTGTAAACGAAAAATATTGTATTATTAAACTTCCAACTTTATTCGGTAGAGGTGTAATCGATACTATGAGAATA 825
PheValAsnGluLysTyrCysIleIleLysLeuProThrLeuPheGlyArgGlyValIleAspThrMetArgIle

TATTGTAGTCTCTTTAAAAATGTTAGACTGCTAAAATGCGTAAGCGATAGCTGGTTGAAAGATAGCGCCATTATG 900
TyrCysSerLeuPheLysAsnValArgLeuLeuLysCysValSerAspSerTrpLeuLysAspSerAlaIleMet

GTGGCTAGTGATGTTTGTAAAAAAAATTTGGATTTATTTATGTCTCATGTTAAGTCCGTCACTAAGTCTTCTTCT 975
ValAlaSerAspValCysLysLysAsnLeuAspLeuPheMetSerHisValLysSerValThrLysSerSerSer

TGGAAGGATGTGAACAGTGTTCAATTTAGTATTTTAAACAATCCAGTGGATACGGAATTC               1035
TrpLysAspValAsnSerValGlnPheSerIleLeuAsnAsnProValAspThrGluPhe

                                                       EcoRI
```

Figure 13. The DNA sequence of the 1035-bp XhoI-EcoRI IHD-W-derived fragment capable of rescuing ts9383. Open reading frame D12 begins at nucleotide 313 in the diagram and extends 141-bp beyond the EcoRI site. This sequence was determined for three individual clones, the WR derivative pC6-44EX, the IHD-W derivative pCEXN1 and the ts9383 derivative pCEXD1 (see text). The single difference between IHD-W and ts9383 is indicated by a star (*). The changes observed between IHD-W/ts9383 and WR are underlined. None of these lead to a change in the corresponding amino acid (see text).

a G in strain IHD-W versus an A in WR at position 882. The third difference at position 277-279 was a TAA stop codon. The WR sequence has four successive stop codons in ORF D13, whereas both IHD-W and ts9383 have five stop codons.

A single point mutation results in both a ts and res⁻ phenotype. Having shown that the single transition mutation in the gene encoding the small subunit of the mRNA capping enzyme is responsible for the ts defect, we next wished to determine whether the same mutation is also responsible for the observed telomere resolution defect. That this might indeed be so was suggested by the observation that both virus lethality and resolution defect were only expressed above the cut-off temperature of approximately 39°C. We set out to test the hypothesis directly by replacing the mutation in ts9383 with the wild-type sequence and testing its effect on viral telomere resolution. To this end, BSC40 cell monolayers were infected with ts9383 at a multiplicity of 0.05 and transfected with plasmid DNA containing either the wild type or the mutated sequence. The infections were allowed to proceed for 72 h prior to harvesting. Rescued virus was used directly (Fig. 14; lanes 1-5), or after a subsequent 48 h passage at 40°C (Fig. 14; lanes 8-12), as inoculum for PFGE analysis. Fig. 14 shows that rescue with pC6-44BX (containing a 1.8-kb wild-type sequence) or pC6-44EX (containing a 1.0-kb wild-type sequence) produced virus that generates predominantly monomeric DNA, which is characteristic of wild-type infections (Fig. 14; compare lanes 1, 2, 3, 8, 9 and 10 with lane 7). In contrast, infection with ts9383 resulted in a ladder of concatemeric DNA molecules, characteristic of res⁻ mutants (Fig. 14; lane 6). Rescue with pCBXD1 (containing a 1.8-kb mutated sequence), was essentially identical to the no-DNA

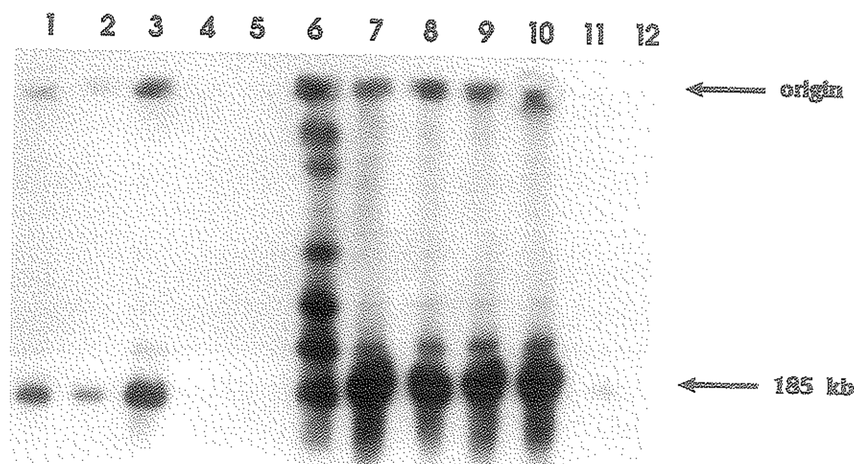

Figure 14. PFGE analysis of viral DNA obtained from marker rescue of ts9383. Mutant ts9383 was rescued by transfecting infected BSC40 cells with 5 μg of plasmid DNA, from which the viral inserts were released. Following a 72 h incubation period at 40°C, cell monolayers were harvested and the rescued virus was passaged for an additional 48 h period at 40°C . Virus obtained after the initial 72 h period (lanes 1-5) or after the additional 48 h passage (lanes 8-12) was then used to infect BSC40 cells for 24 h at 40°C. The progeny DNA was subjected to PFGE analysis as described in the legend of Fig. 10. Mutant ts9383 was rescued with pC6-44EX (performed in duplicate; lanes 1,2,8,9), pC6-44BX (lanes 3,10), pCBXD1 (lanes 4,11), or no DNA (lanes 5,12). Infections with ts9383 (lane 6) and wild-type IHD-W (lane 7) are included as controls.

control: in either case, neither rescued virus nor progeny DNA were observed (Fig. 14; lanes 4, 5, 11 and 12). The results unequivocally demonstrated that rescue with pC6-44BX or pC6-44EX, but not with pCBXD1 or no-DNA controls, had simultaneously repaired the ts and res$^-$ phenotypes. We therefore conclude that the single point mutation in ORF D12 is responsible for both the ts and res$^-$ phenotypes of mutant ts 9383.

Discussion

The resolution of the poxvirus telomere fusion configuration to hairpin termini can be seen as a site-specific recombination event, in which a putative telomere resolvase interacts with two identical inverted copies of a short DNA recognition sequence. The cis elements required for this recombination event are now fairly well-defined, but the component(s) of the putative telomere resolvase remain to be identified. The genetic approach to this problem has met with some difficulty, largely because mutants that are defective in telomere resolution have been found to be simultaneously defective in late gene expression. The defect in such mutants could be explained if one or more late protein(s) is/are required for efficient resolution of telomeres. This mechanism does not appear to be responsible for the resolution defect of mutant ts9383, since we have shown that late protein synthesis of this res$^-$ mutant appears unaffected up to at least 12 h postinfection. Our results are compatible with the view that the protein that is altered in ts9383 normally acts in trans at the telomere resolution target region during the process of telomere resolution. Interestingly, we found this protein to be equivalent to the 33-kDa small subunit of the mRNA capping enzyme, which is encoded by ORF D12 (Niles et al., 1989).

Mutant ts9383 was initially classified as a replication-positive mutant which, when examined by electron microscopy, was shown to be blocked during virion morphogenesis at a stage when replicated nucleoprotein becomes condensed and viral membranes are beginning to enclose the nucleoprotein. As a result, the virosomes formed within ts9383-infected cells at the nonpermissive temperature contain characteristically dense DNA paracrystals and aberrantly-formed virus envelopes (Dales et al., 1978). It is of interest that treatment of vaccinia-infected cells with rifampicin results in an essentially identical block in virion morphogenesis (Moss et al., 1969; Nagayama et al., 1970). Rifampicin resistance mutations have been mapped to ORF D13 (Baldick and Moss, 1987; Thompson and Condit, 1986). Furthermore, we have recently found that three mutants with the same terminal phenotype (category E), ts1085, ts7743, and ts9203 (Lake et al., 1979; Silver and Dales, 1982), and a ts mutant, tsC33, whose lesion has been mapped to ORF D13 (Niles et al., 1986), fail to complement each other (A.M. DeLange, unpublished data). Marker rescue analysis further supported the placement of the lesions within these four ts mutants in ORF D13 (data not shown). Clearly, ORF D13 is implicated to have a role during virion morphogenesis. ORF D13 encodes a 65-kDa protein (Weinrich et al., 1985), which is thought to be involved in proteolytic processing of core polypeptides (Katz and Moss, 1970). Despite the identical morphological phenotype associated with ORF D12 and ORF D13 mutants, telomere resolution is unaffected in the ORF D13 ts mutants (DeLange, 1989; Merchlinsky and Moss, 1989a), or after treatment of wild-type virus with rifampicin (Merchlinsky and Moss, 1989a). It is intriguing that mutations in these adjacent ORFs, D12 and D13, result in a block at the same stage of virion morphogenesis and yet the gene products would appear to be quite

unrelated in function or in the time at which they are expressed, ORF D12 being expressed early (Lee-Chen and Niles, 1988; Lee-Chen et al., 1988) and ORF D13 late and probably intermediate (Weinrich and Hruby, 1987). In contrast to ORF D13 mutants, ts9383 does not have a defect in cleavage of the major core polypeptides (Lake et al., 1979). Could the function of the small subunit of the mRNA capping enzyme during telomere resolution somehow be required for both the resolution event and virion morphogenesis or could proper resolution even be a prerequisite to a specific stage in virion morphogenesis? Such a role of this capping enzyme subunit is plausible, especially since it is known to be a highly stable protein present throughout the infection cycle and is packaged into virion particles (Niles et al., 1989).

Our observation that protein synthesis in ts9383-infected cells is unaffected at the nonpermissive temperature under conditions where the res$^-$ phenotype is expressed, suggests that this res$^-$ phenotype is not caused by a general defect in late protein synthesis. We have, however, found that the res$^-$ phenotype in time course experiments used to determine protein synthesis is slightly less severe than that observed when a single time point is taken at 24 h postinfection (compare Fig. 10B, lane 24 at 40°C and Fig. 14, lane 6). This difference cannot be ascribed to the multiplicity of infection, since essentially identical resolution defects were observed at multiplicities of 1, 5 and 25 (data not shown). We have repeated the experiment under very stringent conditions with essentially identical results and, since the res$^-$ phenotype is still severe under conditions where protein synthesis is essentially normal, we favour the hypothesis that this mutation does not cause a general defect in intermediate and late protein synthesis. These observations pose two interesting questions. (i) Since this gene is known to

be essential (Niles et al., 1989) and the gene product is involved in mRNA capping and transcription termination, why did we fail to detect a defect in protein synthesis? (ii) Is there evidence for an interaction of the mRNA capping enzyme with the telomere fusion elements, and could this interaction somehow mediate or control their resolution to hairpin termini?

The functional mRNA capping enzyme of vaccinia virus constitutes a heterodimeric multifunctional enzyme complex composed of 97-kDa and 33-kDa subunits (reviewed in Moss, 1990a). Both subunits are expressed at early times prior to the onset of viral DNA synthesis. The purified enzyme catalyzes the first three reactions during the formation of a cap structure at the 5' ends of RNA: polynucleotide 5' triphosphatase, mRNA guanylyltransferase and mRNA (guanine-7) methyltransferase (Martin et al., 1975; Venkatesan et al., 1980). The phosphatase and guanylyltransferase activities are performed by the large subunit and the methyltransferase by the small subunit of the mRNA capping enzyme (Shuman, 1989; 1990a, b). The multifunctional nature of this enzyme has been dramatically illustrated by the observation that this enzyme is required in the process of transcription termination (Shuman et al., 1987). The lesion in the small 33-kDa subunit in ts9383 could potentially cause a defect in any or all of the enzymatic activities of the mRNA capping enzyme and/or in some as yet undefined activity of the enzyme subunit. Assuming all functions were abolished by the mutation, one might postulate that a cellular counterpart of the capping enzyme substitutes for the virus-encoded enzyme. However, it is not clear how such a cellular enzyme could also be involved in transcription termination, and since preliminary Northern blots of RNA isolated from ts9383-infected cells showed normal quantities of discrete-length early RNA and heterogeneous-length late RNA (data not shown) it appears that at least the transcription

termination function of the mutant enzyme is unaffected. Whether the capping activities are similarly unaffected awaits *in vitro* testing of the mutant enzyme. Even though transcription termination appears normal, this might be attributed to the normal function of a virus-encapsidated enzyme, if ts9383 is defective in the assembly of a functional enzyme at the nonpermissive temperature, as has been shown for mutants of RNA polymerase subunits (Hooda-Dhingra et al., 1989). In that case, early RNA would be synthesized and processed normally with the virion-carried enzyme complex, but these activities would be defective at late times. Since RNA transcribed from at least most late genes does not appear to utilize the transcription termination function and the *in vivo* requirement of the capping function for these transcripts is unknown, it is possible that an assembly mutant of this type might have the observed phenotype. Preliminary experiments aimed at distinguishing between an assembly and a thermolabile defect in telomere resolution have thus far been nonconclusive.

It was recently shown that the noncoding regions at the ends of the genomes of vaccinia virus and several other orthopoxviruses act as a template for the synthesis of late RNAs (Parsons and Pickup, 1990). Since telomere resolution appears to take place after the completion of DNA synthesis at about the time that these late RNA molecules are synthesized, it is possible that one or more telomeric RNA molecule(s) are somehow involved in the process of telomere resolution. Alternatively, binding of the transcriptional machinery might have a role in the control of the resolution event. The RNA capping enzyme could be involved in this process, either through its transcription termination function or some other activity. Interestingly, this view could also imply a direct role for the viral RNA polymerase in the resolution process. Such a role for RNA polymerase in telomere resolution is consistent

with the observed resolution defect of RNA polymerase mutants. Future experiments are aimed at determining the function of the mRNA capping enzyme during the process of telomere resolution.

CHAPTER III: IDENTIFICATION OF A TEMPERATURE-SENSITIVE MUTANT OF VACCINIA VIRUS DEFECTIVE IN LATE BUT NOT INTERMEDIATE GENE EXPRESSION

Abstract

The vaccinia virus conditional-lethal temperature-sensitive (ts) mutant tsC63 is defective in the synthesis of some but not all postreplicative proteins. Synthesis of the temporal `intermediate' class of proteins was unaffected, whereas `late' proteins were absent at the nonpermissive temperature. At the DNA level, DNA synthesis was unaffected, but telomere resolution was severely inhibited. In order to identify the defective gene responsible for this ts defect, we performed marker rescue and DNA sequencing experiments. We localized the lesion to open reading frame (ORF) A1L, which has recently been identified as one of the three intermediate genes required for the transcription of late genes (J.G. Keck, C.J. Baldick, Jr. and B. Moss, Cell **61**:801-809, 1990). S1 nuclease analysis of viral mRNA demonstrated that the ts defect in late protein synthesis was caused by a defect in the transcription of stable mRNA, and therefore provides evidence for a role of the A1L gene product during *in vivo* transcriptional activation or stabilization of the late class of vaccinia genes. Furthermore, the kinetics of early protein synthesis in tsC63-infected cells suggests that, in addition to its role in trans-activation of late genes, intermediate gene expression mediates suppression of early protein synthesis. The telomere resolution defect of this mutant is presumably a secondary consequence of the defect in late gene expression.

Introduction

Vaccinia virus replicates its linear 185-kilobase (kb) DNA genome in the cytoplasm of a wide range of vertebrate cells. The apparent lack of introns in this genome enables the virus to code up to about 200 polypeptides (for a review, see Moss, 1990a). Transcription proceeds in a temporally controlled manner, and viral DNA synthesis plays a crucial part in its regulation. In agreement with the long-standing belief that most or all factors required for viral DNA replication and transcription are virus-encoded, many such enzymes have now been identified and shown to be encoded by specific viral genes (Moss, 1990b). The added observation that these enzymes are often highly homologous to their cellular counterparts makes a strong case for the use of this virus as a model to study mechanisms of DNA replication and transcription in higher eukaryotes.

The linear double-stranded DNA genome of vaccinia virus is terminated by incompletely base-paired hairpins (Geshelin and Berns, 1974; Baroudy et al., 1982). Upon replication, the genome is converted to high molecular weight concatemers, which consist of unit-length genomes that are joined together by the replicated, inverted repeat configuration of the terminal hairpin (Moyer and Graves, 1981; Moss et al., 1983; DeLange, 1989; Merchlinsky and Moss, 1989a). This inverted repeat structure is resolved in a ppostreplicative step termed telomere resolution. The cis-acting DNA element required for this resolution process, called the telomere resolution target or TRT (DeLange and McFadden, 1990), has been identified (DeLange et al., 1986; Merchlinsky and Moss, 1986; Merchlinsky, 1990). Although some suggestive evidence indicates that a 50-kDa nuclease may be involved in the resolution process (Reddy and Bauer, 1989), no trans-acting resolvase factors have been

positively identified to-date.

Poxvirus genes have traditionally been separated into two classes, those expressed prior to (early or pre-replicative), and those expressed after (late or postreplicative) the onset of viral DNA synthesis. Two classes of postreplicative genes have been recognized on the basis of the temporal appearance of their gene products. One class is expressed immediately after the onset of viral DNA synthesis, whereas the other is expressed after a slight delay (Moss and Salzman, 1968; Pennington, 1974; Vos and Stunnenberg, 1988). While the existence of two distinct temporal postreplicative classes had long been suspected, strong supporting evidence has appeared only recently. Vos and Stunnenberg (1988) used a novel transfection assay in which vaccinia virus-infected cells that were blocked at the onset of DNA replication were transfected with plasmid DNA. Using this *in vivo* assay, only a subset of plasmid-borne, postreplicative genes were expressed. Interestingly, the same subset of genes was selectively expressed from plasmid DNA under *in vitro* conditions when cellular extracts from hydroxyurea-treated, vaccinia-infected cells were used to detect transcription. This class of postreplicative genes is, based on the time of activation of these genes during infection, known as intermediate. The second subclass of postreplicative genes, the true late genes, failed to be expressed from plasmid DNA under the same conditions. These observations inspired a cascade model of gene expression, in which some intermediate proteins would be required to induce the expression of late genes. This hypothesis was confirmed by Keck et al. (1990), who demonstrated that expression of three intermediate genes from naked plasmid DNA was necessary and sufficient to induce expression of a late reporter gene. These three intermediate genes were identified as open reading frames (ORFs)

GK1, A2, and A1, which have coding capacities of 30, 26, and 17-kDa, respectively. The 30-kDa protein comprises part or all of the biochemically-purified late transcription factor VLTF-1 (Wright et al., 1991).

We have recently characterized a class of conditional-lethal temperature-sensitive (ts) mutants of vaccinia virus, which are competent for DNA synthesis but defective in telomere resolution (res^-) (DeLange, 1989). It was found that most res^- mutants are also defective in some aspect of postreplicative gene expression (DeLange, 1989; Merchlinsky and Moss, 1989a). This suggested a cause-and-effect relationship between expression of postreplicative genes and telomere resolution. This view was supported by the finding that RNA instability, caused either by the conditional-lethal mutant tsC22 (Pacha and Condit, 1985) or by the drug isatin-β-thiosemicarbazone (Merchlinsky and Moss, 1989a), also causes a defect in telomere resolution. A number of res^- mutants sharing the same phenotype of abnormal postreplicative gene expression and defective telomere resolution have been shown to contain lesions in various subunits of the virus-encoded RNA polymerase (rpo), namely, rpo147, rpo22, rpo18 (DeLange, 1989; Hooda-Dhingra et al., 1989; Merchlinsky and Moss, 1989a) Ahn et al., 1990), and rpo132 (Hooda-Dhingra et al., 1990; Carpenter and DeLange, 1991). However, two res^- mutants, ts9383 and tsC63, did not appear to have the same dramatic defect in postreplicative gene expression observed in the other res^- mutants. The lesion responsible for the ts and res^- phenotypes of ts9383 has been localized to the small subunit of the heterodimeric virus-encoded mRNA capping enzyme (Carpenter and DeLange, 1991). Yet, this mutant does not appear to be defective in protein synthesis. Given the fact that the mRNA capping enzyme is a multi-functional enzyme which, besides its role in mRNA cap formation

(reviewed in Moss, 1990b), also acts as an early transcription termination factor (Shuman et al., 1987), and an intermediate gene transcription factor (Vos et al., 1991), a direct role of the mRNA capping enzyme during telomere resolution has been postulated (Carpenter and DeLange, 1991).

In this report, we describe the conditional-lethal res⁻ mutant tsC63 which, at the nonpermissive temperature, is partially defective in postreplicative gene expression. We show that, whereas this mutant expresses intermediate genes with high efficiency, it expresses late genes very poorly at the nonpermissive temperature. Marker rescue and DNA sequencing have localized the mutation to ORF A1, which has previously been identified as one of the three intermediate genes required for expression of a plasmid-borne late gene (Keck et al., 1990). Our observations on this mutant provide evidence for a role of the A1 protein during *in vivo* activation of the late class of genes from the viral genome. This mutant should facilitate the study of vaccinia virus postreplicative transcriptional regulation and telomere resolution.

Materials and Methods

Materials. Restriction endonucleases, T4 DNA ligase and S1 nuclease were obtained from Boehringer Mannheim Biochemicals or Pharmacia. Phagemid vectors pTZ18U/R and pTZ19U/R and helper phage M13KO7 were obtained from U.S. Biochemicals. T7 RNA polymerase and ribonucleotides were from Promega. Deoxynucleoside triphosphates, dideoxynucleoside triphosphates and Klenow enzyme were obtained from Bio-Rad Laboratories. [^{35}S]methionine, [^{35}S]deoxyadenosine triphosphate and [^{32}P]cytidine

triphosphate were from Amersham Corp.

Cells and Virus. A continuous line of African green monkey kidney cells (BSC40) was grown as a monolayer culture in Dulbecco's Modified Eagle (DME) medium supplemented with 5% fetal calf serum. Wild-type strain WR and ts mutant tsC63 were kindly provided by Dr. Richard Condit. Mutant tsC63 was twice plaque purified in our laboratory before being used in experiments. Crude virus stocks were obtained as described previously (DeLange, 1989). The permissive temperature for mutant tsC63 was 32°C, the nonpermissive temperature was 40°C.

Molecular Cloning and Marker Rescue. WR and tsC63 viral DNA were purified as described by Esposito et al. (1981). After appropriate restriction endonuclease digestion and gel electrophoresis, DNA fragments were purified from agarose gels by electro-elution and ligated into the multiple cloning site of the Genescribe vectors, pTZ18R, pTZ18U or pTZ19U by standard methods (Maniatis et al., 1982). Constructs were used to transform DH5α or JM101 bacterial strains. Plasmid DNA for marker rescue or restriction enzyme analysis was prepared by the alkaline lysis method of Birnboim and Doly (1979). Plasmid pC6-4 was derived from cosmid pWR93-130 (Thompson and Condit, 1986) by digestion with KpnI plus SacI and ligation into similarly digested pTZ18R. Plasmids pC6-41, pC6-43, and pC6-45 are subclones of pC6-4 (Carpenter and DeLange, 1991). Plasmid pC6-41 was further subcloned by double digestion with HindIII and NdeI (to generate pC6-41-NS) or NdeI and SacI (pC6-41-HN), followed by treatment with Klenow to generate blunt ends and self-

ligation in the presence of T4 DNA ligase. Plasmid pC6-41-A was derived by digestion of pC6-41 with AccI, which removes a 0.75-kb fragment containing most of ORF A2, followed by treatment with Klenow, to generate blunt ends, and self-ligation. Plasmid pTS63-HS was obtained by ligating the 1.7-kb HindIII-SacI fragment from tsC63 viral DNA with similarly digested pTZ18R vector. pTS63-HN was derived from pTS63-HS by double digestion with SacI and NdeI, treatment with Klenow and self-ligation. To test which mutation caused the ts phenotype of tsC63, four plasmids were generated. Plasmids pTS63-HN and pC6-41-HN were each digested with HindIII and AccI, and the digested DNA was electrophoresed in a 5% mini-acrylamide gel. The 364-bp HindIII-AccI fragments thus generated were isolated by gel elution and subjected to an AluI partial digestion. The resulting fragments were electrophoresed in an acrylamide gel and the 195-bp AccI-AluI and 169-bp HindIII-AluI fragments were eluted (see Fig. 19). Each mutant and wild-type AccI-AluI fragment was then combined with either the mutant or wild-type HindIII-AluI fragment and ligated to the 3.1-kb HindIII-AccI fragment of the parent plasmid pTS63-HN. Four plasmids were thus generated: pTS63-HN[$m1^-$,$m2^-$] is identical to pTS63-HN and contains both the m1 and m2 mutation; pTS63-HN[$m1^+$,$m2^+$] contains neither mutation; pTS63-HN[$m1^-$,$m2^+$] contains only the m1 mutation; and pTS63-HN[$m1^+$,$m2^-$] contains only the m2 mutation (see Fig. 19). The identities of the clones were confirmed by DNA sequencing. Marker rescue was performed as described in Carpenter and DeLange (1991).

Protein Labelling. Pulse-labelling of proteins from infected cells, sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography of labelled proteins were

performed as described previously (Condit and Motyczka, 1981; Carpenter and DeLange, 1991). Cells were infected at a multiplicity of infection of 25 PFU per cell.

DNA Sequencing. Sequencing of DNA clones obtained from wild-type WR and mutant tsC63 was performed by the Sanger dideoxynucleotide chain termination method (Sanger et al., 1977). Single-stranded template was prepared according to Dente et al. (1983) and Messing (1983) and has been described previously (Carpenter and DeLange, 1991). For sequencing of double-stranded DNA, templates were prepared according to the method of Hattori and Sakaki (1986).

RNA Isolation. RNA was isolated using the guanidinium isothiocyanate-CsCl method as described in Ausubel et al. (1987). Briefly, 100 mm tissue culture dishes containing monolayers of BSC40 cells were infected at a multiplicity of 10 or 20. Following a 30 min adsorption period at 37°C, the inoculum was removed and replaced with pre-warmed (32°C or 40°C) medium. Plates were then incubated for various times at either the permissive or nonpermissive temperature. At given times postinfection, plates were taken from the incubators and placed on ice. The medium was removed, and the cells rinsed twice with ice cold PBS before being lysed with the guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT, 0.5% N-lauroylsarcosine). The lysates were passaged four times through a 21-G needle, transferred to fresh tubes and flash-frozen in a dry ice ethanol bath prior to being stored at -70°C overnight. The following day, 3.5 ml of the thawed lysate was layered onto 1.5 ml of 5.7 M CsCl in silanized and autoclaved

Beckman tubes. Centrifugation was in a Beckman SW50.1 rotor at 35,000 rpm (150,000 x g) at 18°C for 15-20 h. The resulting supernatant was then drawn off and the pellet resuspended in TES (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1% SDS), extracted with chloroform/1-butanol (4:1) and ethanol precipitated twice. The RNA was resuspended in water and stored at -70°C. Concentration and purity were determined by reading the A_{260} and A_{280} values in a Gilford spectrophotometer.

Riboprobe Construction and S1 Nuclease Analysis. In order to analyze the 5' ends of viral mRNA, specific DNA fragments were cloned downstream of the T7 RNA polymerase promoter in pTZ19U. Clone pTS63-A1 was generated by digesting clone pTS63-HS with HindIII and BglII and ligating this fragment into pTZ19U digested with HindIII and BamHI. pJB307-A4 was constructed by isolating an approximately 1.1-kb ScaI fragment from clone pJB307, which was obtained from a partial MboI library from wild-type WR and contains ORFs A3-A5, treating this fragment with Klenow to generate blunt ends and ligating it into pTZ19U digested with SmaI. pTS63-A1 was linearized with EcoRI while pJB307-A4 was linearized with MluI to give transcripts of discrete length (Fig. 20C). Uniformly ^{32}P-labelled antisense RNA was generated as described in the Promega literature and purified on 5% polyacrylamide/7 M urea denaturing mini-gels. The full length probes were excised and eluted (elution buffer was 2 M ammonium acetate, 1% SDS, 25 μg/ml yeast tRNA) as described by Ausubel et al. (1987) for 3 h at 42°C followed by ethanol precipitation. S1 nuclease analysis of the 5' ends of viral mRNA was performed essentially as described by Weir and Moss (1984) except that uniformly labelled antisense RNA probes were used. Hybridizations were

conducted under conditions of probe excess and contained 10 μg of total RNA in a 30 μl reaction volume containing 80% formamide, 40 mM PIPES, pH 4.6, 400 mM NaCl and 1 mM EDTA. Reactions were heated to 85°C for 5 min, then incubated overnight at 37°C. The RNA duplexes were chilled on ice with 300 μl S1 digestion buffer (0.28 M NaCl, 0.05M Na acetate pH 4.6, 4.5 mM $ZnSO_4$) and digested with 500 U S1 nuclease for 1 h at room temperature followed by phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in loading buffer, heated for 3 min at 85°C and run on a denaturing (7 M urea) 5% polyacrylamide gel. Protected fragments were detected by autoradiography and quantitated by densitometry.

Results

Protein synthesis in tsC63-infected cells. Mutant tsC63 was initially identified as a ts mutant defective in postreplicative protein synthesis (Condit and Motyczka, 1981; Condit et al., 1983). Subsequently, it was also found to belong to a class of mutants(res$^-$) which, while not defective in DNA synthesis, is defective in the process of telomere resolution (DeLange, 1989; Merchlinsky and Moss, 1989a). A closer analysis of protein synthesis at the permissive (32°C) and nonpermissive (40°C) temperatures suggested that at least some late proteins were expressed normally at the nonpermissive temperature (DeLange, 1989). Following this initial analysis, firm evidence was obtained for the existence of two classes of postreplicative proteins: intermediate proteins, which are expressed immediately after the onset of viral DNA synthesis, and late proteins, which are expressed with a delay (Vos and Stunnenberg, 1988; Keck et al., 1990; Vos et al., 1991). Since at least one subclass of

postreplicative proteins in tsC63 was expressed normally at the nonpermissive temperature, we hypothesized that these might be equivalent to the intermediate class and that a second class of proteins which was expressed poorly or not at all might represent the late class of proteins. We thus reexamined in detail the pattern of protein synthesis in both tsC63- and parental strain WR-infected cells.

Time course analysis of proteins produced during mutant infection confirmed the absence of some, but not all, postreplicative proteins at the nonpermissive temperature (Fig. 15A). One of these classes of proteins (labelled "inter" in Fig. 15) was produced beginning at 4 h postinfection at the nonpermissive temperature and approximately 1 h later at the permissive temperature (a short lag is usually observed in infections performed at lower temperatures). However, whereas the second class of postreplicative proteins (labelled "late" in Fig. 15) appeared at approximately 8 h postinfection at the permissive temperature, this class of polypeptides was absent at the nonpermissive temperature (Fig. 15A). The arrows on the right-hand side of Fig. 15 indicate representative examples of the three classes of proteins based on their temporal appearance in the WR infection (see below). We designate the two classes of postreplicative proteins as intermediate (inter) and late and will provide further evidence in subsequent sections that the two temporal classes are indeed equivalent to intermediate and late proteins.

At the same time as proteins were being harvested from the tsC63 infection, we also examined proteins synthesis from wild-type WR-infected cells (Fig. 15B). In addition to the normal harvesting time-course, proteins were harvested every 30 min between 2 h and 5 h postinfection since it is during this time that the temporal switch between the intermediate and

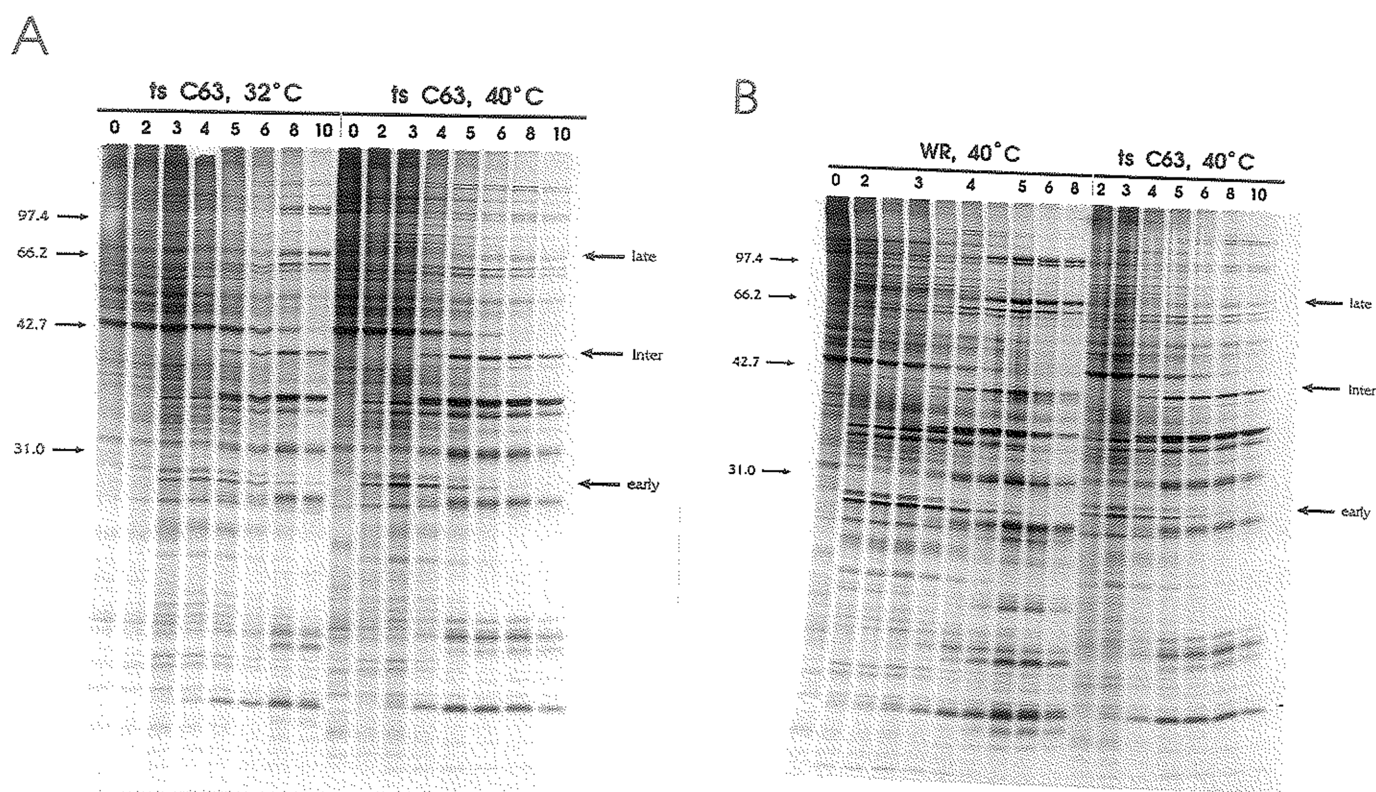

Figure 15. Protein synthesis in cells infected with vaccinia virus mutant tsC63 (A, B) and wild-type strain WR (B) infected cells. Monolayers of BSC40 cells were infected at a multiplicity of 25 PFU per cell and incubated at 32°C or 40°C. At various times postinfection, cells were pulse-labelled with [^{35}S]methionine. Additional time points were obtained for WR at 2.5, 3.5 and 4.5 h postinfection. Samples were harvested and separated on an SDS-12.5% polyacrylamide gel and exposed to X-ray film. Molecular weight markers as well as representative early, intermediate and late proteins are indicated.

the late class of proteins takes place. In keeping with the previous results, one class of polypeptides (indicated as inter) appeared by 3.5 h postinfection which is similar to the results obtained with the mutant infection at 40°C. A second class of postreplicative proteins (Fig. 15B, indicated as late) appeared shortly after the intermediate proteins and is evident by 4 h postinfection in the WR infection. The short lag between the appearance of the two classes of postreplicative proteins is characteristic of wild-type infections. Even at 32°C, the temporal late class of proteins invariably appears 4-5 h postinfection (not shown). The lag period was much extended in mutant infections at the permissive temperature (32°C), and late proteins did not appear until 8 h postinfection. Significantly, the late class of proteins was essentially absent in the mutant infection even by 10 h postinfection at the nonpermissive temperature (Fig. 15B). These data are consistent with the interpretation that tsC63 is defective in the synthesis of late but not intermediate proteins.

When comparing the intensities of the "inter" bands in mutant and wild-type infections, it appears that synthesis of this class of proteins peaks at around 5 h postinfection in wild-type infections and then levels off. In contrast, this class of proteins remains highly expressed up to at least 10 h postinfection in tsC63-infected cells. It is not clear whether this apparent partial turnoff of intermediate protein synthesis at late times is the result of active suppression by a late gene product or reflects competition between the synthesis of intermediate and late proteins.

Marker rescue of tsC63. Utilizing cloned viral DNA from a cosmid library, Thompson and Condit (1986) roughly located the tsC63 mutation site to an approximately 10-kb region

spanning the right side of HindIII D and the left side of HindIII A. In a preliminary experiment we determined that cosmid pWR93-130 and a 12.5-kb subclone, pC6-4, rescued tsC63 with high efficiency (Fig. 16). pC6-4 was therefore further subcloned into three plasmids: pC6-43, pC6-45 and pC6-41. All three subclones, including plasmid pC6-41 which contains a 1.7-kb HindIII-SacI fragment derived from the extreme left end of the HindIII A fragment, still rescued with high efficiency. The pC6-41 fragment contains three complete ORFs, A1, A2, and A3, and part of ORF A4 (Weinrich and Hruby, 1986). To localize the mutation to a specific ORF, various subclones of pC6-41 were obtained and tested in marker rescue experiments. Positive rescue with plasmids pC6-41-HN and pC6-41-A, but not with plasmid pC6-41-NS, indicated that the ts lesion was located in ORF A1 (Fig. 16).

tsC63 contains two point mutations in ORF A1. To determine the mutation(s) in ORF A1 that cause(s) the ts phenotype, we cloned the 1.7-kb HindIII-SacI fragment from tsC63 DNA. The resulting clone pTS63-HS (the mutant equivalent of the wild-type pC6-41 clone) and its HindIII-NdeI derived subclone, pTS63-HN (the mutant equivalent of pC6-41-HN) were tested for their ability to rescue the ts phenotype. As expected, plasmids containing the mutant DNA were not able to rescue the ts phenotype of tsC63. We next determined the DNA sequence of the HindIII-NdeI fragments of both wild-type WR and mutant tsC63. This 566-bp fragment was found to contain two nucleotide substitutions, a G→A transition causing a change from valine to methionine at amino acid position 41, and a C→A transversion causing a change from proline to glutamine at position 100 (Fig. 17). For convenience, we termed these mutations m1 and m2, respectively. The sequence of the wild-type clone was found to

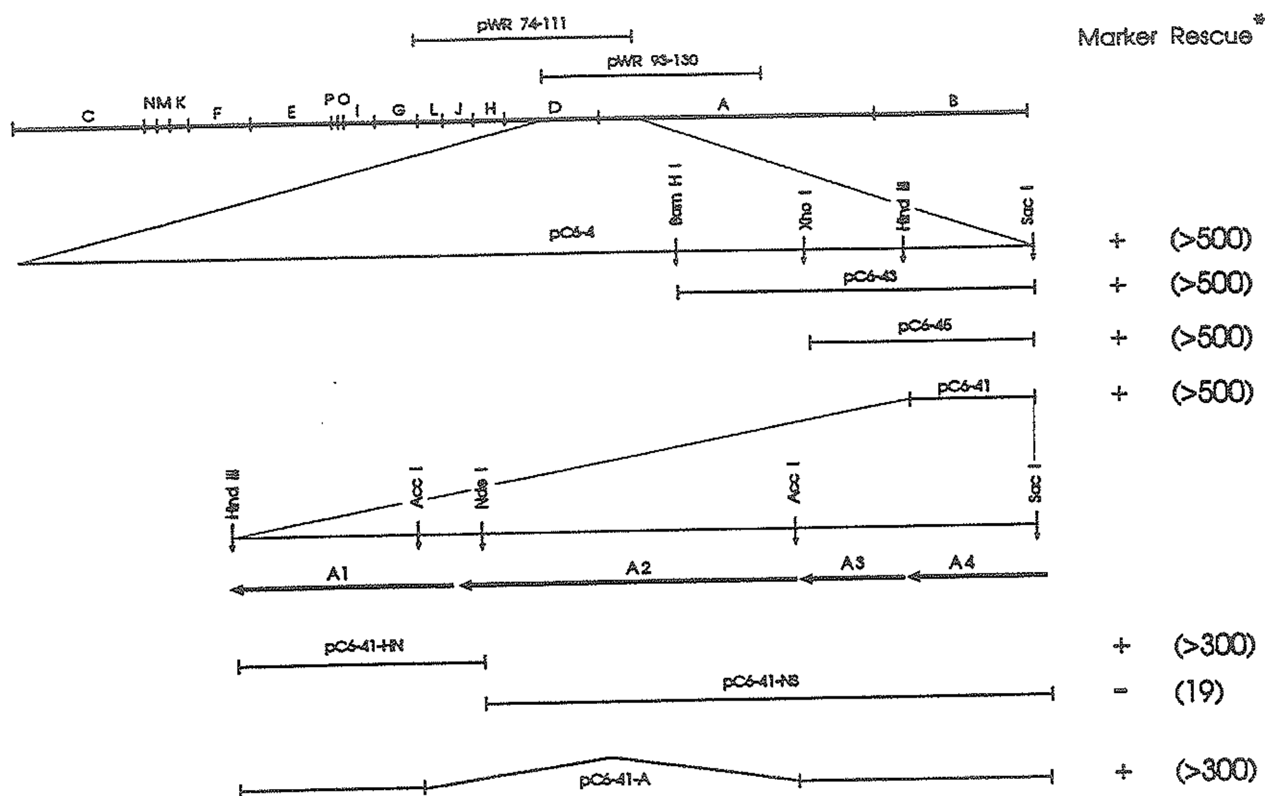

Figure 16. Marker rescue of mutant tsC63. Confluent monolayers of BSC40 cells were infected at a multiplicity of 0.05 PFU per cell. Each rescue was performed with 10 μg of linear wild-type viral DNA obtained from subclones of cosmid pWR93-130. Calcium phosphate-precipitated DNA was added to the infected monolayers as described in Materials and Methods and the infections were allowed to proceed for 72 h prior to visualizing plaque formation by staining with crystal violet. The vaccinia genome is indicated at the top of the figure with the letters A to P representing HindIII restriction fragments. The location and direction of transcription of genes A1 to A4 is indicated as are relevant restriction sites and positive or negative marker rescue results (*) in number of plaques formed per 10 μg DNA.

```
           NdeI
           CATATGAGATCTAAAAAACGTGTAACTATGGTAGAGGATTATGGACACGAGTA   53

TGTTTTTGTAGATGAGAGGTTTTCTACTTGCTCATTAGAAGTATAAAAAAATAGTTCCGT 113

AATTAAATGGCTAAGCGAGTAAGCCTTCCAGATGTGGTTATTTCAGCACCTAAAGCAGTT 173
       MetAlaLysArgValSerLeuProAspValValIleSerAlaProLysAlaVal
                    5                  10                  15

                                  AccI
TTTAAGCCCGCTAAAGAAGAAGCACTCGCTTGTATACTACCAAAGTATTATAAATCTATG 233
PheLysProAlaLysGluGluAlaLeuAlaCysIleLeuProLysTyrTyrLysSerMet
   20                 25                  30                 35
      A
      ↑
GCAGATGTGTCTATTAAGACAAATAGTGTAATTGATAAGTGTTGGTTTTGTAATCAAGAT 293
AlaAspValSerIleLysThrAsnSerValIleAspLysCysTrpPheCysAsnGlnAsp
   40 ↓               45                  50                 55
     Met

TTGGTTTTTAAACCTATTAGTATTGAGACATTCAAGGGTGGTGAAGTTGGGTATTTCTGT 353
LeuValPheLysProIleSerIleGluThrPheLysGlyGlyGluValGlyTyrPheCys
   60                 65                  70                 75

                                                     AluI
TCTAAAATATGTAGGGATTCGTTGGCGTCTATGGTTAAGTCTCACGTAGCTCTTAGAGAA 413
SerLysIleCysArgAspSerLeuAlaSerMetValLysSerHisValAlaLeuArgGlu
   80                 85                  90                 95
   A
   ↑
GAACCAAAAAATTTCTTTGTTGCCTTTAGTATTCTATGAAGATAAGGAAAAGGTCATAAAT 473
GluProLysIleSerLeuLeuProLeuValPheTyrGluAspLysGluLysValIleAsn
   ↓                 105                 110                115
  Gln
                                              AluI
ACAATAAACTTACTAAGAGATAAAGACGGCGTTTACGGAAGCTGTTACTTTAAGGAAAAC 533
ThrIleAsnLeuLeuArgAspLysAspGlyValTyrGlySerCysTyrPheLysGluAsn
   120                125                130                135

                                               HindIII
TCACAAATTATAGATATTTCTCTACGGAGTTTATTGTAAGCTT                 573
SerGlnIleIleAspIleSerLeuArgSerLeuLeuEnd
   140                145                150
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Figure 17. DNA sequence of the 573-bp NdeI-HindIII wild-type fragment capable of rescuing tsC63 at 40°C and the location of the mutations in the tsC63 mutant DNA. The two differences which exist between the wild-type and mutant DNA are indicated by arrows along with the predicted change in amino acid sequence.

match exactly the sequence described by Weinrich and Hruby (1986).

It has been previously reported that the 35-amino acid sequence 51-KCWFCNQDLVFKPISIETFKGGEVGYFCSKICRNS-85 within the 17-kDa protein encoded by ORF A1 resembles a zinc finger of the C4 type (Keck et al., 1990). The two amino acid changes in tsC63 are both located outside this putative zinc finger domain, one 10 amino acids to the left and the second 15 amino acids to the right. In an attempt to further elucidate the effects of each of the amino acid substitutions on the structure of the A1 protein, we constructed hydropathic plots of the relevant region according to the procedure described by Hopp and Woods (1981) (Fig. 18). Only the m2 mutation was seen to alter the hydrophilicity profile of the A1 protein. While the extent of change is marginal, the fact that the m2 mutation (Fig. 18; arrow) is located immediately adjacent to the most hydrophobic region of the protein may have important implications for its function.

A C→A base change in ORF A1 confers temperature-sensitivity. To determine which of the two mutations was responsible for the ts phenotype, we constructed plasmids that contained either one or the other mutation. This was achieved by substituting either a 194-bp AccI-AluI fragment (containing mutation m1) or a 169-bp HindIII-AluI fragment (containing m2) from tsC63 with corresponding fragments from wild-type WR (see Fig. 19). These plasmids were then used to rescue the ts phenotype of tsC63. Using this approach we demonstrated that DNA containing only mutation m1 rescued with high efficiency, comparable to that of wild-type DNA, whereas sequences containing the m2 mutation failed to rescue (Fig. 19). Thus it is the C→A mutation at position +299 in ORF A1, causing a

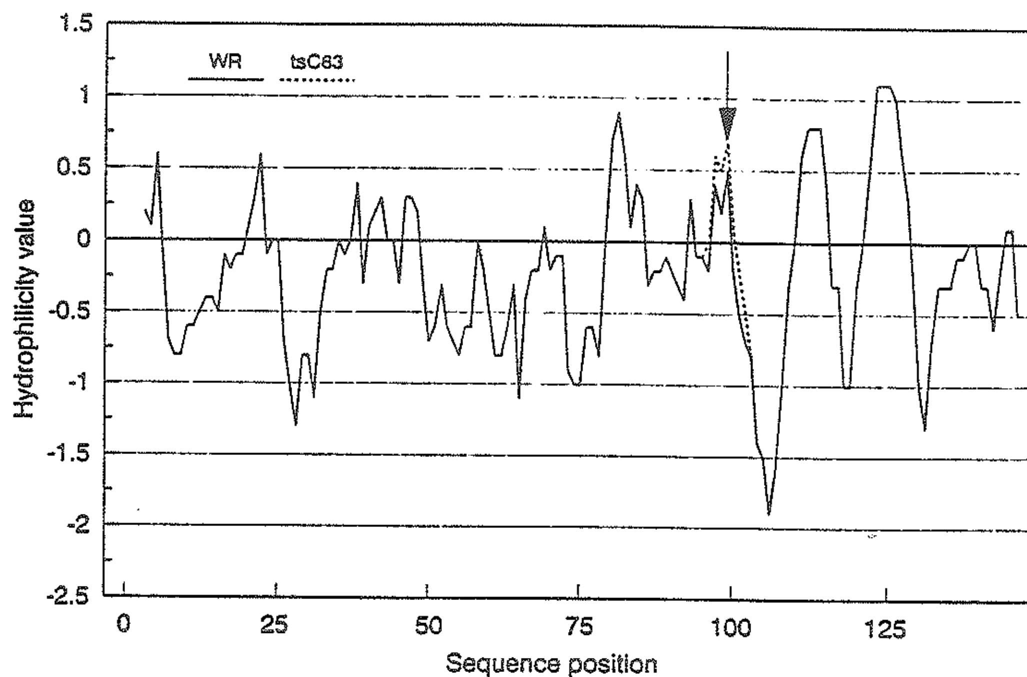


Figure 18. Hydrophilicity plot of the wild-type and mutant A1 gene product using the Hopp and Woods algorithm (Hopp and Woods, 1981). Positive values indicate regions with a tendency for hydrophilicity while negative values indicate regions of hydrophobicity. The arrow indicates the location of the m2 (ts) lesion in mutant tsC63. Dashed lines correspond to the predicted hydrophilicity change the mutant sequence would have on the A1 protein .

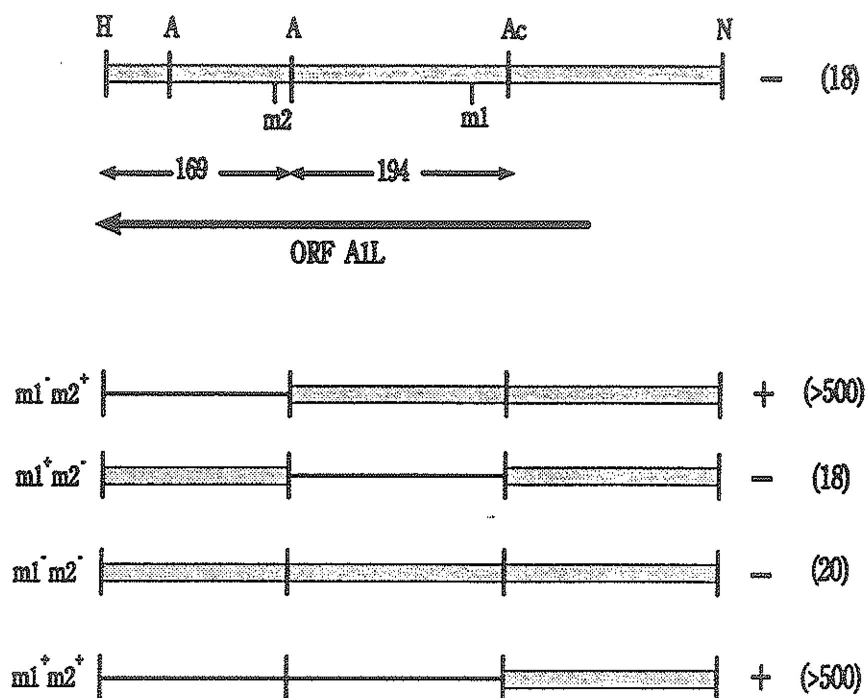


Figure 19. Marker rescue of tsC63 with plasmids containing single mutations in the A1 open reading frame. Parental plasmid pTS63-HN and the location and direction of transcription of open reading frame (ORF) A1 are indicated at the top of the figure. Relevant restriction sites are H, HindIII; A, AluI; Ac, AccI and N, NdeI. The mutant DNA of plasmid pTS63-HN (thick stippled bar) was replaced with either a 169-bp HindIII-AluI fragment, a 194-bp AluI-AccI fragment or both derived from WR DNA clone pC6-41-HN (thin solid bar). Marker rescue was performed with 10 µg of linearized plasmid DNA as described in Materials and Methods and results are indicated as positive or negative rescue with the number of plaques per 10 µg DNA indicated in brackets (*).

change from proline to glutamine at amino acid position 100, that is responsible for the ts phenotype of mutant tsC63.

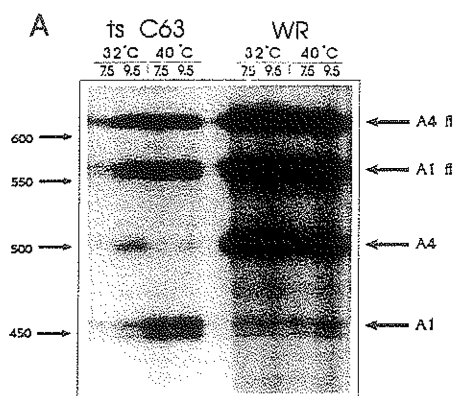
Mutant tsC63 produces distinct small plaques at the permissive temperature. To determine whether the m1 mutation had any effect on phenotype, we examined the small-plaque phenotype of virus that had been rescued with either $m1^-,m2^+$ or $m1^+,m2^+$. Both types of rescued virus produced plaques which were significantly larger at both 32°C and 40°C than those produced by mutant tsC63 (data not shown). We conclude that the m1 mutation is neutral, and that both ts and small-plaque phenotypes are caused by the m2 mutation. These data suggest that the 17-kDa protein containing the m2 lesion, while nonfunctional at the nonpermissive temperature, is only partially active at the permissive temperature.

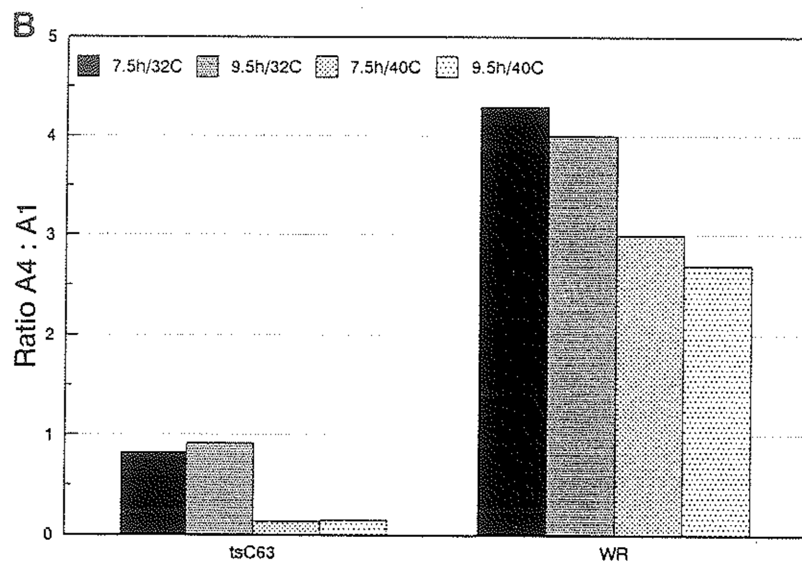
Transcription in tsC63-infected cells. Marker rescue and DNA sequencing experiments described in the previous sections localized the ts lesion in tsC63 to ORF A1. The A1 gene product has recently been shown to be involved in the trans-activation of a plasmid-borne late gene (Keck et al., 1990). Our *in vivo* analysis of protein synthesis in tsC63-infected cells is consistent with such a function, since it appears that only the early and intermediate proteins are synthesized at the nonpermisssive temperature. To determine whether the absence of late proteins was caused by a defect in transcription of late genes, we examined the *in vivo* expression of intermediate and late RNA in tsC63-infected cells. For this experiment we selected the A1 gene as the intermediate, and the A4 gene as a representative late probe (Weir and Moss, 1984; Weinrich and Hruby, 1986). We chose ORF A4 as a late representative for two reasons: i) it has been well-characterized and has been shown to

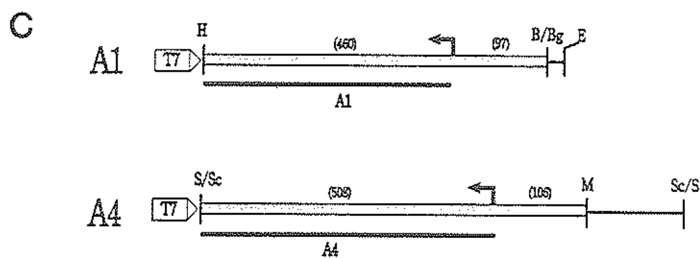
contain a typical late promoter motif TAAAT (Davison and Moss, 1989) from which transcription proceeds (Rosel and Moss, 1985); ii) it is known to encode the strongly expressed late major core polypeptide 4b (Wittek et al., 1984; Rosel and Moss, 1985) which is readily distinguishable on a polyacrylamide gel at late but not intermediate times (Fig. 15B, late arrow). From the protein analysis, we anticipated that the A1 intermediate mRNA would be expressed in an approximately normal fashion at either temperature, whereas the late A4 mRNA would be expressed only at the permissive temperature.

In order to test this hypothesis, we isolated total RNA from tsC63- and WR-infected cells at 7.5 h and 9.5 h postinfection and assayed for the presence of A1 and A4 transcripts by the S1 nuclease protection assay. In order to exclude the possibility of loading errors, differences in RNA samples or hybridization conditions, we assayed each RNA sample with an excess of both antisense probes concomitantly. Probe A1 is 557 nt in length and is expected to protect an RNA fragment of 460 nt following S1 nuclease treatment. Probe A4 is 614 nt in length and is expected to protect a fragment of 508 nt (Fig. 20C). The RNA protection experiment demonstrated that the intermediate (A1) transcript was present at both temperatures in both WR- and tsC63-infected cells (Fig. 20A). Because vaccinia intermediate and late transcripts lack defined 3' ends, the observed full length protection of probes in RNA protection experiments is the expected result of read-through from upstream genes (reviewed in Moss, 1990b). In agreement with our hypothesis, the expression of the late gene A4 in mutant infections was much reduced at the nonpermissive temperature even as late as 9.5 h postinfection. At the permissive temperature, accumulation of the A4 transcript in mutant infections was also lower than wild-type controls, though not reduced to the same extent as

Figure 20. Transcriptional analysis of tsC63 and WR virus at 32°C and 40°C. (A) BSC40 cells were infected at a multiplicity of 20 PFU/cell with either wild-type WR or mutant tsC63. Following 7.5 and 9.5 h of incubation, RNA was isolated. Ten μg of total RNA was hybridized to ^{32}P-labelled antisense RNA probes containing ORF A1 (intermediate) and ORF A4 (late) sequences. The RNA hybrids were then digested with S1 nuclease and run on a 5% polyacrylamide, 7 M urea gel, which was dried and exposed to X-ray film. A1 and A4 arrows indicate the specific protected transcripts initiated from the A1 and A4 promoters while A1fl and A4fl refer to the full length protected probes derived from upstream read-through transcription. Sizes in DNA nucleotides were determined by running an M13 phage sequencing ladder alongside. (B) densitometric scan of the autoradiograph in (A). Gels were scanned and the areas under the curve determined. The values A4 to A1 (A4/A1) ratios are shown. (C) restriction maps of DNA templates used to prepare antisense probes. Relevant restriction sites are H, HindIII; B, BamHI; Bg, BglII; E, EcoRI; S, SalI; Sc, ScaI; and M, MluI. DNA fragment sizes (between brackets) are indicated in base pairs. The T7 polymerase promoter is indicated as a large open arrow, and the vaccinia transcription initiation sites are shown as solid arrows.


A
ts C63
WR
32°C
40°C
7.5
9.5
600
550
500
450
A4 fl
A1 fl
A4
A1


B
7.5h/32C
9.5h/32C
7.5h/40C
9.5h/40C
Ratio A4 : A1
0
1
2
3
4
5
tsC63
WR


C
A1
T7
H
(460)
(97)
B/Bg
E
A1
A4
T7
S/Sc
(508)
(106)
M
Sc/S
A4

at the nonpermissive temperature. To obtain a quantitative measure of the defect in tsC63, we scanned each sample lane and determined the ratio of A4 and A1 transcripts at the permissive and nonpermissive temperatures (Fig. 20B). While there appears to be a small amount of expression of the late A4 gene in tsC63 at 40°C, the ratio of A4 to A1 is fully 6-fold lower than the comparable ratio at 32°C and is almost 20-fold lower than that seen in wild-type infections. We have repeated this experiment using an antisense probe to the adjacent ORF A3 late gene and have found similar results to those documented here. Specifically, following infection with tsC63, the A1 transcript accumulated to significant levels at both temperatures. In contrast, the A3 transcript was absent or present at very low levels at the nonpermissive temperature (data not shown). The observation that tsC63 produced reduced levels of the late mRNA even at the permissive temperature might explain the small-plaque phenotype observed with tsC63 infections at 32°C. Our data support the hypothesis that the defect in mutant tsC63 initially observed by the absence of late-appearing proteins at the nonpermissive temperature is caused by a defect in transcription of late but not intermediate genes.

Temporal appearance of transcripts in tsC63-infected cells. We next determined the temporal appearance of A1 and A4 mRNA in cells infected with either wild-type WR or mutant tsC63 (Fig. 21). At 40°C, both wild-type and mutant virus produced detectable quantities of the intermediate A1 transcript as early as 3 h postinfection. In tsC63-infected cells at 32°C, this transcript was produced by 4 h postinfection. The temporal appearance of the intermediate transcript A1 (Fig. 21) and intermediate proteins (Fig. 15) are in excellent

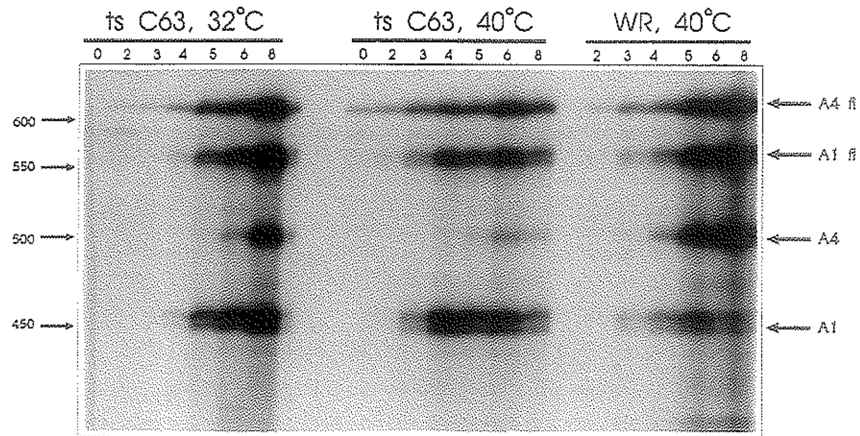


Figure 21. Time course analysis of A1 and A4 transcripts for tsC63 and WR. Total RNA was harvested at various times, indicated in hours (h) postinfection, at both 32°C and 40°C. Ten μg of total RNA was hybridized with ^{32}P-labelled antisense A1 and A4 transcripts and analyzed as described in Fig. 20.

agreement. Compared to the intermediate gene A1, the late gene A4 was expressed with a delay. Thus, during infection with wild-type WR at 40°C, the A4 transcript was first detected at 4 h postinfection. Similarly, infection with tsC63 at either 32°C or 40°C generated detectable amounts of the A4 transcript by 6 h and 5 h postinfection, respectively. In the case of WR at 40°C and tsC63 at 32°C, the amount of mRNA increased rapidly as a function of time postinfection. In contrast, when tsC63-infected cells were incubated at 40°C, the level of A4 mRNA remained very low at all times. We conclude that the pattern of intermediate and late proteins shown in Fig. 15 is an accurate reflection of the temporal appearance of intermediate (A1) and late (A4) mRNA transcripts. These data therefore provide strong evidence for a function of the intermediate A1 protein during the transcriptional activation of late genes.

Discussion

Expression of a large proportion of vaccinia virus genes is dependent on viral DNA synthesis. One subclass of these postreplicative genes can overcome this DNA synthesis dependence when a plasmid-borne gene is transfected into virus-infected cells in which DNA synthesis is blocked. This observation has inspired a cascade model of gene expression in which some early gene products activate transcription of intermediate genes, intermediate proteins activate late gene expression, and several late proteins activate transcription of early genes during the subsequent infection cycle (Vos and Stunnenberg, 1988). This transfection assay was subsequently used to identify three intermediate genes, A1, A2, and GK1, whose gene products are required for trans-activation of a plasmid-borne late reporter gene (Keck

et al., 1990). We have now shown that mutant tsC63 contains a ts lesion in ORF A1 which, at the nonpermissive temperature, causes a defect in the synthesis of the temporal class of late but not intermediate proteins. Transcriptional analysis of one intermediate gene (A1) and two late genes (A3 and A4) demonstrated that the defect occurred at the RNA level. These data provide strong evidence that this 17-kDa intermediate A1 protein is not only required for expression of plasmid-borne late genes under conditions where DNA synthesis is blocked, but it also stimulates gene expression of late genes from the viral genome under normal *in vivo* infection conditions. Available evidence is compatible with the notion that the A1 protein serves as a transcription initiation factor. However, we can not as yet rule out the possibility that this protein might be needed during elongation or stabilization of RNA in a late gene specific manner. In either case, the conditional lethality of mutant tsC63 implies that the A1 protein performs an essential function during the viral replication cycle.

When comparing the efficiency of transcription and protein synthesis in wild-type WR and mutant tsC63, we observed wild-type levels of intermediate gene expression in tsC63-infected cells at both the permissive and nonpermissive temperature. In contrast, synthesis of late RNA and late proteins was defective at both temperatures, although the defect was much more extreme at the nonpermissive temperature. In this respect it is of interest that tsC63 has a small-plaque phenotype at the permissive temperature. Our finding that this plaque phenotype is corrected by rescue of the ts phenotype indicates that the single mutation in ORF A1L, which we have shown to be the cause of the observed defect in the expression of the late class of genes, results in lethality at the nonpermissive temperature and a small-plaque phenotype at the permissive temperature. These data also suggest that a productive infection

may require a certain threshold level of late gene expression. Temperature-sensitivity might at least in part be explained if the value of this threshold level were to vary at different temperatures.

The observation that mutant tsC63 is defective in late but not intermediate gene expression should make it a useful tool in the study of several aspects of the vaccinia virus replication cycle. It offers a means to study pre- and postreplicative control of gene expression in an *in vivo* setting, without the need to block DNA synthesis. For example, it is known that early protein synthesis, which is normally suppressed after the onset of viral DNA replication, continues for an extended time if either DNA replication or postreplicative gene expression is inhibited at a gross level (Condit et al., 1983). Our finding that early protein synthesis is suppressed under conditions where viral DNA synthesis and intermediate gene expression are unaffected but late gene expression is inhibited, suggests that, in addition to a role in the trans-activation of late genes, intermediate gene expression may mediate the suppression of early gene expression. Similarly, using tsC63 and specific antibodies, it should be possible to determine whether the production of intermediate proteins is suppressed by late gene expression. With regards to the switch from early to intermediate gene expression it may be significant that the mRNA capping enzyme, which is required during transcriptional termination of early genes, functions as an intermediate transcription factor. Sequestering of the mRNA capping enzyme or another element of the transcription complex might be sufficient to account for the observed switch in gene expression. Alternatively, and possibility in combination with transcriptional suppression, one or more intermediate protein(s) might stimulate mRNA degradation.

In addition to providing a means of studying switching mechanisms during viral gene expression, tsC63 should provide a rapid and definitive means of assigning genes as intermediate or late, thereby facilitating definition of control elements used during intermediate and late gene expression. Our observation that both the A4 gene transcript and its gene product, the major virion core polypeptide 4b, are late rather than intermediate suggests that late gene expression is controlled at the transcriptional rather than the translational level. Furthermore, definition of cis-acting sequences used by intermediate and late genes, and the derivation of consensus sequences should be greatly facilitated by this mutant. From the limited number of genes assigned on the basis of the transfection assay, and using linker scanner mutagenesis, Stunnenberg and co-workers have identified a short promoter element containing two regions that appear to be required in cis for transcription of an intermediate gene (Hirschmann et al., 1990). One essential region required for expression of gene I3 was found at -20 to -9, the other at +1 to +9 relative to the mRNA start site. Both are highly A-rich, with the second region having the sequence TCAAAT which is similar to the prototypal late promoter sequence TAAAT (Rosel and Moss, 1985; Hanggi et al., 1986; Davison and Moss, 1989). Keck et al. (1990) noted that four of the five currently described intermediate genes have a sequence TNAAAT (where N may be A, T or C) with the exception being I8R which has the sequence TAGAAT. There are several examples of postreplicative promoters having the TNAAAT motif including N1L (Kotwal et al., 1989) I1L, H5L, D13L, A1L, A2L, and A3L (Davison and Moss, 1989). However, the linker scanning experiments of Hirschmann et al. (1990) suggest that this is not sufficient to dictate an intermediate promoter. Our finding that ORF A3, which contains the TNAAAT motif near

the RNA start site, is a late gene supports this view (this study).

We have recently identified a subclass of ts mutants that is defective in telomere resolution (res⁻). It was found that most of these res⁻ mutants are simultaneously defective in postreplicative gene expression (DeLange, 1989; Merchlinsky and Moss, 1989a), suggesting that either intermediate or late gene expression, or both, are required for efficient telomere resolution. Our observation that only late gene expression is severely defective in the res⁻ mutant tsC63 indicates that the late postreplicative class of gene expression is essential for resolution. These data do not as yet allow us to distinguish whether a late protein is required or if telomere resolution involves some essential function of the transcription apparatus at the telomere, such as the synthesis of specific telomeric noncoding transcripts (Parsons and Pickup, 1990; Hu and Pickup, 1991). At least one of the telomeric transcripts initiates at a conserved sequence required for telomere resolution which is known as the telomere resolution target (reviewed in DeLange and McFadden, 1990) and which has been shown to function as a late promoter (Stuart et al., 1991). These transcripts, which appear after DNA replication has initiated, have as yet not been ascribed a function. It has, however, been postulated that they may play a role in telomere resolution, perhaps by eliciting a conformational change essential for this process (Parsons and Pickup, 1990). We are currently addressing the function and temporal appearance of these telomeric transcripts by examining tsC63 and other res⁻ mutants.

CHAPTER IV: ALTERED VACCINIA VIRUS mRNA CAPPING ENZYME IN A TELOMERE RESOLUTION DEFECTIVE MUTANT

Abstract

Vaccinia virus conditional lethal temperature-sensitive mutant ts9383 is currently the only known telomere resolution defective mutant which does not exhibit a defect in late gene expression. The protein (D12) responsible for this defect, the small subunit of the virus-encoded heterodimeric mRNA capping enzyme, is stably produced in mutant infections at both permissive and nonpermissive temperatures. Intriguingly, at the nonpermissive temperature, the ability of this protein to form a physical association with the large subunit of the capping enzyme is drastically reduced as shown by antibody co-immunoprecipitation experiments. Association of the two subunits is believed to be important for 5' mRNA cap methyltransferase activity. However, RNA species produced in mutant infections were comparable in quantity, temporal appearance and size to that seen in wild-type infections although, there was a slight difference in the migration pattern of one discrete sized late telomeric RNA. This difference in migration is not temperature-sensitive. Collectively, the data suggest that while this physically altered D12 protein can be tolerated with regard to activities normally associated with the viral mRNA capping enzyme, the physical alteration is deleterious for telomere resolution.

Introduction

Vaccinia virus, a structurally and developmentally complex virus of vertebrate hosts, replicates its genome in the cytoplasm of infected host cells. Because of its high level of autonomy from host nucleus activities, the virus is believed to encode essentially all of the factors required for the transcription and replication of its genome (Moss, 1990a).

The virus replication cycle can be summarized as follows: upon entry into the host cell cytoplasm and an initial uncoating phase, early genes are expressed inside the virus core by the actions of a number of viral factors including a multisubunit RNA polymerase, mRNA capping enzyme, poly(A) polymerase and VETF, an early gene specific transcription factor (Moss, 1990b). A second uncoating step releases the viral genome which is replicated by a virus-encoded DNA polymerase. The onset of viral DNA replication results in the expression of intermediate genes, some of whose products are responsible for the activation of a late class of genes. Factors essential for virus propagation, including the transcriptional machinery, are packaged into progeny virions for the next infection cycle. In this manner, virus gene expression can be viewed as a cascade pathway in which the previous temporal class is responsible for activation of a subsequent temporal class (Vos and Stunnenberg, 1988; Keck et al., 1990).

DNA replication plays an important role in the viral life cycle since blocking DNA synthesis also blocks the expression of intermediate genes (Keck et al., 1990). The vaccinia 185-kb linear, double-stranded DNA genome is bounded by hairpin telomeres which are incompletely base-paired and exist in two inverted but complementary configurations (Geshelin and Berns, 1974; Baroudy et al., 1982). Replication is believed to initiate with the

introduction of a nick at or near the hairpin terminus. This nicked hairpin end could serve as a primer for DNA replication and synthesis would proceed through the opposite hairpin. Continued rounds of synthesis can generate large concatemeric arrays of genomes linked by the telomere fusion (reviewed in Traktman, 1990).

Regeneration of monomeric genomes with hairpin termini from these concatemers can be viewed as a site-specific recombination event and is termed telomere resolution (for review see DeLange and McFadden, 1990). The cis-acting elements required for resolution have been well characterized through the use of cloned plasmid copies of the telomere fusion junction which, when transfected into poxvirus-infected cells, are converted to linear minichromosomes with hairpin termini (DeLange et al., 1986; Merchlinsky and Moss, 1986). The telomere resolution target (TRT) sequence represents the cis-element required for this event. The minimal core sequence is a highly conserved, 20 base-pair A-T rich sequence present near the hairpin termini. For resolution in the plasmid assay, this sequence must be present as two copies in an inverted repeat orientation at the telomere fusion (DeLange and McFadden, 1987; Merchlinsky and Moss, 1989a). The physical distance between the two TRT regions and the degree of sequence similarity between each TRT is also important for efficient resolution (McFadden et al., 1988; Merchlinsky and Moss, 1989a). Furthermore, the TRT sequence bears strong similarity to typical poxvirus late promoter sequences (Davison and Moss, 1989b). In fact, this sequence, when placed upstream of a CAT (chloramphenicol-acetyl transferase) reporter gene and then transfected into virus-infected cells, directs efficient gene expression late in an infection (Stuart et al., 1991).

Both biochemical and molecular genetic approaches have been utilized in an attempt

to characterize the trans-acting elements involved in telomere resolution but have enjoyed only limited success. A 50-kDa nuclease present in the infecting virions is believed to play a role in the resolution event, however the gene encoding the nuclease has not been identified. For this reason, and because of a lack of antibodies to the 50-kDa protein, it has been difficult to determine if this enzyme has an actual role in telomere resolution in an *in vivo* setting (Merchlinsky et al., 1988; Reddy and Bauer, 1989). Genetic analysis has been hampered by the fact that virtually all conditionally-lethal, resolution defective mutants (res⁻ are also defective in either intermediate and late, or late gene expression. This has suggested a requirement for some late gene product(s) in the resolution event (DeLange, 1989; Merchlinsky and Moss, 1989b; Carpenter and Delange, 1992; Zhang et al., 1992). Alternatively, or possibly in addition, some aspect of late gene expression aside from a late protein may be directly involved in telomere resolution. Such an hypothesis could account for the presence of non-coding transcripts in this region of the viral genome (Parsons and Pickup, 1990).

Recently, we identified a temperature-sensitive (ts) mutant, ts9383, which is unique from all other res⁻ mutants in that neither intermediate nor late gene expression appeared significantly affected during infection under nonpermissive conditions (Carpenter and DeLange, 1991). The lesion was mapped to the small subunit of the viral heterodimeric mRNA capping enzyme. Since general late protein synthesis appeared normal, we hypothesized that the entire capping enzyme or the small subunit itself plays a more direct role in telomere resolution.

The 127-kDa vaccinia virus mRNA capping enzyme is composed of two polypeptides:

a large, 95-kDa subunit and a small, 31-kDa subunit (Martin et al., 1975). The genes encoding these proteins, ORFs D1 and D12, respectively, have been shown to contain typical virus early promoters and indeed, transcription of these genes has been shown to occur at early times during the virus life cycle (Lee-Chen et al., 1988). Three activities required for 5' mRNA cap formation are performed by this enzyme: RNA triphosphatase, RNA guanylyltransferase and RNA (guanine-7)-methyltransferase. Along with its role in 5' end processing, the capping enzyme also acts as a transcription termination factor for virus early transcripts (Shuman et al., 1987), and as a transcription initiation factor for the expression of intermediate genes (Vos et al., 1991).

A specific structure/function analysis of the mRNA capping enzyme has only been undertaken with respect to the capping reaction. The 60-kDa amino-terminal region of the large subunit contains the triphosphatase and guanylyltransferase functions (Guo and Moss, 1990; Shuman, 1990a; Shuman and Morham, 1990; Higman et al., 1992). The 35-kDa carboxyl terminal end of the D1 protein forms a physical association with the D12 subunit and this association appears to be essential for methyltransferase activity (Cong and Shuman, 1992; Higman et al., 1992). No function has been ascribed to the purified D12 polypeptide in the absence of any D1 protein.

In this paper, we have continued our characterization of the telomere resolution defective mutant ts9383. Our previous observations demonstrated that in this mutant, late proteins are produced even during nonpermissive condition infections. Consistent with this, we now show that both the quantity and temporal appearance of early and late mRNAs appear unaffected at the nonpermissive temperature by the presence of the ts9383 mutation.

Pulse-chase analysis established that both the small and the large subunits of the mRNA capping enzyme were essentially stable at both permissive and nonpermissive temperatures. However, our data suggest an aberrant physical conformation of the D12 protein which interferes with its association with the D1 subunit at the nonpermissive temperature. Specifically, co-immunoprecipitation of the small D12 subunit with antisera directed against the large D1 subunit was seen in ts9383 lysates from permissive-, but not from nonpermissive-temperature infections. This co-immunoprecipitation difference was not seen in comparable wild-type infection lysates. This observation may provide an explanation for the ts defect in telomere resolution seen in this mutant virus.

Materials and Methods

Materials: [^{35}S]-methionine and [^{32}P] radiolabelled-nucleotides were obtained from New England Nuclear. [^{14}C] protein molecular weight markers were from Amersham. Prestained protein molecular weight markers, goat anti-rabbit antibody and polyvinylidene difluoride (PVDF) membrane were from BioRad. Immunoblot visualisation components were from Sigma Chemical Co. Protein-A sepharose CL4-B beads were from Pharmacia. Polyclonal antisera to vaccinia virus protein p4b was a gift from Dr. D. Hruby, Oregon State, and polyclonal antiseras to D1 and D12 were from Dr. E. Niles, SUNY, Buffalo.

Cells and Virus: For all experiments, a continuous line of African Green Monkey kidney (BSC40) cells were grown as a monolayer culture in Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% fetal calf serum. Wild-type virus strain IHD-W and

mutant ts9383 were provided by Dr. G. McFadden and Dr. S. Dales, respectively. Wild-type strain WR and mutant tsC63 were provided by Dr. R. Condit. The permissive temperature was 32°C, the nonpermissive temperature 40°C.

DNA Clones: Plasmid pJB319 contains an approximately 5.5-kb fragment of vaccinia strain WR DNA. The plasmid construct includes the 3'-end of ORF D1, the entire coding sequence of ORFs D2, D3 and D4, and the 5'-end of ORF D5 inserted into vector pTZ18R (Millns et al., 1994).

***Protein Labelling*:** Pulse-labelling of proteins from virus-infected cells and polyacrylamide gel electrophoresis was performed essentially as described previously (Condit and Motyczka, 1981; Carpenter and Delange, 1992). Confluent monolayers of BSC40 cells were infected by adsorption of virus at a multiplicity of 10-20 pfu/cell for 30 min. Following this incubation, monolayers were rinsed and fresh DMEM added. At specific times post-infection, medium was removed, cells were rinsed with prewarmed PBS then pulse-labelled for 30 min in prewarmed DMEM (minus methionine) supplemented with 100 μCi/ml [^{35}S]-methionine. Cells were harvested immediately or, for pulse-chase analysis, were washed 2 x with PBS before adding fresh DMEM and returning the cells to the incubator. Lysis was achieved using either SDS buffer (12.5 mM Tris-HCl, pH 7.5, 2% SDS, 100 mM β-mercaptoethanol) or modified MAXI buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, 0.2% sodium deoxycholate, 0.5% NP-40, 1% bovine hemoglobin, 50 mM β-mercaptoethanol, 1 mM PMSF [phenylmethyl-sulfonylfluoride])(Niles et al., 1989).

Samples were frozen overnight at -80°C to assist in lysis. SDS samples were boiled for 4 min and then clarified by a 5 min microcentrifugation step at room temperature. MAXI buffer lysates were spun for 15 min at 4°C to remove insoluble material. All samples were stored at -80°C.

Immunoprecipitations: 25-60 μl of lysate was brought to a total volume of 200 μl with dilution buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl; immunoprecipitations performed using SDS-lysed samples were further supplemented with 0.4% (w/v) bovine serum albumin). Antiserum (3 - 7.5 μl) was added and samples incubated at 4°C overnight with gentle end-over-end rocking. Ten μg of protein A-Sepharose prepared in dilution buffer was added to each sample and rocking continued for 90 min followed by a 10 sec microcentrifugation to pellet the complexes. Pellets were washed with 1 ml each of dilution buffer + 0.1% Triton X-100 (2x), dilution buffer (1x) and finally 50 mM Tris-HCl, pH 6.8 (1x) essentially as described in Ausubel et al. (1987). The resulting pellet was diluted in SDS buffer and boiled prior to gel electrophoresis. Fluorography was performed using the commercial kit, Enhance (New England Nuclear Corp.). Protein quantitations were performed by the Bradford method as described in the Biorad literature.

Immunoblotting and Detection: Proteins were transferred from SDS-polyacrylamide gels to PVDF membrane in an LKB/Pharmacia tank electroblotter (LKB 2005) for 1 h at 0.7 - 1 amp, 4°C as described in the manufacturer's literature. The transfer buffer was 25 mM Tris-HCl, pH 8.3, 150 mM glycine, 20% (v/v) methanol. Following transfer, the membrane was

immediately blocked in TTBS (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20) plus 5% skim milk powder and incubated with appropriate antiserum overnight. D12 and p4b antisera were used at a 1/5000 dilution, and secondary goat antirabbit antibody (BioRad) was used at a 1/3000 dilution. Alkaline phosphatase-linked secondary antibody detection was performed as described in Ausubel et al. (1987).

***Pulsed-Field Gel Electrophoresis (PFGE)*:** PFGE, southern blotting, PVT-1 probe synthesis and hybridization was performed as described previously (DeLange, 1989; Carpenter and DeLange, 1991).

RNA Methods: RNA was isolated by the guanidinium-isothiocyanate CsCl method as described in Ausubel et al. (1987). Ten μg of total RNA was electrophoresed in a 2.2 M formaldehyde, 1.2% agarose gel as per Sambrook et al. (1989) prior to transfer to Zetaprobe membrane according to the manufacturer's instructions (BioRad). Synthesis of ^{32}P-labelled probes was by the random hexanucleotide primer method (Feinberg and Vogelstein, 1983). Hybridization and washing conditions were as described in the BioRad literature. Autoradiography was performed using Kodak X-OMAT AR film and intensifying screens.

Results

We previously demonstrated that both temperature sensitivity and the telomere resolution defect in mutant ts9383 were due to a proline to serine alteration at amino acid 23 in the small subunit of the viral mRNA capping enzyme (D12 protein). Given the

multifunctional nature of the capping enzyme, with regards to RNA metabolism and processing, it was perhaps surprising to find no significant defect in the expression of viral genes. That synthesis of early, intermediate and late proteins is unabated in 40°C infections suggests that: i) the ts9383 mRNA synthesized was competent for translation, and ii) the intermediate gene transcription factor activity of the capping enzyme was not significantly impaired (Carpenter and DeLange, 1991). We therefore hypothesized that the D12 protein either on its own, or in combination with the D1 protein in an active capping enzyme complex, played a more direct role in the telomere resolution process.

Transcription in mutant ts9383. Our initial analysis examining gene expression in ts9383 involved pulse-labelling of proteins in infected cells. Even though we took precautions to maintain temperatures at 40°C at all times, this type of analysis is invasive and does not completely exclude the possibility that a temperature drop during labelling allowed slip-through. Therefore, we have re-examined the phenotype at the transcriptional level. Since RNA is isolated by the immediate lysis of cells (without any labelling step), there is no opportunity for a phenotypic slip-through. The transcripts obtained should accurately reflect the state of the infection at the time of lysis. Our principal goal was to determine whether the steady-state level of RNA was affected at the nonpermissive temperature. In addition, since the viral capping enzyme has a role in transcription termination of early genes, it was of interest to determine whether the discrete early transcript length was affected. We also wished to determine if RNA species showed any gross degradation as this might reflect a physical alteration or lack of a cap structure at the 5' termini of these transcripts.

In order to study these possibilities, BSC40 cells were infected with either IHD-W or ts9383 at 15 pfu/cell and infections were allowed to proceed at the nonpermissive temperature of 40°C. Total RNA was isolated at 2, 4 and 6 h post-infection, electrophoresed in a formaldehyde-agarose gel, transferred to nylon membrane and probed with labelled plasmid pJB319 which detects transcripts D1-D5. Of these, only the D1, D4 and D5 transcripts are detectable as early, discretely-terminated bands by Northern analysis. The D1 transcript has a size of 2.75-kb and the D4 and D5 transcripts, which share a common transcription termination site, are 3.6 and 2.9-kb in size respectively (Evans and Traktman, 1987; Roseman and Hruby, 1987; Lee-Chen et al., 1988). The D3 transcript is produced at later times in virus infections and appears as a smear by Northern analysis while the D2 transcript which is produced at both early and late times is not generally detectable by Northern analysis (Lee-Chen and Niles, 1988).

When the Northern blot was probed with this clone, unique-sized transcripts of 2.7, 2.9 and 3.6-kb were detected in both IHD-W and ts9383 derived samples at each time point (Fig. 22). The 3.6-kb transcript is only expressed to a low level in either the IHD-W or ts9383 samples. However, it is detectable in 2 h samples with over-exposures of the autoradiograph shown. By 6 h p.i., lanes also displayed a characteristic smearing. This is due to the fact that poxvirus intermediate and late mRNAs are generally not discretely terminated and as a result read through early coding regions (Vos and Stunnenberg, 1988; Moss, 1990b). The probe did not hybridize to mock-infected samples (not shown). That the telomere resolution defective phenotype was expressed in this experiment was determined by isolating an infected cell sample for PFGE, transferring the separated products to nylon, then probing the blots with

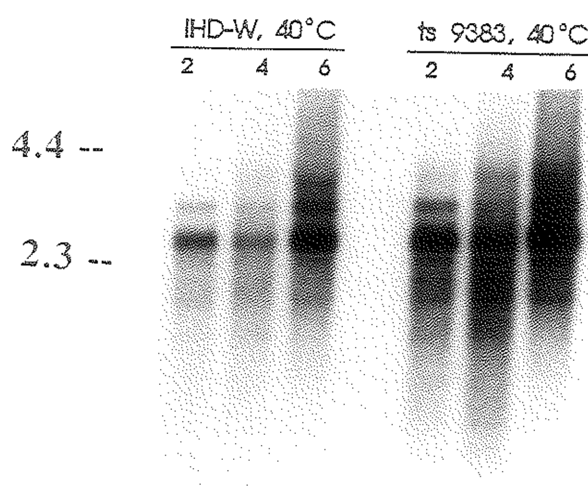


Figure 22. Northern analysis of IHD-W and ts9383 early transcripts. BSC40 monolayers were infected at a multiplicity of 15 pfu/cell with strains IHD-W or ts9383 and incubated at 40°C. RNA was isolated by the guanidinium lysis/CsCl centrifugation method at 2, 4 and 6 h p.i. Ten µg of total RNA was electrophoresed in agarose-formaldehyde gels, transferred to nylon membranes and probed with radiolabelled clone pJB319 which includes parts of the vaccinia D1 and D5 genes and all of the vaccinia D2, D3 and D4 genes. Migration positions of bacteriophage lambda DNA digested with Hind III are indicated as size markers.

a near-telomeric probe pVT-1 as described previously (Carpenter and DeLange, 1991). This control was performed each time RNA or protein experiments were performed with ts9383 and in every case, the res$^-$ phenotype was apparent at the nonpermissive temperature.

These results suggest two things: i) early mRNA is discretely terminated at both permissive and nonpermissive temperatures, and ii) representative early (D1, D4, D5) and postreplicative (indicated by the smearing in the 6 h p.i. lanes) transcripts produced in ts9383 at the nonpermissive temperature do not appear to be significantly degraded in comparison to wild-type mRNA species. As a consequence, the data suggest that two functions specifically associated with the viral mRNA capping enzyme, early gene transcription termination and the intermediate transcription transactivation activity, are present in the mutant infections under nonpermissive temperature conditions.

Telomeric transcripts. The critical nature of the telomere in DNA replication and telomere resolution is certain. However, the function the capping enzyme plays is unclear; what role does an RNA initiating/processing enzyme play in what is essentially a site-specific recombination event? Two recent observations have suggested a potential link between transcription and telomere resolution: (i) the TRT sequence can act as a late promoter (Stuart et al., 1991); and ii) late non-coding telomeric transcripts are present at the telomeres (Parsons and Pickup, 1990), and in fact at least one transcript has been shown to originate from the TRT (Hu and Pickup, 1991). Interestingly, unlike the vast majority of late transcripts, these transcripts appear to contain discrete 3' termini, like early mRNAs. It is not known whether these discrete transcripts are produced by an endoribonuclease cleavage as

is seen for other late discretely-terminated transcripts (Antczak et al., 1992).

We were interested to know if ts9383 was defective in the synthesis of these recently described telomeric transcripts. Before looking at ts9383, we wished to determine whether these transcripts belonged to the intermediate or late gene class. We therefore isolated RNA from mutant tsC63 and wild-type WR strains. Mutant tsC63 is defective in the production of late but not intermediate transcripts at the nonpermissive temperature and can therefore be used as a tool to differentiate between intermediate and late transcripts. A mutant transcription factor is responsible for the ts phenotype (Keck et al., 1990; Carpenter and Delange, 1992).

Total RNA was isolated at 2.5, 5, 7.5 and 10 h p.i. at both 32°C and 40°C. Northern blots were prepared and probed with radiolabelled plasmid pVD12. This construct contains approximately 200-bp of the cloned vaccinia virus telomere fusion (DeLange et al., 1986). The probe detected a single weak-intensity band of approximately 1.7-kb by 5 h p.i. at 32°C and a strong signal by 5 h p.i. at 40°C in WR infected samples, confirming that transcript belongs to the postreplicative class (Fig. 23A). In tsC63 infections, while the transcript was clearly present by 7.5 h at 32°C, only a low amount of transcript appeared in the 40°C infection, even by 10 h p.i. (Fig. 23A). This may be due to residual activity of the mutant A1 protein at the nonpermissive temperature, or to a basal level of transcription independent of the A1 protein. The data indicate that this telomeric transcript belongs to the late class. Interestingly, while Parsons and Pickup (1989) detected two transcripts of 1.8 and 3.5 kb, we detected only a single transcript of approximately 1.7 kb. The reason for the discrepancy regarding this larger transcript is unclear at this time (see Discussion, this chapter).

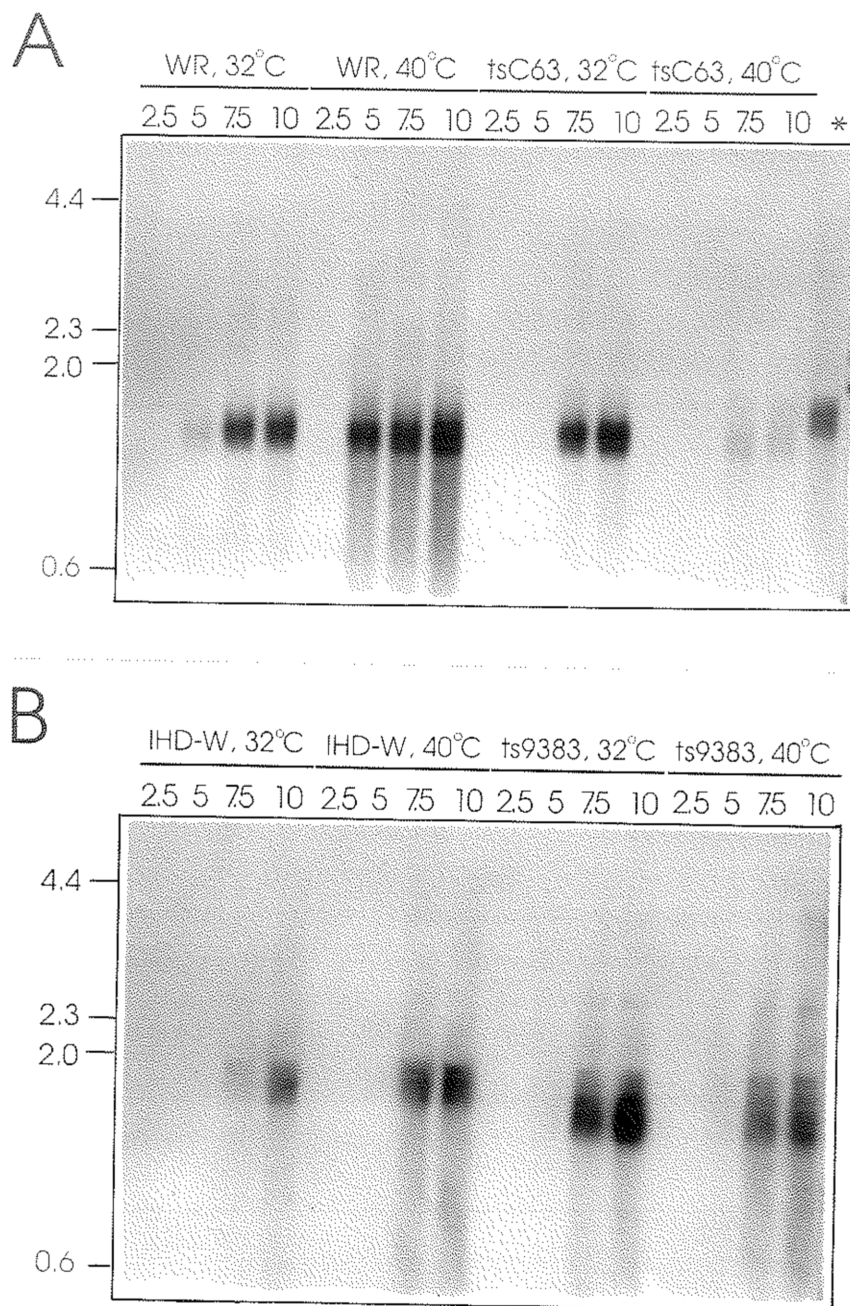


Figure 23. Northern analysis of postreplicative telomeric transcripts. BSC40 monolayers were infected at a multiplicity of 15 pfu/cell with strains WR or tsC63 (A) or IHD-W or ts9383 (B) and incubated at 32°C or 40°C for 2.5, 5, 7.5 and 10 h p.i. after which RNA was isolated by the guanidinium lysis/CsCl centrifugation method. Ten μg of total RNA was electrophoresed in agarose-formaldehyde gels, transferred to nylon membranes and probed with radiolabelled clone pVD12 whose insert maps to the vaccinia hairpin terminus. Migration positions of bacteriophage lambda DNA digested with Hind III are indicated as size markers. The * in (A) indicates a sample of IHD-W derived RNA.

In order to determine if there were any abnormalities in this late telomeric transcript in ts9383, RNA was isolated as described above but from IHD-W- and ts9383-infected cells. The RNA was subjected to electrophoresis, transferred to nylon membrane and the blot probed with radiolabelled plasmid pVD12. This probe detected a single unique transcript of approximately 1.8 kb by 7.5 h p.i. in wild-type infections at both 32°C and 40°C infections. ts9383-infected samples also displayed a single band but it was slightly smaller in size (approximately 1.7 kb) and may be more smeared-out (Fig. 23B). We have run IHD-W samples alongside tsC63 and WR samples (Fig. 23A, lane *) and have determined that the telomeric transcripts detected for WR and tsC63 and ts9383 (the major band detected), are all essentially the same size and that the IHD-W transcripts are slightly larger. This may reflect an increase in the size of the telomeric region due to recombination at the tandem repeats. Alternatively, RNA in ts9383-infected cells may indeed be smaller than that of IHD-W wild-type. If this telomeric transcript is defective, the defect is not expressed in a temperature-sensitive fashion and therefore is not likely the cause of the ts defect in this mutant. We can still not rule out the possibility that this anomaly might be a contributing factor.

We have also probed this blot with radiolabelled plasmid pVT-1 which includes sequences from the inverted repeat region of the virus telomere (Carpenter and DeLange, 1991) and find that the probe detects heterogeneous smears in all late virus samples irrespective of temperature. This observation along with the data in Fig. 22 offers further evidence that late gene transcription is not fundamentally affected in ts9383 infections at the nonpermissive temperature.

Stability of the D12 protein during ts9383 infections. We wished to determine if the molecular lesion in the small subunit of the capping enzyme resulted in any physical instability of the synthesized protein *in vivo*. Preliminary experiments with both IHD-W and ts9383 suggested that D12 peak synthesis occurred at approximately 2-3 h p.i. at 40°C with 32°C infections lagging behind only by approximately 30 minutes (not shown). Therefore, for pulse-chase studies, we used 2.5 h p.i. as a labelling point for 40°C infections and 3 h p.i. for 32°C infections.

BSC40 cells infected with wild-type IHD-W or mutant ts9383 at either 32°C or 40°C were pulse-labelled for 30 min then harvested immediately, or returned to the appropriate incubators. At 0.5, 1, 2.5 and 4 h post-pulse, samples were lysed in SDS buffer and the polypeptides separated by electrophoresis in an SDS-polyacrylamide gel. As Fig. 24 A demonstrates, there was little loss of any of the prevalent viral polypeptides over this period suggesting that general stability of early proteins in mutant-infected cells was not significantly altered in comparison to IHD-W infections. The D1 and D12 proteins were then isolated from these extracts by immunoprecipitation using two antisera; one prepared against D1, the other prepared against D12 antigens (Niles et al., 1989; Higman et al., 1992). The resulting polypeptides were fractionated on an SDS-12.5% polyacrylamide gel and detected by fluorography (Fig. 24 B). In support of previous observations (Niles et al., 1989; Christen et al., 1992), we find that newly synthesized D12 and D1 polypeptide from wild-type infections are stable over the chase period. The D1 and D12 proteins produced in ts9383 infections were similarly stable (Fig. 24 B). There is a slight drop seen from the ts9383-40°C pulse sample (P) to the 1/2 h sample, however this drop occurs in both D1 and D12 proteins and we believe

Figure 24. Pulse-chase analysis of IHD-W and ts9383 D12 and D1 polypeptides. Confluent monolayers of BSC40 cells were infected at an m.o.i. of 15 pfu/cell and incubated at either 32°C or 40°C. At 2.5 h (40°C) or 3 h (32°C) p.i., infected cells were labelled for 30 min with [^{35}S] methionine then harvested immediately (P) or chased with unlabelled DMEM for up to 4 h prior to harvest. Cells were lysed with the addition of a buffer containing 2% SDS. (A) Total proteins separated on an SDS-12.5% polyacrylamide gel. (B) Stability of the D12 and D1 proteins as determined by immunoprecipitation of samples from above with D12 and D1 antisera. Immunoprecipitated proteins were resolved by electrophoresis by SDS-PAGE and visualized by fluorography. M, mock-infected cells; PI, IHD-W infected pulse lysates immunoprecipitated with D1 and D12 antisera. The positions of [^{14}C] molecular weight markers are indicated as are the expected migration positions of the D1 and D12 polypeptides.

it reflects an overloaded sample. Furthermore, repeats of the experiment support this interpretation. That quantitative precipitation was achieved for all of these samples was demonstrated by an inability to further immunoprecipitate antigen from the above samples (not shown). Therefore the single amino acid alteration present in the ts9383 D12 protein does not lead to an instability in newly synthesized D12 polypeptides.

Antibody co-immunoprecipitation of capping enzyme subunits. We were next interested in determining whether the known interaction between the D1 and the D12 subunits was affected in ts9383 infections. We attempted to resolve this question by using antibody co-immunopreciptiation as it has been previously shown that antibodies directed against the D1 antigen can co-immunoprecipitate the D12 subunit (Niles et al., 1989; Cong and Shuman, 1992).

A number of different antisera have been prepared against the D1 antigen (Higman et al., 1992; Niles, unpublished). These include antisera D1-N generated against the 27-kDa amino-terminal fragment, D1-M generated against a middle 52-kDa fragment (amino acids 147-621) and D1-C generated against a 25-kDa carboxy terminal fragment (amino acids 620-845). We wished to determine which of these antisera would be appropriate for co-immunoprecipitation of the D12 subunit. To test these antisera, we pulse-labelled IHD-W infected cells at 2.5 h p.i. and harvested the cell monolayers in nondenaturing MAXI buffer. The infections were maintained at 37°C for this trial experiment. Next, different antisera were mixed with the IHD-W lysate and immunoprecipitations performed (Fig. 25).

Initially, we used both D1 and D12 antisera together in a single-sample

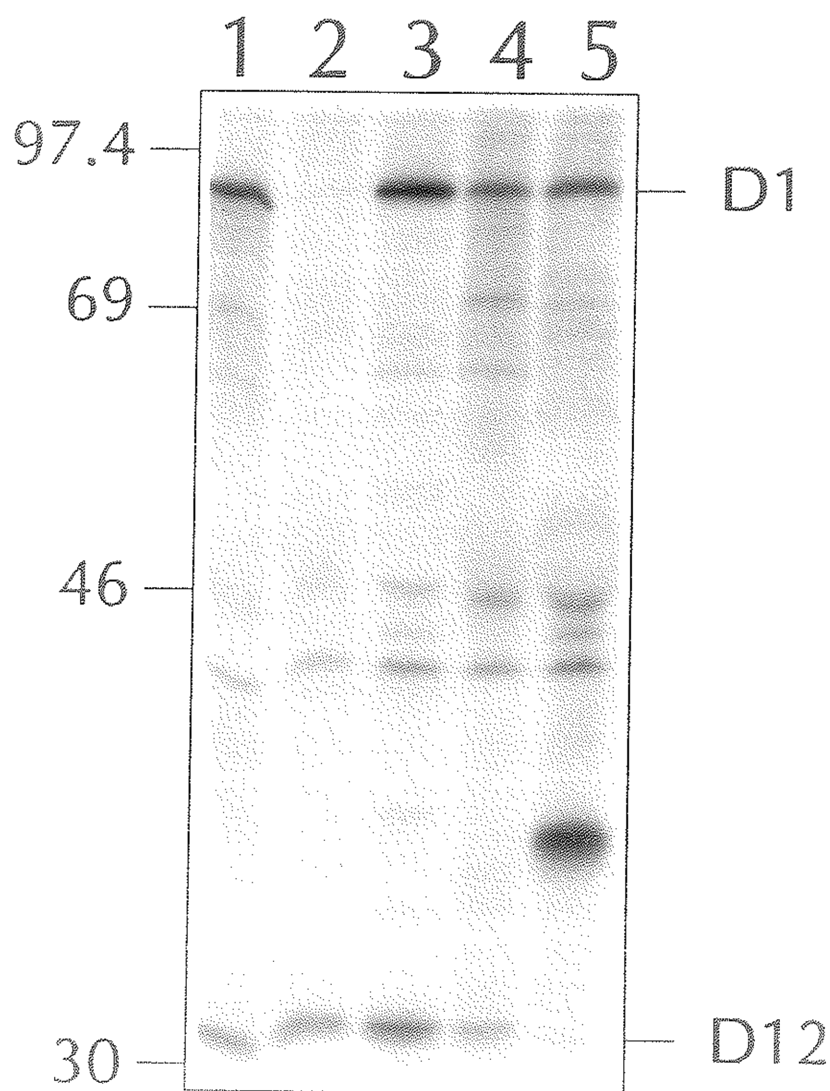


Figure 25. Co-immunoprecipitation of the viral D12 protein with D1 antisera. BSC40 monolayers were infected with IHD-W virus at a multiplicity of 20 pfu/cell and the infection maintained at 37°C. At 2.5 h p.i., cells were labelled with [^{35}S] methionine and harvested immediately in non-denaturing MAXI buffer. Lysates were incubated with different antisera, the immune complexes harvested with protein A sepharose, electrophoresed on SDS-polyacrylamide gels and the radiolabelled proteins detected by fluorography. Extract was incubated with the following antisera D1-M (lane 1), D12 (lane 2), D1-M and D12 (lane 3), D1-N (lane 4) or D1-C (lane 5). Migration positions of [^{14}C] molecular weight markers are indicated as are the expected migration positions of the D1 and D12 polypeptides.

immunoprecipitation to demonstrate the relevant migration positions of the D1 and D12 polypeptides (Fig. 25, lane 3). Mock-infected samples incubated with immune serum, or infected samples incubated with preimmune serum did not detect any D1 or D12 polypeptide (not shown; see Fig. 24 B, lane PI and M). In the presence of antiserum generated against the internal 1/3 of the D1 polypeptide (D1-M), two bands were specifically detected; the D1 polypeptide at 95-kDa and a second band at 31-kDa (Fig. 25, lane 1). This band migrates at the same position as samples incubated with only the D12 antiserum (Fig. 25, lane 2). Further proof that this polypeptide corresponds to the D12 protein was obtained by transferring duplicate gels to PVDF membrane and detecting the D12 antigen with D12 antiserum in a standard Western blotting procedure (not shown). Co-immunoprecipitation of the D12 protein is not seen in samples isolated in SDS buffer and incubated with D1-N antiserum demonstrating that maintenance of the protein's native state is critical for the co-immunoprecipitation (not shown). Antiserum directed against the N-terminal fragment of D1 is also capable of co-immunoprecipitating the small subunit (Fig. 25, lane 4) but antiserum directed against the C-terminal region of D1 does not co-immunoprecipitate any significant quantities of D12 (Fig. 25, lane 5). Since the D12 subunit has previously been shown to interact with the C-terminal region of the D1 subunit (Cong and Shuman, 1992; Higman et al., 1992), it is likely that the D1-C antibodies displace the D12 subunit from the D1-D12 complex.

ts9383 D12 does not interact with D1 at 40°C. Having determined that either anti D1-N or anti D1-M generated antiserum could co-immunoprecipitate the D12 subunit in a wild-

type infection, we turned our attention to ts9383-infected lysates. IHD-W and ts9383-infected BSC40 cells maintained at 32°C, 37°C or 40°C were pulse-labelled for 30 min at 2.5 h p.i. and the samples lysed immediately thereafter in MAXI buffer. Following incubation with D1-N antiserum, and harvesting by protein-A sepharose precipitation, the proteins were separated by SDS-PAGE and the specific bands detected by fluorography (Fig. 26).

Neither mock-infected cells incubated with D1-N antiserum (Fig. 26A, lane M) nor IHD-W-infected samples incubated with preimmune serum (not shown) detected the D1 protein or any co-immunoprecipitating D12. IHD-W-infected cell lysates from infections incubated at either 32°C, 37°C or 40°C (Fig. 26A, lanes 1-3) displayed a prominent 95-kDa band (D1) and a co-immunoprecipitating 31-kDa band (D12) demonstrating that the physical association of D1 and D12 is evident in extracts obtained not only at 37°C, but also at low (32°C) or high (40°C) temperatures. ts9383-derived lysates incubated with D1-N antiserum recovered D1 protein from all three temperature lysates and co-immunoprecipitation of the D12 protein was seen in the 32°C and 37°C samples (Fig. 26A, lanes 4, 5). In contrast, co-immunoprecipitation of the D12 antigen was not appreciable in samples from 40°C derived lysates (Fig. 26A, lane 6). To determine if this was due to an absence of soluble D12 protein altogether, supernatants from each of the D1 immunoprecipitation experiments were re-precipitated with anti-D12 specific antiserum and the resulting samples separated by SDS-PAGE (Fig. 26B). In all six samples, a significant amount of D12 protein was present.

The co-immunoprecipitation results were also borne out in a separate experiment in which D1-M antiserum was used to co-immunoprecipitate the D12 subunit. Again, whereas co-immunoprecipitation of D12 was seen in IHD-W samples prepared at both 32°C and at

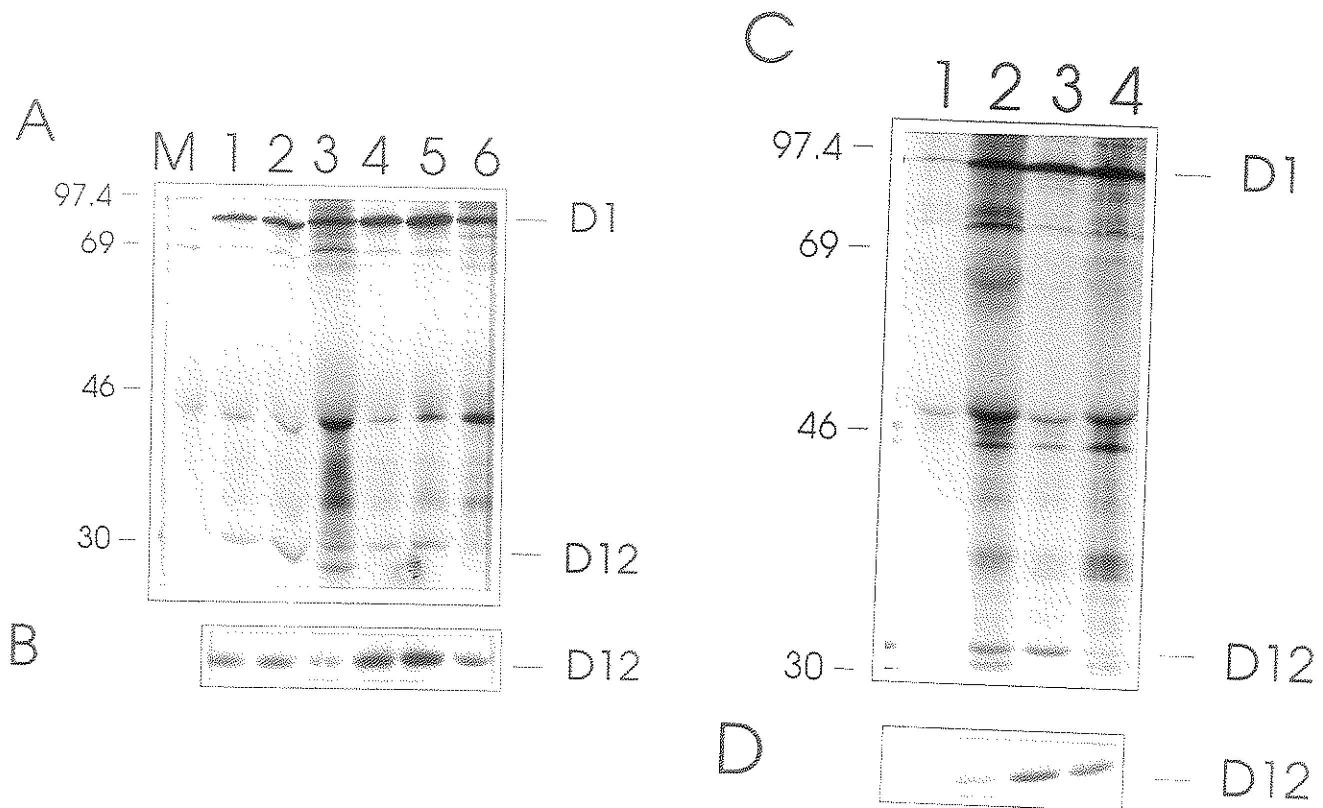


Figure 26. Subunit association of D1 and D12 polypeptides as determined by co-immunoprecipitation. BSC40 monolayers were infected with IHD-W (A, lanes M, 1-3; B, lanes 1-3; C,D lanes 1,2) or ts9383 (A,B lanes 4-6; C,D lanes 3,4) at 32°C (A,B lanes 1,4; C,D lanes 1,3), 37°C (A,B lanes 2,5) or 40°C (A, B lanes 3,6; C,D lanes 2,4). Samples were incubated with D1-N antiserum (A) D1-M antiserum (C). Figures B and D depict the immunoprecipitation of supernatants from samples in A and C with D12 antiserum. Immune complexes were isolated with protein A sepharose beads, electrophoresed on polyacrylamide gels and the proteins detected following fluorography. [^{14}C] molecular weight markers are indicated as are the expected migration positions of the D1 and D12 polypeptides.

40°C (Fig. 26C, lanes 1, 2), and in ts9383-32°C lysates (lane 3), co-immunoprecipitation was not significant in ts9383-40°C infected lysates (Fig. 26C lane 4). These data indicate that *de novo* synthesized D12 produced at 40°C does not have a conformation typical of wild-type D12 and therefore, may not form a stable physical interaction with D1 protein at the nonpermissive temperature.

Packaging of the D12 polypeptide in ts9383 virions. From the above experiments, it is clear that D12 produced at 40°C does not behave like wild-type D12 or ts9383 D12 produced at 32°C. Yet, it is not clear if the mutant polypeptide synthesized at 32°C behaves entirely like its wild-type counterpart. Since D1 and D12 proteins are normally packaged into progeny virions, we wished to know if, at the permissive temperature, the ts9383 D12 protein was properly packaged. To address this, mutant and wild-type virus were grown at 32°C and the virus purified by sucrose gradient sedimentation. Twenty-five µg of each of the purified virions were denatured in SDS load buffer and electrophoresed in an SDS-12.5% polyacrylamide gel, transferred to PVDF membrane and D12 protein visualized by immunodetection (Fig. 27). Anti-D12 antiserum, but not preimmune serum, detected a single band of 31-kDa in both IHD-W (Fig. 27, lane 2) and ts9383 samples (Fig.27, lane 1) proving that ts9383 D12 was normally packaged in the permissive temperature infection. Based on the intensity of the D12 signal, we conclude that a comparable amount of D12 is packaged into both mutant and wild-type virions. As a quantitative control, we also performed an analysis on the amount of major core polypeptide 4b present in each sample and found the amounts to be comparable (not shown). Therefore, the mutant D12 capping enzyme subunit

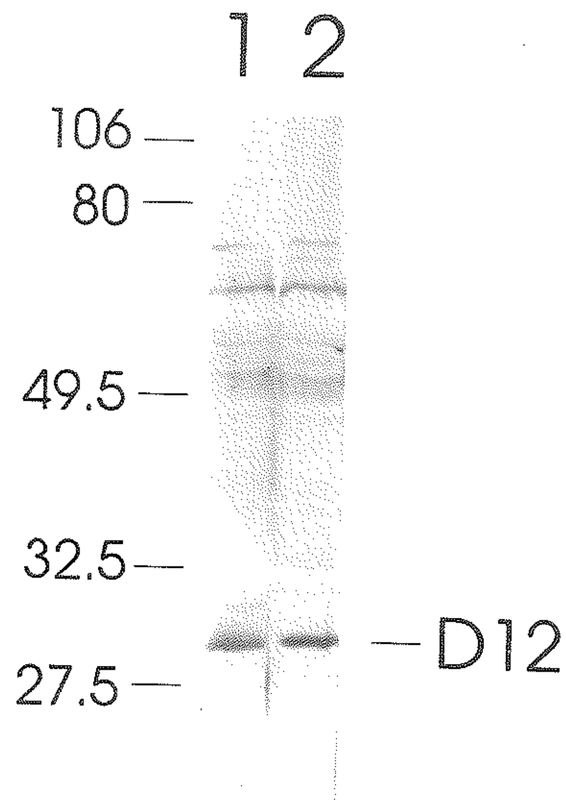


Figure 27. Detection of packaged D12 polypeptides in purified IHD-W and ts9383 virions as determined by Western detection. Mutant and wild-type virus grown at 32°C was purified by sucrose gradient sedimentation. Twenty-five µg of ts9383 (lane 1) or IHD-W (lane 2) was denatured in SDS load buffer and electrophoresed in SDS-12.5% polyacrylamide gels, transferred to PVDF membrane and the D12 protein detected by immunoblotting with D12 specific antiserum as described in Materials and Methods. Migration positions of molecular weight markers are indicated as is the protein D12 specifically detected by the antiserum.

has normal packaging characteristics at the permissive temperature.

Discussion

In this communication, we have continued our analysis of vaccinia virus mutant ts9383, a telomere resolution defective mutant harbouring a defect in the small subunit of the mRNA capping enzyme. Consistent with previous observations, specifically that intermediate and late proteins are produced at the nonpermissive temperature in this mutant, we have now shown that representatives of the early class (D1, D4 and D5) appear normal in size and quantity as compared to wild-type RNA when produced at 40°C. Postreplicatively expressed genes are also produced as indicated by the heterogeneous smears at late times in infection. Furthermore, one late transcript which includes in its sequence the telomeric hairpin was shown to be unaffected in either temporal size or quantity in comparison to wild-type samples. There was a slight difference in the size of this transcript such that the IHD-W transcript appeared to be larger than the ts9383, tsC63 or WR transcript sizes. It will be necessary to examine the DNA from these strains to determine if the difference in transcript length corresponds to a difference in DNA length within the inverted repeat region. With respect to the telomeric transcript, it is interesting that whereas Pickup's group detected two transcripts, a larger and a smaller one, we detected only the smaller one. Although different cell lines were used, Parsons and Pickup (1990) demonstrated that the two transcripts appeared in a number of different poxvirus infections performed in several different cell lines. Therefore, we do not believe that the difference in host cells is a likely explanation for our inability to detect the larger transcript. A second possibility, that the 3.6-kb transcript is not

readily apparent until much later times, was also tested by isolating RNA from vaccinia-infected cells at 16 h p.i. and subjecting these samples to Northern analysis (not shown). We have still been unable to detect any significant quantities of this larger transcript. Parsons and Pickup also used poly (A) mRNA in their analysis. We have not addressed the possibility that ribosomal RNA bands may be obscuring this transcript however, the sizes for the 28S and 18S ribosomal RNA bands, 4.7 and 1.9-kb respectively (Ausubel et al., 1987), do not appear to be in the proper size range to obscure this band. Finally, neither of the probes used by Parsons and Pickup (1990) includes hairpin sequences, rather they include near-terminal sequences; probe pVD12 used in this study does include hairpin ends. Is it possible that the larger transcript described by Parsons and Pickup (1990) does not pass through the telomere? We are attempting to address some of these questions using poly (A) mRNA and different probes.

At the protein level, we have shown that in ts9383 nonpermissive infections, the small and large subunits of the capping enzyme are stable during the early phases of the viral life cycle. However, the single amino acid alteration was shown to affect the ability of newly-synthesized D12 and D1 subunits to interact at the nonpermissive temperature as assayed by a co-immunoprecipitation. It is possible that failure to detect the D12 co-immunoprecipitation is due either to stripping of D12 from D1 by the D1 antibodies or due to the extraction process in MAXI buffer. Irrespective of this, IHD-W samples prepared in the same buffer under identical conditions did not show a variation in co-immunoprecipitation and therefore the data suggest a true physical alteration in the mutant D12 protein. Second, the failure to interact is not due to insolubility of the mutant protein since re-precipitation of these samples

showed plenty of soluble D12 polypeptide remaining (Fig. 26 B,D). Intriguingly, experiments performed using the yeast two-hybrid system (Fields and Song, 1989; Chien et al., 1991) have determined that whereas wild-type D1 and D12 polypeptides do interact, mutant polypeptides do not (DeLange et al., in preparation). To address the above concerns regarding antibody stripping and examine subunit interaction in a less invasive fashion, we are examining the association of the two subunits during column chromatography purification.

If the interaction defect is proven to be true, the implications for the mutant infection may be significant, especially considering the numerous roles that the capping enzyme plays in the viral life cycle. For example, it is possible that the cap structure itself could be altered. In eukaryotic mRNAs, the cap structure is important in increasing mRNA stability by protecting against 5'→3' exonuclease degradation (Furuichi et al., 1977; Shimotohno et al., 1977). The D1 polypeptide by itself should be sufficient to perform triphosphatase and guanyltransferase activities (Guo and Moss, 1990; Shuman, 1990a; Shuman and Morham, 1990), but a tight physical association with the small subunit appears to be required for (guanine-7)-methyltransferase activity (Cong and Shuman, 1992; Higman et al., 1992). The data presented here could suggest that newly-synthesized mutant capping enzyme may not be able to methylate the terminal guanine cap residue. The presence of the methyl group in mRNAs is important for at least two reasons: 1) it halts reversal of the transguanylation reaction; and 2) it is important in stimulating translation of mRNA since unmethylated (GpppN) cap structures cannot substitute for the methylated cap in translation stimulation activity (Banerjee, 1980). This is likely due to the strict physical/structural requirement of mRNA cap-binding proteins (Shatkin, 1985). With regards to the first point, unmethylated

caps can stabilize transcripts, however there is a tendency for hydrolysis of the cap structure to occur (from m^7GpppN to (p)pN) (Martin and Moss, 1975). The presence of a methyl group donor has also been shown to stimulate incorporation of GTP into mRNA cap structures possibly by decreasing reversal of the capping reaction.

The importance of the methylated cap structure is well-defined during translation in promoting initiation complex formation. However, there are a number of other factors which are important in translation, one being the 5' untranslated region. For example, poliovirus mRNA does not contain a 5' cap structure. Instead, the 5' untranslated region may itself facilitate ribosome binding. Furthermore, adenovirus and picornaviruses destroy or alter factors important for cap-dependent translation. In fact many virus families use a cap-independent translation technique to promote the translation of their own transcripts at the expense of host cell mRNAs (Sonenberg, 1988). The mechanism by which vaccinia virus shuts down host translation is currently unknown. Bablanian et al. (1991) have found a preferential translation of viral over host mRNAs in the presence of small poly(A) tracts. Furthermore, they found that the cap analogue m^7GTP is a less efficient inhibitor of vaccinia mRNA than of host HeLa cell mRNA. Translation in the presence of cap analogue suggests that cap-independent translation may play some role in virus gene expression. Whatever the mechanism, the selective translation of vaccinia virus mRNA is not due to alteration or degradation of the cap binding complex eIF-4F (Gierman et al., 1992; Schnierle and Moss, 1992).

The vaccinia virus mRNA capping enzyme also plays a role in transcript termination and initiation, and so far all *in vitro* tests have been performed with both subunits present.

This is in part due to the difficulty in obtaining enough soluble D1 protein through bacterial expression to perform these tests with the individual subunits (Guo and Moss, 1990; Shuman and Morham, 1990; Higman et al., 1992). It is clear that in ts9383 nonpermissive infections, both functions are operative since early RNAs are discretely terminated and production of a late telomeric transcript is seen. If the *de novo* synthesized mutant capping enzyme is physically different from a wild-type equivalent at 40°C, how are these functions fulfilled? While a host-encoded capping/methylating activity might catalyze the processing of the RNA at the 5'-end, the other roles for the virus capping enzyme such as intermediate transcription termination factor activity likely requires virus-encoded proteins. That the intermediate transcription factor activity is present is shown by the appearance of a late transcript detected by probe pVD12 and intermediate/late transcripts detected in the HindIII D fragment (by probe pJB319). For the telomeric transcript in particular to appear, late transcription factors (and therefore intermediate mRNA) would have to have been produced. Based on this, it is likely that other intermediate and late transcripts are also produced and functional.

We have shown here that in a permissive temperature infection, the D12 protein has normal packaging characteristics and it is possible that the capping enzyme packaged during the previous infection cycle is not thermolabile. This could for example explain transcription termination at early times in ts9383-40°C infections. Alternatively, all of these RNA-involving functions may only require the D1 subunit. In this respect, it is interesting to note that cross-linking studies have shown that it is the D1 subunit of the capping enzyme which makes contact with RNA (Luo and Shuman, 1993).

The role that the capping enzyme plays in telomere resolution remains unclear. At this

point in time, the data are consistent with all known functions of the ts9383 capping enzyme being unaffected during nonpermissive conditions. We are considering the possibility that the small subunit (possibly in combination with the D1 or some as yet unidentified protein) plays a structural role in telomere resolution, perhaps in bringing together elements of the putative "resolvase" to the telomere resolution target (TRT) site or in maintaining the telomere fusion in a form conducive to telomere resolution.

CHAPTER V: FINAL COMMENTS

In poxviruses, telomere resolution is the process by which concatemeric replicative intermediate structures are converted to monomeric hairpin terminated forms. While the cis-acting sequences required for this process have been relatively well defined in an *in vivo* plasmid resolution assay, the trans-acting factors involved have remained largely unknown. From a genetic standpoint, the primary stumbling block has been the lack of appropriate conditional lethal mutants (i.e. mutants defective only in telomere resolution). Prior to this work, all resolution defective (res$^-$) mutants characterized also displayed a defect in postreplicative gene expression (DeLange, 1989; Merchlinsky and Moss, 1989a). Therefore, while it was clear that some aspect of intermediate or late gene expression was required for the resolution event, the particular element(s) required was (were) unknown. In this thesis, two res$^-$ mutants tsC63 and ts9383 were characterized. Unlike all other res$^-$ mutants, these two isolates are not wholly defective in postreplicative gene expression.

Mutant tsC63

This mutant, at a gross level analysis, was shown to be defective in the production of late but not intermediate proteins due to an absence of late mRNA species. The defective gene, ORF A1 had previously been shown to encode a factor required for late gene transactivation (Keck et al., 1990). Three significant findings have resulted from the analysis of this mutant: i) the A1 gene product is required for late gene transactivation in a normal *in vivo* setting; ii) late gene expression is essential for telomere resolution, and iii) analysis of telomeric transcripts in this mutant confirmed the suspected late, rather than the intermediate,

nature of this noncoding transcript.

First, the ORF A1 gene product is required for the transactivation of late genes in an *in vivo* situation. Keck et al. (1990), using a plasmid transfection assay, originally demonstrated that the transactivation of a late reporter gene required the ORF A1, A2 and G8 gene products. However, the experiment was performed under unnatural conditions in that DNA synthesis was blocked with the drug hydroxyurea and therefore only the three transfected intermediate genes and the transfected reporter gene would be expressed. Our results, obtained in a more natural *in vivo* setting, support the results seen by Keck et al. (1990) namely that the ORF A1 gene product is required for late gene transactivation. Subsequently, Zhang et al. (1992) demonstrated that repression of ORF G8 (performed by placing the gene under the control of an *Escherichia coli lac* promoter) also inhibited late gene expression without affecting early or intermediate gene expression. *In vitro* studies have also demonstrated the importance of the A1 protein in late gene transactivation (Wright et al., 1991; Keck et al., 1993; Wright and Coroneos, 1993).

The phenotypes that these two viruses (ts9383 and tsC63) exhibit make them useful for identifying late-class specific genes. For example, S1 nuclease protection experiments performed in this work demonstrated that ORFs A3 and A4 are late-expressed genes. By Northern blot analysis, it was shown that the telomeric transcript was also a member of the late class. Other postreplicative genes could similarly be classified based on the presence or absence of transcripts from tsC63 nonpermissive infections.

Secondly, and of greater significance for this work, it was shown that early and intermediate gene expression is insufficient for telomere resolution - some aspect of late gene

expression is still required. This is not intended to imply that early or intermediate products do not play a role in a late-occurring event. For example, Wright and Coroneos (1993) recently identified a novel protein (32-kDa) which is required for late transcriptional transactivation *in vitro*, but which is expressed early in infections. This polypeptide does not appear to correspond to any of the factors (A1, A2, G8), identified by the previously described transfection assay, that are required for expression of late genes. Besides their obvious role in activating subsequent classes of genes, early and/or intermediate gene factors may also be required for the resolution event either through a direct involvement or after an early/intermediate suppressed activity is unmasked at late times.

In this respect, it is also relevant to note that the nicking-joining (N-J) enzyme is present in virions prior to early gene expression yet is likely not required until after early gene expression takes place. Reddy and Bauer (1989) have demonstrated that proteolysis renders the protein more active for cross-linking activity. Could this activity be unmasked by proteolysis at a specific stage in the viral life cycle or could the activity be induced through an interaction with other proteins which act as regulators or activators? These questions remain unanswered and as yet, no specific evidence proving a role for the N-J enzyme in telomere resolution exists.

While it is certain that late gene expression is required for resolution, it is not clear what specific aspect of late gene expression is required. The finding that the TRT sequence's ability to function as a late promoter correlates with its ability to function as a resolution sequence in the plasmid assay (Stuart et al., 1991) begs the question: does resolution require the production of late proteins or would the action of transcription itself be sufficient? For

that matter, would the binding of transcription factors or the transcriptional machinery to the TRT be sufficient? White et al. (1993) have shown that meiotic recombination at the *HIS4* locus of *S. cerevisiae* is dependent on the binding of transcription factors BAS1, BAS2 and RAP1 but is not necessarily dependent on transcription from this promoter. It would be difficult to ascertain the involvement of the virus transcriptional machinery in telomere resolution because late gene activation is not only required for telomere resolution but is also required for the production of progeny virus. In essence, one would have to selectively block late transcription without affecting the interaction of the late transcriptional machinery with the late promoter. Furthermore, even if binding of transcription factors is required for telomere resolution, there is the possible added complexity of late protein requirements for efficient telomere resolution.

Mutant ts9383

Without question, the most significant finding in this work is that a defect in the small subunit of the mRNA capping enzyme (ORF D12 gene product) results in a res$^-$ phenotype. Proof that the D12 mutation was responsible for res$^-$ phenotype came from the fact that replacement of the defective allele with a wild-type copy resulted in correction of the resolution defect. Interestingly, neither gross transcription or translation appeared significantly affected in this mutant.

One of the major concerns during this study, which has still not entirely been resolved, is the possibility that transcription and translation are actually defective in the mutant at the

nonpermissive temperature and that an absence of late gene expression would thereby explain the telomere resolution defect. We are now confident that at the transcriptional level, there is no gross defect, but proof that these transcripts are actually translated *in vivo* would require a thorough examination of late protein production by Western blotting analysis. We have attempted to use the late synthesized p4b protein as a marker but interpretation has been difficult given the fact that p4a and p4b are already present in infecting virions as components of the virus core. As a consequence, p4b protein, as detected by immunobloting, is present at all stages of the virus life-cycle.

In an attempt to perform a non-invasive test, we turned to studies of virus RNA. In these experiments, there is no incubation period and the RNA present therefore accurately reflects the state of the infection at the time of cell lysis. Our studies have shown that at least one late transcript (the telomeric transcript detected by pVD12) appears at essentially the same time and at the same quantity when compared with wild-type transcripts. It is likely that other late transcripts are also produced, but this must be demonstrated by analysing a number of representative late transcripts by S1 nuclease protection. Because of the problems intrinsic to the *in vivo* approach, we are as yet not sure whether the late RNA produced in ts9383-40°C infections is actually translated *in vivo*. It would be interesting to test translation of this mRNA *in vitro* and compare the quantity of protein produced with that from similarly isolated wild-type mRNA. Ultimately, and of greatest significance, it will be necessary to examine the 5' ends of representative early and late mRNA transcripts from ts9383 infections to determine if any alteration in the 5' cap structure exists.

Examination of the D1 and D12 proteins by pulse-chase analysis of SDS-extracted

lysates determined that newly synthesized D12 and D1 proteins were not significantly degraded over the pulse period for either mutant or wild-type infections. Significantly, a physical basis for the ts9383 phenotype was suggested by co-immunoprecipitation experiments demonstrating that whereas D1 antisera could co-precipitate D12 from 32°C-infected/incubated ts9383 extracts, co-immunoprecipitation was not seen in 40°C-derived ts9383 lysates. What this observation means for the virus is unclear. However, the fact that the D12 protein's physical alteration correlates with the temperature at which defective telomere resolution occurs suggests that this is the primary effect of the proline to serine mutation. It is intriguing that although incubation of the extracts with D1 antisera during the co-immunoprecipitation experiment was performed at a low temperature (4°C), interaction between D1 and D12 was not seen in the ts9383-40°C extract. It is possible that once a physical conformation has been adopted by the mutant D12 protein, it cannot necessarily reform back to wild-type even when shifted to a lower temperature. Our attempts to determine if this observation correlates with a temperature-sensitive synthesis (assembly) phenotype have been indeterminate (see Discussion section, Chapter II Results).

Another major problem in identifying the trans-acting factors required for telomere resolution has been the lack of an *in vitro* system by which to purify factors required for resolution. Stuart et al. (1992), using a fairly crude cytoplasmic extract from vaccinia-infected cells, were able to demonstrate resolution of supercoiled circular input plasmid to hairpin terminated minichromosomes similar to that described for the *in vivo* plasmid transfection assay. Unlike the *in vivo* assay, the *in vitro* activity does not show a requirement for the TRT sequence in order to generate linear hairpin terminated products. There are a number of

possibilities to explain this observation and the interpretation may be significant for future genetic and biochemical efforts.

(i) Loss of true "resolvase activity" due to lability or removal of proteins during preparation of the extract. Uninfected HeLa cells already contain a Holliday junction endonuclease which is present in both nuclear and cytoplasmic fractions (Waldman and Liskay, 1988; Carpenter and DeLange, unpublished observations). Yet something in the extraction protocol used by Stuart et al. (1992) led to a loss of the cell-encoded activity. Likewise, the non-specific activity detected may be due to loss or inactivation of specificity factors required for resolution.

(ii) The DNA substrate is not present in a conformation acceptable for telomere resolution. Both the *in vitro* and *in vivo* plasmid assays utilize a supercoiled plasmid which contains the inverted repeat configuration of the hairpin fusion. During the *in vivo* assay, the transfected plasmids are replicated into high molecular weight concatemers which then serve as substrates for telomere resolution. In the *in vitro* assay, plasmids which are incubated with the extract do not appear to be replicated. Could either DNA replication or the physical conformation of the replicated DNA be essential for proper resolution? Merchlinsky and Moss (1989) have shown, using ts mutants, that concomitant DNA synthesis is not required for resolution. However, this says nothing about the physical conformation of the DNA prior to inhibiting DNA synthesis.

(iii) A third possibility is somewhat more speculative and concerns the physical site of virus DNA replication and telomere resolution. Evidence has been accumulating which suggests that in higher eukaryotes, transcription and DNA replication events are localized to a

structure known as the nuclear matrix/nucleoskeleton (Jackson and Cook, 1986a,b; Cook, 1989; Georgiev et al., 1991). In such a model, greater organization and efficiency can be brought to bear upon both transcription and DNA replication by having the machinery in place on an insoluble matrix rather than in a soluble-soup form. A number of experiments have suggested that interaction with the nucleoskeleton may not be restricted to just host protein factors, but that this may also apply to viruses. Proteins from a number of nuclear localizing viruses appear to interact with the host matrix. Included in this list are SV40 (Schirmbeck and Deppert, 1989), adenoviruses (Chatterjee and Flint, 1986; Bodnar et al., 1989), HIV (Muller et al., 1989) and herpes simplex virus (Bibor-Hardy et al., 1982; Ben-Ze'ev et al., 1983; Quinlan et al., 1984). While it is possible that the physical association of virus-encoded proteins may simply reflect a mechanism used by the virus to regulate host functions, it may also reflect a requirement for virus morphogenesis. For vaccinia virus which replicates in the cytoplasm of host cells, the situation must be different from that of the nuclear viruses. However, one could envision that the micronuclei or viral factories in which DNA replication takes place may have an architecture remotely akin to the eukaryotic nuclear matrix. Disruption of this structure might also explain the loss of TRT specificity and might also help explain the efficiency of telomere resolution *in vivo*.

(iv) The telomere resolution activity may represent only a small fraction of recombinase activity. Therefore, the 'resolvase' function may be masked by an otherwise more prominent recombination activity. Vaccinia and SFV are both known to exhibit high levels of recombination (Fathi et al., 1981; Ball, 1987; Evans et al., 1988; Spyropoulos et al., 1988). These recombination activities which are present prior to late gene expression

(Merchlinsky, 1989) may form part of the resolution activity but possibly in combination with other factors. It has been suggested that like T4 bacteriophage, recombination may play a role in the initiation as well as the resolution of vaccinia DNA replication (Evans et al., 1988; Merchlinsky, 1989; Zhang and Evans, 1993).

Aside from ts9383, no other vaccinia mutant has been isolated which is defective apparently only in telomere resolution. One simple explanation as to why this may be so is that the elements involved in resolution play roles in other essential processes. For example, the N-J enzyme may play a role both in initiation of DNA synthesis and in resolution. This appears to be the case for the transcriptional machinery and telomere resolution.

Evidence for a link between transcription and telomere resolution includes: i) the TRT sequence is capable of acting as a poxvirus late promoter (Stuart et al., 1991); ii) non-coding telomeric transcripts have been located which appear to pass through the telomere (Parsons and Pickup, 1990, this work) and at least one of these is initiated from the TRT sequence (Hu and Pickup, 1991); iii) a component of the transcriptional machinery (the small subunit of the mRNA capping enzyme) has a role in telomere resolution (Carpenter and DeLange, 1991).

The process of transcription and the involvement of the transcriptional machinery have been shown to play important roles in the initiation of DNA replication (DePamphilis, 1988) and in site-specific and homologous recombination. In *S. cerevisiae*, the HOT-1 ribosomal DNA enhancer/transcription initiation site can stimulate mitotic recombination when placed at different sites along the yeast chromosome (Keil and Roeder, 1984). This recombination activity appears to depend on high levels of transcription (Voekel-Meiman et al., 1987; Kim and Wang, 1989; Stewart and Roeder, 1989). Also in *S. cerevisiae,* recombination between

GAL10 direct repeats, has been shown to be directly related to transcription (Thomas and Rothstein, 1989). In mammals, the rearrangement of immunoglobulin genes is enhanced by transcription (Blackwell et al., 1986; Sclissel and Baltimore, 1989). Site-specific recombination coupled to transcription has also been noted with respect to the yeast mating type locus in which only transcriptionally active copies of the locus are cleaved by the *HO* endonuclease thus allowing them to function as recipients during *Mat a/α* switching (Strathern, 1988). Finally, there is evidence that RNA polymerase II transcription in general enhances recombination (Thomas and Rothstein, 1989). This incomplete list gives a clear indication that transcription can play a significant role in recombination.

To examine more fully the possibility that transcription plays a role in the resolution process, the TRT sequences (in the plasmid normally utilized in a transfection assay) were replaced with T7 bacteriophage promoters. Transfection into T7 RNA polymerase-expressing poxvirus-infected cells did not result in the resolution of these constructs (Dr. G. McFadden, cited in Traktman, 1990b). The experiment might suggest that while transcription may play a role in the resolution, it is likely that the activity requires the true poxvirus machinery and that transcription by itself is not sufficient.

The actual role that the mRNA capping enzyme plays in telomere resolution is still unclear. The capping enzyme is a very complex protein and current models regarding functional domains favour at least three independent active sites which must accommodate guanyltransferase, triphosphatase and methyltransferase activity (Shuman, 1989; Niles and Christen, 1993). Regions of the capping enzyme must also be able to interact with different proteins and nucleic acids. For example, the capping enzyme is able to form a binary complex

with RNA polymerase in the absence of nucleic acids or associated proteins (Vos et al., 1991; Hagler and Shuman, 1992). Also, this enzyme is able to interact in a non-sequence-specific manner with an RNA substrate *in vitro*. Interestingly, the interaction does not depend on a specific 5' terminus suggesting that RNA binding is also independent from the three ascribed enzymatic activities (Luo and Shuman, 1993). It is also interesting that cap synthesis and methylation are so tightly associated in vaccinia, yet no such tight association is seen in enzymes derived from cellular counterparts (Ensinger and Moss, 1976; Mizumoto and Lipmann, 1979). That the capping enzyme in other systems is likely essential has been shown by disruption studies in yeast (Mizumoto, 1990).

The cap structure is important for many reasons including transcript stability and translatability. The methyl group also appears to be important for interaction with cap binding proteins. It is believed that the cap may mediate melting of the 5' secondary structure of mRNA through the activity of these cap-binding protein complexes (Sonenberg, 1988). Yet there is a body of evidence which suggests that the extent of reduction in translation efficiency of uncapped mRNAs varies considerably among mRNAs and among different translation systems (see Banerjee, 1980 for review). For example, de-capping of alpha mosaic virus 4 RNA or addition of a cap analogue has only a slightly detrimental effect (maximum reduction <50%) on translation or ribosome binding.

Regardless of these observations, we as yet have no evidence that any of the functions normally ascribed to the capping enzyme are affected in ts9383-40°C infections. To the contrary, it is highly possible that none are affected (the sole exception may be the methyltransferase activity which requires the physical association between the D1 and D12

subunits; an answer to this question will require further studies).

At this time, a model for the direct role that the capping enzyme has in telomere resolution is premature. We do not anticipate that the capping enzyme (or the D12 subunit alone) participates in the actual cleavage event at the telomere fusion but instead is involved at an earlier stage. Perhaps the enzyme plays a structural role as part of a larger protein complex or is an element of a hypothetical `scaffold or matrix superstructure'. In this case, the aberrant physical structure of the D12 subunit might interfere with the assembly of the complex or with the organization of a matrix either of whose structures might be critical for resolution and subsequent organized generation of new virions.

One other explanation for the ts9383 res$^-$ defect is that a very early stage of packaging might be affected by the aberrant capping enzyme conformation and since packaging cannot proceed, perhaps telomere resolution cannot proceed either. Although Merchlinsky and Moss (1989) have shown that resolution occurs in the presence of rifampicin (a drug which inhibits envelope formation), this does not prove that an earlier stage of packaging does not play a role. It is important to note that some proteins (including the capping enzyme) are specifically packaged into newly formed virions by a mechanism which is poorly understood.

Irrespective of these hypotheses, it is clear that the link between transcription, DNA replication and recombination is certainly more complex than one could have imagined.

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APPENDIX: DETAILED MATERIALS AND METHODS

Source of Materials

Restriction enzymes, and DNA and RNA modifying enzymes were purchased from Boehringer-Mannheim Canada (Laval, Quebec), Pharmacia Canada Inc. (Baie d'Urfe, Quebec) or Gibco/BRL (Burlington, Ontario). Radiolabelled nucleotides and radiolabelled methionine was purchased from Dupont/New England Nuclear (Mississauga, Ontario) or Amersham (Dateville, Ontario). Nitrocellulose and nylon membranes for nucleic acid transfer were from BioRad (Mississauga, Ontario). Polyvinylidene difluoride (PVDF) membrane used in Western blotting was also obtained from Biorad. Plasmids pTZ18R and pTZ18U, as well as the Sequenase T7 polymerase sequencing kit, were obtained from United States Biochemicals (Cleveland, Ohio). All other chemicals were obtained from Sigma Biochemicals (St. Louis, Mo.).

BSC40 African Green Monkey kidney cells were obtained from Dr. R. Condit (University of Florida, Gainseville, Florida), Hela S3 human epitheloid carcinoma (cervix) cells were obtained from the American Type Culture Collection (ATTC, Rockville MD) or from Dr. G. McFadden (University of Alberta, Edmonton, Alberta). Vaccinia virus wild-type strain WR and IHD-W were obtained from Dr. R. Condit and Dr. G. McFadden respectively. Viral strain ts9383 was obtained from Dr. S. Dales (University of Western Ontario, London, Ontario) and strain tsC63 was obtained from Dr. R. Condit. Cell culture media was purchased from Gibco/BRL.

Cell Culture

A continuous line of African green monkey kidney (BSC40) cells was maintained as a monolayer culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine (all from Gibco), 50 IU/ml penicillin, 50 μg/ml G-streptomycin (Flow Laboratories), and 15 mg/litre phenol red sodium salt (Gibco) as a pH indicator. Stock cultures were typically grown in T-150 mm flasks and incubated at 37°C in a 5% CO_2/95% air environment.

All cell culture manipulations were performed under sterile conditions in a laminar flow hood. BSC40 cells exhibited a doubling time of approximately 18 h. At confluency, cells were split by the following method: medium was removed and cells rinsed with 10 ml PBS (PBS is: 137 mM NaCl, 8 mM Na_2HPO_4, 3 mM KCl, 1.5 mM KH_2PO_4) containing 2 mM EDTA. The rinse solution was removed and 5 ml of PBS added. Next, 0.5 ml of 10 x (2.5%) trypsin was added and the flask returned to the 37°C incubator until cells rounded-up (30-120 sec). To inactivate the trypsin, 5 ml of medium was added and the cells collected by shaking. One tenth of the cells were retained for the continuous line, fresh medium added and cells returned to the 37°C incubator.

HeLa S3 cells were grown and maintained either as monolayer cultures in DMEM as described above, except supplemented with 10% fetal calf serum, or as a suspension culture in S-MEM (suspension-MEM) supplemented with 5% fetal calf serum. Suspension cultures were maintained at 37°C in a non-CO_2 injecting incubator. Cell counts were determined with a haemocytometer and densities maintained at 3-5 x 10^5 cells/ml by dilution with medium.

Virus Isolation

When preparing large scale stocks of vaccinia virus, we initially used monolayers of BSC40 cells (20x150 mm plates). However, we eventually turned to preparations derived from HeLa S3 suspension cultures (1 litre) due to the ease of harvesting and high yields of virus produced.

i) Infections

BSC40 monolayer culture infections: The media from 20x150mm plates of confluent BSC40 cells (2 x 10^7 cells/ 150 mm plate) was removed and the cells infected at a m.o.i. of 0.1 PFU/cell (2 ml/plate). The plates were incubated for 1 h with rocking every 15 min. The inoculum was then removed and medium added to each plate. Incubation was at 32°C for ts strains and 37°C for wild-type strains. Plates were incubated for 2-3 days prior to being harvested.

HeLa S3 suspension culture infections: One litre of HeLa S3 cells (at 5 x 10^5 cells/ml) were centrifuged for 10 min at 3000 rpm (1800 x g) in a Beckman JA-10 rotor at room temperature. The cells were resuspended in medium to 1 x10^7 cells/ml and placed in a T-150 mm flask. Cells were infected at 0.2-1.0 pfu/cell with gentle rocking for 1 h at room temperature. The cells were returned to spinner flasks, made to 1 litre with fresh medium and incubated at 32°C or 37°C for 2-3 days.

ii) Purified Virus Preparation

BSC40 cells were harvested by scraping the cells and medium into 50 ml plastic

Corning tubes kept on ice. This was followed by a low speed (2,000 rpm; 700 x g) centrifugation for 5 min in a clinical centrifuge to pellet the cells and virus. The media was removed and the cell pellet resuspended in 20 ml ice-cold TM swelling buffer (10 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$). For HeLa infections, cells were harvested by centrifugation for 10 min as described in the previous section and resuspended in 20 ml cold TM buffer. From this point on, viral purification procedures were identical for either BSC40 or HeLa-S3 cells.

Cells were incubated on ice for an additional 15 min before being transferred to pre-chilled Dounce homogenizers. A slide of the cell preparation served as a control to monitor cell breakage. The cell prep was then subjected to 20 slow strokes with an A-type plunger to break up clumps of cells and with 20 strokes of a B-type plunger to break up cell plasma membranes. Cell breakage was determined by viewing small aliquots of the preparation under phase contrast microscopy. Occasionally, staining with 0.1% trypan blue was used to identify dead/broken cells. B-type homogenization was continued until most cells were broken-open. The suspension was transferred to a 50 ml corning tube and centrifuged in a clinical centrifuge at 2,000 rpm (700 x g) for 8 min at 4°C. The supernatant was transferred to a second tube and the cell pellet re-extracted with 20 ml of TM buffer, re-homogenized and re-centrifuged. The supernatants were layered onto a 36% sucrose solution (16 ml) in Beckman open top ultracentrifuge tubes (final total volume of each tube was 38 ml), and centrifuged in a Beckman SW28 rotor at 18,000 rpm for 80 min at 4°C. Acceleration and deceleration profiles were set to 4 (slow acceleration from 0 to 170 rpm; deceleration from 170 rpm to 0 without brake). After centrifugation, the supernatant was poured off and the tubes inverted on paper towels to drain. Pellets were resuspended in 0.5 ml 10 mM Tris-Cl, pH 9.0 and stored in snap

cap tubes at 4°C overnight.

The following day, 36 ml 10-40% (w/v) continuous potassium tartrate gradients in 10 mM Tris-HCl, pH 9.0 were made with a small gradient maker. The virus preparation was layered onto the gradients and centrifuged in a Beckman SW28 rotor at 20,000 rpm for 90 min at 15°C; acceleration and deceleration profiles were set to 4. At the end of the run the top layer was removed and the clearly flocculent band containing virus was transferred to a separate tube. Three volumes of 10 mM Tris-HCl, pH 9.0 were added to the centrifuge tube and the sample mixed. Centrifugation was in a Beckman SS34 rotor at 16000 rpm for 40 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 ml 10 mM Tris-HCl, pH 9.0 and recentrifuged. The final pellet was resuspended in 1 ml of 10 mM Tris-Cl, pH 9.0, aliquoted into microcentrifuge tubes and stored at -80°C.

iii) Crude Virus Preparation

Infection and initial harvesting were carried out as described for a purified virus preparation. After the first centrifugation to isolate the cells and virus, the pellet was resuspended in approximately 3-5 ml of PBS and the sample subjected to 3 cycles of freeze thawing to lyse the cells and release the virus. One cycle involves freezing at -80°C for 30 min to overnight followed by thawing in a 37°C water bath. Cellular debris was spun out by a 5 min centrifugation at 2000 rpm (700 x g) and the supernatant was aliquoted into microcentrifuge tubes prior to being frozen at -80°C for storage.

iv) Virus Titres

Serial dilutions of virus preparations, usually in the range 10^{-1} to 10^{-9} were prepared in Hanks balanced salt solution + 0.1% BSA. Virus titres were determined using monolayers of BSC40 cells. Medium was removed from cells and virus preparations added; 150 µl for 24-well dish samples and 350 µl for 6-well (35 mm) dish samples. Virus was adsorbed for 30 min to 1 h depending on the experiment. Plates were rocked every 15 min to ensure that the cells did not dry out. Following the adsorption period, the virus inoculum was removed and fresh medium added to plates. The plates were returned to the incubators (for ts mutant virus, duplicate infections were prepared and one placed at 32°C, the other at 40°C). After 2 days, cells were fixed with neutral buffered formalin (Sigma) and stained with 0.1% crystal violet. The results were expressed as plaque forming units/ml of virus preparation (pfu/ml). If plaques appeared in ts 40°C infections, these were reported as # revertants/ml virus preparation.

Isolation and Purification of Viral Genomic DNA

Isolation of full length vaccinia virus genomic DNA was performed according to the protocol of Esposito et al. (1981). To prepare for the isolation, 8 x 150 mm tissue culture plates were used and cells were infected at a m.o.i. of 0.05-0.1 as per standard methods. Prewarmed media was then added and the plates incubated at 37°C (for wild-type) or 32°C (for ts mutants) for 2-3 days until a noticeable cytopathic effect had taken place. Cells were harvested by scraping with a rubber policeman into 50 ml Corning tubes and pelleted by centrifugation at 2,000 rpm (700 x g) for 15 min. The media was discarded and the pooled

cell pellets were resuspended in 20 ml of ice-cold isotonic buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA), mixed and re-pelleted at 2000 rpm for 5 min. The cell pellet was resuspended in 9 ml of hypotonic buffer (10 mM Tris-HCl pH 8.0, 10 mM KCl, 5 mM EDTA) by mixing and then incubated on ice for 10 minutes. To this suspension, 25 μl of β-mercaptoethanol and 1 ml Triton X-100 were added with gentle mixing followed by incubation on ice for an additional 10 min. This procedure solubilizes the plasma membrane thereby releasing the cell nuclei, viral cores and cellular organelles. Nuclei were spun out by centrifugation at 2000 rpm for 5 minutes at 4°C.

The resulting supernatant was transferred to a fresh tube and recentrifuged if visible cellular debris was still present. The final supernatant was spun in a Beckman JA-20 rotor at 1600 rpm for 60 min at 4°C. The pellet (containing viral cores) was resuspended in 0.8 ml ice-cold core buffer (100 mM Tris, pH 8.0, 1 mM EDTA) and mixed to get an even suspension. To this solution 15 μl β-mercaptoethanol, 50 μl Proteinase K (10 mg/ml) and 200 μl 20% (w/v) N-lauroyl sarcosine was added and the mixture incubated for 30 min at 4°C. This solution served to lyse the viral cores and release the DNA. Next, 1.4 ml of a 54% (w/v) sucrose solution in water was added to the lysate and incubated for 2 h at 55°C to allow digestion of proteins. In some cases where the preparation was difficult to solubilize, SDS was added to a final concentration of 0.2%. Next 0.4 ml of 5 M NaCl was added to the preparation to inhibit nonspecific binding of proteins to the DNA. The lysate was then divided into microcentrifuge tubes, gently extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1) and spun for 2 min at room temperature. The aqueous phase was re-extracted twice more with phenol-chloroform-isoamylalcohol, and once with chloroform-

isoamylalcohol (49:1). The resulting aqueous solution was placed in a spectra/por cellulose dialysis bag (MW cut-off 12,000-14,000 Da) and dialysed against an excess of 25 mM Tris-HCl, pH 7.5 for 24 h with one change of the dialysate. The purified DNA was stored at 4°C.

Plasmid DNA Amplification and Purification

i) Growth Conditions

Bacterial strains were grown in Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10g NaCl per litre, pH to 7.5 and sterilized by autoclaving). To make media for plates, 14.4 g of agar was added per litre of media prior to autoclaving. Antibiotic addition was dependent on the selectable marker and could include: ampicillin or carbenicillin (final concentration at 40 μg/ml), chloramphenicol (20 μg/ml) or kanamycin (30 μg/ml).

ii) Plasmid Purification

Plasmid purification was performed as described by Birnboim and Doly (1979). Typically, 5 ml cultures were grown for routine screening, however, the procedure could be scaled up (linearly) for any culture volume. Overnight cultures were placed on ice for approximately 5 min before being centrifuged either at 7000 rpm for 5 min in a Sorvall JA-20 rotor or in a microcentrifuge for 1 min at room temperature. The supernatant was discarded and the pellet resuspended by mixing in 100 μl of cold Solution I (50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0). The cells were incubated for 5 min at room temperature after which 200 μl of freshly prepared SDS solution (1% SDS in 0.2 M NaOH)

was added and the samples gently mixed by inversion. Each tube then received 150 µl of a 3 M potassium-acetate (pH 5.2) solution; the slurry was gently mixed, incubated on ice for 15 min and then centrifuged for 5 min at room temperature to pellet insoluble material. The supernatant was transferred to a fresh tube, subjected to a phenol-chloroform-isoamylalcohol (25:24:1) extraction and centrifuged as above. The DNA in the aqueous phase was ethanol-precipitated by the addition of 2 volumes of absolute ethanol at room temperature, followed by a 5 min incubation at room temperature then centrifugation at 7000 rpm for 15 min.

The DNA was rinsed once with 70% ethanol and dried briefly under vacuum. The resulting pellet was resuspended in 100 µl TE to which 1 µl of an RNAse A solution (10 mg/ml RNAse A, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl) was added. Incubation was at room temperature for 1 h. The sample was made to 400 µl total volume by the addition of 260 µl of H_2O and 40 µl of 3 M Na-acetate, pH 5.2, extracted with phenol-chloroform-isoamylalcohol then chloroform-isoamylalcohol. Finally, the DNA-containing aqueous phase was precipitated with 800 µl of absolute ethanol. The sample was chilled at -80°C for 10 min prior to a 15 min microcentrifugation at 4°C. The sample was rinsed with 70% ethanol, dried under vacuum, then resuspended in 20-100 µl TE and stored at 4°C.

Quantitation of DNA, RNA and Proteins

DNA and RNA quantitations were determined with the use of a Gilson Response Spectrophotometer. Samples were appropriately diluted and placed in quartz cuvettes and absorbance values at 260 nm and 280 nm were obtained. One A_{260} unit was considered to be equivalent to 50 µg of dsDNA or 37 µg of ssRNA. An $A_{260/280}$ ratio indicated levels of purity

with a value of 1.8 being considered essentially free of contaminating protein in a ds DNA preparation.

Protein concentrations were determined using the BioRad Bradford Protein Assay kit. A standard curve was prepared using various dilutions of either IgG or BSA and by determining absorbance at 595nm.

Restriction Enzyme Digestion and Gel Electrophoresis

i) Digestion of DNA with Restriction Endonucleases

Restriction enzyme digestion was performed according to the manufacturer's conditions with the assumption than 1 U of enzyme could digest 1 μg of DNA in 1 h under optimal conditions. Typically digests involved 30-1000 ng of DNA in a total volume of 20-50 μl of the manufacturer's 1 x buffer. Following digestion, products were either analyzed directly by gel electrophoresis or in circumstances where the removal of restriction enzymes was necessary, samples were phenol-chloroform-extracted and alcohol-precipitated as described for plasmid purification.

ii) DNA Gel Electrophoresis

Electrophoresis of DNA fragments was performed in either agarose or polyacrylamide gels depending on the size of the fragments being separated:

Gel Type	Resolution Rnge
0.7% agarose	0.8 - 12.0 kb
1.2% agarose	0.4 - 7.0 kb
5.0% polyacrylamide	0.1 - 0.5 kb
8.0% polyacrylamide	0.06 - 0.4 kb
12.0% polyacrylamide	0.05 - 0.2 kb

For agarose gels, the appropriate amount of agarose was added to 1x TAE (40 mM Tris acetate, 1 mM EDTA, final pH approx. 8.5) and the solution boiled until dissolved. The solution was cooled to 60°C, and then gels were cast in plastic trays as described in Maniatis et al. (1982). Typically, agarose gels were run at 12 V/cm in 7 cm x 7 cm minigels (approximate running time 20-30 min) or at 5 V/cm in 14 cm x 15 cm medium sized gels (overnight running time) in TAE buffer. Following electrophoresis, gels were stained in 0.5 μg/ml ethidium bromide in TAE.

To prepare 5% polyacrylamide gels (which were most commonly used), 5 ml 10 x TBE (900 mM Tris-HCl, 900 mM boric acid, 20 mM EDTA) was mixed with 8 ml 29:1 acrylamide:bis-acrylamide (w/w) and 36 ml ddH_2O. To this, 25 μl TEMED and 250 μl 10% ammonium persulfate was added and the gels cast in a vertical position and allowed to set for 45-60 min. Miniacrylamide gels (7 cm x 9 cm) were run at 15 V/cm in 1 x TBE buffer for approximately 45 min and then stained in 0.5 μg/ml ethidium bromide in TBE.

DNA Cloning and Bacterial Transformation

i) Cloning

Typical sub-cloning reactions were performed as described by Maniatis et al. (1982). DNA fragments to be cloned were purified from agarose gels by the following method. After

electrophoresis, the band of interest was excised with a scalpel and placed in a centrifuge tube. A second thick agarose gel was prepared and a cube cut out of the gel. A layer of Spectra/por-4 membrane (MW cutoff of 12-14-kDa) was added and shaped into the form of a container. The agarose plug was placed in this box along with a small amount of TAE. Electrophoresis at 110 V was conducted for approximately 5-15 min (depending on the size of the band) and progress was monitored by detection of ethidium-stained DNA with a hand-held long-wavelength UV light. The agarose plug was removed and the current briefly reversed to remove DNA from the membrane. The aqueous material was collected in a microcentrifuge tube and the DNA recovered by addition of 3M Na-acetate (pH 5.2) and alcohol precipitation.

Once purified fragments were obtained, both fragments and vector were electrophoresed to check quantity. In some cases, cut-vector was treated with calf intestinal phosphatase (1 U/5μg of DNA) according to manufacturer's instructions (Boehringer). For "sticky-end" ligations, a final reaction volume (typically 10 μl) contained: insert and vector (2:1 molar ratio, approximately 100 ng of vector), 1 μl of 10 x ligase mix (10 x buffer is 200 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM dithiothreitol), 1 μl 5 mM ATP and 0.2 units T4 DNA ligase. Reactions were incubated at 14°C for 4 h to overnight. For "blunt-end ligations", reactions were similar except that higher concentrations of insert and vector were used and approximately 0.6-1 U of T4 DNA ligase was added to each ligation mixture.

In cases where 5' sticky ends had to be blunt-ended, following restriction enzyme digestion, DNA was treated with Klenow enzyme along with 0.1 mM of each dNTP for 15 min at 37°C. In cases of 3' overhangs to be made blunt, DNA was first treated with Klenow

for 5 min without dNTPs and then 15 min with dNTPs.

ii) Transformation of Bacteria by Chemical Method

This standard transformation protocol has been described by Hanahan (1985). Bacterial cells (JM101, JM109 or DH5α) were grown to an OD_{550} of 0.5 (approx. 4-7 x 10^7cells/ml) in SOB (2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 $MgSO_4$) then chilled on ice for 10-15 min. Cells were pelleted in a clinical centrifuge (750-1000 x g; 2500 r.p.m.) for 15 min at 4°C, the supernatant drained and the cells resuspended in 1/3 the initial culture volume in filtered TFB (100 mM KCl, 45 mM $MnCl_2$-$4H_2O$, 10 mM $CaCl_2$-$2H_2O$, 3 mM $HACoCl_3$, 10 mM K-MES, final pH 6.2) and chilled on ice for 15 min. The cells were re-pelleted, and resuspended in TFB to 1/12 the original volume. DnD (1M dithiothreitol, 90% dimethylsulfoxide, 10 mM potassium acetate) was added to 3.5% (v/v), the cultures swirled and incubated on ice for 10 min. A second aliquot of DnD was added and another incubation on ice was performed. 200 μl aliquots of cells were placed in Falcon 2059 snap cap tubes and 20-40% of a ligation mixture (2-4 μl) added. The mixture was left on ice for a further 30 min and then heat-shocked for 90 sec at 42°C prior to a 2 min cooling on ice. 800 μl of SOC media (SOB containing 20 mM glucose) was added and the cells incubated at 37°C with gentle rocking for 30 min. Aliquots of the ligation mix (2, 20 and 200 μl) were plated onto LB plates containing appropriate antibiotics and the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for colour detection. Plates were incubated at 37°C overnight to allow colonies to form. White colonies were selected as potential positive clones and grown up as described for a small scale

plasmid preparation. Products were determined by gel electrophoresis. Typical transformation frequencies for this procedure were 2-5 x 10^8 transformants/μg of circular plasmid added.

iii) Transformation of Bacteria by Electroporation

The technique of electroporation is a much more rapid procedure since cells can be prepared prior to use then frozen at -80°C for prolonged periods. Cells were prepared according to Dower et al. (1988). This involved growing cells to mid-log phase and performing several washes in distilled water prior to flash-freezing cells with liquid nitrogen in 10% glycerol. Transformations were done with a BioRad electroporator set to 1.25 kV (12.5 kV/cm) and 25 μF, pulse controller = 400 ohms. Generally, 10-50 pg of DNA was added to tubes containing 25 μl of competent cells on ice. The cells and DNA were then transferred to sterile, prechilled electroporation cuvettes and the samples pulsed in the apparatus. One ml of SOC (as above for chemical transformation) was immediately added and the cells gently pipetted up and down. Cells were transferred to snap cap tubes and incubated at 37°C for 30 min prior to plating. Transformation efficiencies were typically 1 x 10^8 transformants/ug plasmid DNA.

DNA Sequencing

i) DNA Sample Preparation and Sequencing Reactions

Single-stranded DNA templates were prepared as described by Dente et al. (1983) and Messing (1983). Briefly, overnight cultures of JM101 (F') bacterial cells harbouring the appropriate plasmids were diluted 1:10 in fresh LB broth and grown to mid log phase at 37°C.

The cultures were then infected with M13K07 helper phage at a multiplicity of 20 PFU/cell and incubation continued for 30 min. Kanamycin (Sigma) was added to a concentration of 30 μg/ml and incubation continued for another 8-12 h. Cells were harvested by centrifugation and the supernatant treated with 50 μg/ml RNAse A for 20 min at room temperature. Single-stranded DNA phage particles were precipitated by adding an equal volume of a 20% polyethylene glycol (PEG), 3.75 M ammonium acetate solution to the supernatant and chilling on ice for 30 min (Carlson and Messing, 1984). After centrifugation, the DNA was extracted several times with phenol:chloroform followed by chloroform extractions and ethanol precipitation. Double-stranded DNA was prepared according to the method of Hattori and Sakaki (1986). Briefly, DNA was denatured in 0.2 M NaOH for 5 min at room temperature then neutralized in 1.4 M ammonium acetate, pH 7.4. The DNA was precipitated in 2.5 vols of ethanol prior to use with the Sequenase kit. Sequencing was performed by the Sanger dideoxy chain termination method (Sanger, 1977) using either the Klenow fragment of *E. coli* DNA polymerase I (BioRad), or a T7 polymerase based kit (Sequenase version 1) from United States Biochemicals, according to the manufacturer's instructions.

ii) Gel Preparation and Electrophoresis Conditions

Denaturing polyacrylamide sequencing gels (50 x 21 cm) were prepared as described in the BioRad literature. Typically 5% polyacrylamide gels containing 7 M urea were used. Stock solutions of 30% acrylamide (19:1 acrylamide/bis-acrylamide) were used in preparing gels. Prior to electrophoresis, gels were pre-run until the temperature of the gel reached 50°C. Electrophoresis buffer was 1 x TBE (900 mM Tris-HCl, 900 mM boric acid, 20 mM EDTA).

Samples were denatured in loading buffer (80% formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol) and electrophoresed at 1800-2000 V for 1-3 h. Gel temperature was maintained at 50°C over the running period.

Marker Rescue

A modified protocol to that described by DeLange and McFadden (1986) was used to perform the marker rescues described in this work. For each rescue, 10 µg of cloned DNA was used for 3 x 10^6 cells (T25 culture flask) or 3 µg of DNA for 1 x 10^6 cells (35 mm monolayer dish). The DNA was used either undigested or after digestion with appropriate restriction enzymes to either linearize or release the insert from the vector. The digested DNA was extracted once with phenol-chloroform-isoamylalcohol (25:24:1), once with chloroform-isoamylalcohol (49:1) and then spin-dialysed on 0.2 µm Millipore disks against an excess of 2 x NNH (50 mM HEPES [N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic acid], pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 min. As an alternative to spin dialysis, the DNA was precipitated by the addition of 1/10th volume 3M Na acetate, pH 5.2 and 2 volumes of absolute ethanol, chilled briefly at -80°C and spun in a microcentrifuge for 15 minutes at 4°C. The pellet was rinsed with 70% ethanol and the sample lyophilized in a Savant Speed-Vac until dry. The pellet was then resuspended in 2 x NNH (350 µl for a T25 flask or 150 µl for a 35 mm dish). One hour before transfection of the DNA (see below) the 2 x NNH solution was mixed with an equal volume of 250 mM $CaCl_2$ under conditions of continuous agitation created by immersing a plugged glass pasteur pipette into the test tube and bubbling a fine stream of air through the liquid. The test tubes were capped and a DNA-calcium phosphate

precipitate was allowed to form at room temperature.

Confluent monolayers of BSC40 cells in T25 tissue culture flasks or 35mm dishes were infected with a temperature-sensitive virus diluted in Hanks balanced salt solution (Gibco) containing 0.1% BSA at a multiplicity of infection of 0.05 PFU per cell. After 1.5 h of adsorption at 37°C, the inoculum was aspirated and the calcium phosphate DNA precipitate was added to the cells. After 30 min at room temperature, 10 volumes of fresh medium (DMEM/5% FCS) prewarmed to 40°C was added and the flasks were placed in a 40°C incubator. At 5.5 h postinfection, the medium was replaced and the flasks were returned to the 40°C incubator. After 72 h of incubation, the infected monolayers were fixed with neutral buffered formalin and stained with 0.1% crystal violet. The results were expressed as #plaques formed/µg of DNA.

Pulsed-Field Gel Electrophoresis

i) Preparation of Samples

Monolayers of BSC40 cells ($1x10^6$ cells) were harvested into a 15 ml Corning tube on ice by scraping the cells and medium with a rubber policeman. A low speed centrifugation step (2000 rpm = 700 x g) for 5 minutes in a clinical centrifuge, was employed to pellet the cells and virus.

Cell pellets were resuspended by gentle mixing in 100 µl of PBS/40 mM EDTA prewarmed to 40°C then incubated for 5 min at 40°C. 125µl of 1.4% low melting point agarose (Incert Agarose, FMC Corporation), 125mM EDTA at 45°C was added to the samples followed by mixing with a few up and down strokes of a micropipettor. The samples

were then immediately transferred to plastic block molds (2 x 5 x 10 mm - each holding approximately 100 µl of volume). After 10 minutes at room temperature to allow the agarose plugs to solidify, they were transferred to 15 ml disposable snap cap tubes containing 3 ml of protease solution (10 mM Tris-HCl pH 7.5, 180 mM EDTA, 1.0% Sarkosyl, 100 µg/ml Proteinase K [Boehringer-Mannheim]). The samples were incubated overnight in a water bath at 50°C. The following day, the protease-containing solution was removed and the agarose plugs treated with 10 ml of 0.1M TBE (1 M TBE is 1 M Tris-HCl 1.0 M boric acid, 20 mM EDTA, final pH 8.3-8.5) for 12 h with gentle rocking at 4°C. This 0.1 M TBE equilibriation process was repeated 3 times in total. Plugs were stored in 0.1 M TBE at 4°C.

ii) Gel Formation and Running Conditions

Pulsed-field gel electrophoresis (PFGE) was performed in an LKB 2015 Pulsaphor electrophoresis apparatus as described by DeLange (1989). Cooling coils underneath the apparatus were connected to a Haake circulating water cooler set to 8°C. PFGE plugs were cut in half and loaded into 1.5% agarose/0.1 M TBE gels (15 x 15 x 0.7 cm = 160 ml of agarose solution). Plugs were sealed into position by topping-off each well with 1.4% low melting point agarose. Electrophoresis was in 0.1 M TBE and was performed in two stages. First, to chase the DNA into the gel, a uniform field of 5 V/cm was applied to the gel for 45 minutes using two sets of point electrodes. Second, the gel was pulsed at 200 mA for 20-24 h at 100 sec intervals with a hexagonal attachment (LKB). A homogeneous field was achieved by running at a 110° angle between the alternating currents.

iii) Southern Transfer of Pulsed-Field Gel

Following electrophoresis, DNA in the gel was prepared for transfer to a nylon or nitrocellulose membrane by the following method. The DNA was initially detected by immersing the gel in 0.5 μg/ml ethidium bromide/0.1 M TBE for 1 h to overnight followed by several hours of de-staining in 0.1 M TBE. A photograph was taken, the gel cut to an appropriate size and the DNA nicked by a 5 min exposure to short wavelength UV light. The DNA was then partially depurinated by a 15 min incubation in 0.25 N HCl, denatured by 2 changes of 20 min each in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and finally neutralized by a 1 h soak in neutralizing solution (0.5 M Tris-HCl, 3.0 M NaCl). The DNA was then transferred from the gel to either a nitocellulose or nylon membrane as described in the Southern Transfer section.

Synthesis of Radiolabelled DNA Probes

Probes were constructed by a modification of the random primer method of Feinberg and Vogelstein (1983). Fifty ng of DNA to be labelled was boiled for 2 min in a total volume of 6.25 μl of H_2O followed by chilling on ice for 5 min. 11.75 μl of reaction buffer (425 mM Hepes, pH 6.6, 10.5 mM $MgCl_2$, 21 mM β-mercaptoethanol, 105 mM Tris-Cl, pH 8.0, 42.5 μM dGTP, 42.5 μM dTTP, 1.7 μg/ml BSA, and 0.02 U/ml $Pd(N)_6$-mer primers), 2.5 μl [$\alpha^{32}P$] dCTP, 2.5 μl [$\alpha^{32}P$] dATP and 1 μl (10U) Klenow enzyme were added. The cocktail was incubated at room temperature for 2 h after which unincorporated nucleotides were separated from the mix by gel filtration chromatography on Sephadex G-50 Nick columns (9 x 20 mm, Pharmacia) equilibrated with TE.

Southern Transfer and Detection of DNA

i) Transfer of DNA to Membrane

The general method for the transfer of DNA from a 0.6-1.0% agarose gel to a solid support was performed essentially as described by Southern (1975). The DNA in the gel was first depurinated by treatment with 0.25 M HCl for 15 min and then denatured by treatment with 0.5 M NaOH, 1 M NaCl for 30 min. Finally, the gel was neutralized in 1.5 M Tris-HCl, pH 7.4, 3 M NaCl for 30 min. Gels were placed on Whatmann 3MM paper whose wick ends were placed in 10 x SSC (1.5 M NaCl, 150 mM sodium-citrate). A sheet of nitrocellulose or nylon Zetaprobe membrane (BioRad) was placed in contact with the gel. Then, two sheets of 3MM paper and an approximately 15 cm high stack of paper towel were placed on top of the membrane. The transfer tower was pressed down lightly with a sheet of glass or a light book. Following overnight transfer, membranes were rinsed briefly in 2 x SSC then air-dried. Blots were baked at 80°C in a vacuum oven for 30 min (nylon) to 2 hours (nitrocellulose). Blots were then sealed in plastic bags and stored at 4°C until ready for prehybridization.

ii) Hybridization Conditions

Areas of the nylon membranes not containing DNA were blocked for 15 min - overnight at 60°C in a high SDS solution (7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4) as recommended by the manufacturer. After this prehybridization step, the solution was removed and replaced with more of the same solution containing radiolabelled probe. Both prehybridization and hybridization volumes were the same (150 $\mu l/cm^2$ of membrane) and

purified probe concentrations were 1 x 10^6cpm/ml. Hybridization was performed at 60°C overnight with gentle agitation.

Nitrocellulose membranes were prehybridized in a solution containing 200 µg/ml yeast tRNA, 20 µg/ml single-stranded salmon sperm DNA, 3 x SSC, 1 x Dernhardts (50 x Dernhardts solution is 1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin - all w/w) and 50% formamide. Prehybridization was at 42°C overnight. For hybridization conditions, probe was added to fresh solution and incubation continued again overnight at 42°C.

iii) Washing Conditions

Following the hybridization step, membranes were washed with agitation as follows:

Nylon: 2 x 1 h at 60°C in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS
2 x 1 h at 60°C in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 1% SDS

Nitrocellulose: 2 x 1 h at 50°C in 2 x SSC, 0.1% SDS
1 x 30 min at room temperature in 0.2 x SSC

Following the final washes, the membranes were air-dried briefly on Whatmann 3 MM paper, resealed in fresh plastic bags and placed on X-ray cassettes ready for exposure to film. Exposures were at -80°C.

Isolation of Total RNA

The procedure for total RNA isolation by lysis in guanidinium solution followed by purification through a CsCl cushion is taken from Ausubel et al. (1987). At the time of harvest, medium was removed from virus-infected BSC40 cells (5 x 10^6 cells in 100 mm

dishes) and the cells were washed twice with 10 ml of cold PBS. The PBS was removed and the cells lysed by the addition of 3.5 ml of guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT, 0.5% *N*-lauroylsarcosine [Sarkosyl]). The viscous lysate was scraped to the bottom of the tissue culture plates and drawn up in a 10 cc syringe fitted with a 21 gauge needle. The lysate was drawn up and down through the needle five times in order to shear chromosomal DNA and was then placed into 15 ml disposable plastic test tubes. The samples were flash frozen in a dry ice ethanol bath and stored frozen at -80°C. The following day, a step gradient was formed by layering 3.5 ml of the thawed samples on top of 1.5 ml of 5.7 M CsCl placed into silanized and autoclaved polyallomer ultracentrifuge tubes (13 x 51 mm). Tubes were silanized by immersion in a dichloro-dimethylsilane/chloroform bath followed by air drying and autoclaving.

Tubes were balanced and centrifugation was performed in a Beckman L8-80M ultracentrifuge in an SW50.1 rotor for 15 - 20 h at 35 000 rpm (150,000 x g) at 18°C. Acceleration and deceleration profile values were set to 0 to allow slow acceleration and deceleration. Once the centrifugation step was completed, the supernatant was removed with a sterile pasteur pipette from the top. The tubes were inverted onto Kimwipe towels and allowed to drain for 15 min. The RNA was then resuspended in 360 µl TES solution (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1% SDS) by pipetting up and down several times with a 1 ml micropipettor. The mixture was allowed to sit at room temperature for an additional 20 min to allow complete resuspension of the RNA and was then transferred to a microcentrifuge tube and extracted with an equal volume of chloroform/1-butanol (4:1 v/v). The aqueous phase was made to 0.3 M sodium acetate (pH 5.2) and 1 ml ethanol was added to allow

precipitation of the nucleic acid. The tubes were chilled at -80°C for 10 min and then spun in a microcentrifuge for 15 min at 4°C. The RNA pellet was resuspended in 360 µl DEPC-treated water and re-precipitated as described above. The final ethanol supernatant was discarded and the pellet air-dried for 20 min at room temperature before being resuspended in 150 µl of DEPC-H_2O. RNA was stored at -80°C.

S1 Nuclease Protection

i) Synthesis of Antisense Riboprobes

Labelled RNA products were synthesized by cloning the DNA region of interest downstream of a T7 RNA polymerase promoter site in pTZ19U. The plasmid was linearized, in order that discreetly sized transcripts would be produced during the *in vitro* reaction. Following digestion, the DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1), chloroform-isoamylalcohol (49:1) and then alcohol-precipitated and resuspended in DEPC-treated H_2O. A transcription protocol based on that described in the Promega literature was used and the 20 µl reaction included the following components:

4.0 µl 5 x transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl)
2.0 µl 100 mM DTT
20 U RNA guard (24 U/µl, Pharmacia)
4.0 µl NTP mix (includes 2.5 mM each of ATP, UTP, GTP)
2.5 µl 100 µM CTP
1.0 µl linearized template (approx 1 µg/reaction)
5.0 µl [α-^{32}P]-CTP (50 µCi at 10 mCi/ml; specific activity > 400 Ci/mmol)
1.0 µl T7 RNA polymerase (15 U; Pharmacia)

The mixture was incubated for 60 min at 37°C after which 1 U of RNAse-free DNase

was added to destroy the template. Incubation was continued for an additional 15 min. The synthesized RNA was then purified on 5% polyacrylamide/7M urea sequencing gels. The location of the full length probes was determined by autoradiography and the samples excised with a scalpel in as small an acrylamide slice as possible. After macerating the slice, 200 μl of elution buffer (2 M ammonium acetate, 1% SDS, 25 μg/ml yeast tRNA) was added to the microcentrifuge tube containing the slice and elution allowed to proceed with vigorous agitation for 3 h at 42°C as described by Ausubel et al. (1987). Next, the aqueous fraction was removed to a fresh tube and the RNA ethanol precipitated and air dried. The RNA pellet was resuspended in hybridization buffer (described below) in preparation for the annealing stage.

ii) Hybridization and digestion conditions

S1 nuclease analysis was performed essentially as described by Weir and Moss (1984) except that uniformly-labelled RNA probes were used rather than end-labelled DNA probes. Hybridizations were conducted under conditions of probe excess and the 30 μl reactions contained 10 μg of total RNA + appropriate probe(s) in hybridization buffer (80% v/v deionized formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA). Samples were heated to 85°C for 5 min then incubated at 37°C overnight. The following morning 300 μl of prechilled S1 nuclease buffer (280 mM NaCl, 50 mM Na acetate, pH 4.6, and 4.5 mM $ZnSO_4$) was added to the reaction tubes and the ssRNA was digested with 500 U S1 nuclease (Boehringer Mannheim) for 1 h at room temperature followed by phenol-chloroform-extraction and ethanol-precipitation. Pellets were resuspended in loading buffer (80% formamide, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol), heated at

85°C for 3 min and then run on a 5% polyacrylamide/7 M urea sequencing gel. Gels were dried under vacuum for 2 h at 80°C on a BioRad gel drier, then exposed to Kodak X-OMAT AR film to detect the bands.

Gel Electrophoresis of RNA and Northern Transfer

i) Gel Electrophoresis of RNA

Ten µg of total RNA was prepared for electrophoresis by mixing with a pre-load buffer containing 1 x MOPS gel running buffer (20 mM 3-[N-morpholino]-propanesulfonic acid, pH 7.0, 8 mM Na acetate, 1 mM EDTA, pH 8.0), 20% formaldehyde and 50% formamide. The RNA was then incubated at 65°C for 15 min to eliminate secondary structure, then chilled on ice. Gel loading buffer (50% glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to 10% of the final sample volume. The RNA was fractionated in a 1.2% agarose gel containing 2.2 M formaldehyde prepared in 1 x gel running buffer. Gels were electrophoresed in 1 x MOPS gel running buffer at 5 V/cm (distance between electrodes). Buffer in the electrophoresis tank was constantly recirculated with a pump mechanism. After electrophoresis, the gel was extensively washed in distilled-deionized water (ddH_2O) then stained briefly in 0.5 µg ethidium bromide in water to detect ribosomal bands. The gel was then rinsed extensively again in ddH_2O prior to Northern transfer.

ii) Transfer of RNA to Membrane

Northern transfers were performed identically to the Southern transfer for nylon membrane described previously except that following detection of ribosomal bands by ethidium bromide no further treatment to the gel was performed. Hybridization and washing conditions were as described for nylon membranes as in the Southern blotting section.

Radiolabelling of Proteins and Immunoprecipitations

i) ^{35}S-Methionine labelling of proteins

BSC40 cells in 35 mm dishes were infected for 30 min as described by Condit and Motyczka (1981) and DeLange (1989). The inoculum was removed and medium (prewarmed to 32°C or 40°C) was added. At various times postinfection, the medium was removed and the monolayer washed with 1 ml prewarmed PBS. Next, 0.5 ml of [^{35}S]-methionine in prewarmed DMEM lacking methionine (Gibco) was added to the monolayer (radioisotope concentration was 100 μCi/ml; specific activity = 1200 Ci/mmol). The cells were placed back in the appropriate incubator for 15-30 min depending on the experiment being performed. After this incubation period, the radioactive medium was removed and the cells lysed in place by the addition of 350 μl of either SDS buffer (2.0% SDS, 12.5 mM Tris-HCl, pH 7.5, 100 mM β-mercaptoethanol), or MAXI buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, 0.2% sodium desoxycholate, 0.5% NP-40, 1% bovine hemoglobin, 50 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride). The lysates were then frozen overnight at -80°C to assist cell lysis. The following day, lysates were thawed and transferred to 1.5 ml microcentrifuge tubes. MAXI lysates were spun for 15 min at 4°C in a

microcentrifuge to remove insoluble material. Samples prepared in SDS lysis buffer were boiled for 3 min and insoluble material, of which there was essentially none, was removed by microcentrifugation for 5 min at room temperature. All samples were stored at -80°C.

For pulse-chase experiments, samples were radiolabelled as described above. However, after the incubation period, the radiolabel was removed, the cells washed twice with prewarmed PBS (1 ml each time) and then overlayed with 2 ml complete DMEM/5% FCS. At the end of the chase period, medium was removed and the cells lysed by addition of 0.4 ml of one of the above buffers.

ii) Immunoprecipitations

Samples (25-60 μl) were made to 200 μl total volume with dilution buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl; for SDS immunoprecipitations, dilution buffer was supplemented with 0.4% (w/v) BSA). Appropriate antibody was added (generally 3- 7.5 μl per sample) and the samples were gently rocked on an end-over-end mixer overnight at 4°C. The following morning, 100 μl of a Protein A-Sepharose CL4b mixture in dilution buffer (100 μg/ml) was added to each sample and rocking continued for an additional 2 h. The bead/protein A/antigen complexes were pelleted by a 10 sec spin in a microcentrifuge. The supernatant was removed and the pellets washed four times essentially as described in Ausubel et al. (1987) with 1 ml each of dilution buffer + 0.1% Triton X-100, no BSA (2 x), dilution buffer (1 x) and finally 50 mM Tris-HCl, pH 6.8 (1 x). Each wash was removed with the aid of a drawn fine-tipped glass pasteur pipette. After the final wash, the samples were

spun a final time and any recalcitrant liquid was removed from the sides of the tube. The samples were then diluted with 50 μl protein sample loading buffer and boiled for 4 min prior to loading on 12% SDS-polyacrylamide gels (as described in the section on polyacrylamide gel electrophoresis and detection of proteins).

Polyacrylamide Gel Electrophoresis and Detection of Proteins

i) Preparation of Samples

Protein samples were diluted at least 2 fold in sample buffer (65 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, 0.05% (w/v) bromophenol blue) then boiled for 4 min prior to loading on polyacrylamide gels.

ii) Preparation of SDS-Polyacrylamide Gels and Electrophoresis

Discontinuous SDS-polyacrylamide gels were prepared essentially according to the method of Laemmli, 1970). A Protean II vertical slab cell apparatus (BioRad) was used for all protein electrophoresis. Gel sizes were 16 (w) x 20 (h) x 0.1 cm. Separating gels were 12% acrylamide/bis (30% total cross-linked plus non-cross-linked monomer), 2.67% cross-linked monomer), 375 mM Tris-HCl pH 8.8, 0.1% SDS. Stacking gels were 4% acrylamide/bis, 125 mM Tris-HCl, pH 6.8 and 0.1% SDS.

Electrophoresis conditions were 16 mA/gel during the stacking phase (run time of approximately 45 min) and 25 mA/gel during the separating phase (3-4 h). The running buffer was 125 mM Tris-HCl, 1 M glycine, 0.5% SDS.

iii) Detection of Proteins by Coomassie Blue Staining or Fluorography

Proteins were detected by Coomassie brilliant blue (R-250) staining by soaking the gels in a 0.1% Coomassie Blue, 40% methanol, 10% acetic acid stain for 30 min with gentle rocking at room temperature followed by destaining in 30% methanol, 10% acetic acid for 2 h to overnight. Gels were dried on a gel dryer at 63°C for 2 hours.

For detection of radiolabelled proteins, gels were soaked for 45 min in a fixative solution followed by a 45 min saturation in a fluor (Entensify, NEN/Dupont) as described by the manufacturer. Polyacrylamide gels were dried as described above prior to being placed in contact with Kodak X-Omat AR film in metal cassette holders. Exposures were at -80°C.

Western Blotting and Immunodetection

i) Transfer of Proteins to Membrane

Protein samples were electrophoresed as described in the Polyacrylamide Gel Electrophoresis section. Immediately following this, gels were equilibrated in transfer buffer (25 mM Tris-HCl, pH 8.3, 150 mM glycine, 20% v/v methanol) for 30 min. Polyvinylidene difluoride (PVDF) membrane was placed briefly in 100% methanol to wet the support, then placed in transfer buffer for approximately 15 min. A clamped sandwich assembly was prepared containing in order: one scotchbrite pad, 2 sheets of 3MM Whatman paper, the gel, PVDF membrane, 2 sheets of Whatman paper and a final scotchbrite pad. This assembly was put together while submerged in transfer buffer, then transferred to an LKB/Pharmacia tank electroblotter (LKB 2005) filled with buffer. Transfer was for 1 h at approximately 1

ampere. Buffer temperature was kept at 4°C with the addition of a cooling core attached to a Hakke cold water circulating pump.

Following transfer, the sandwich was disassembled and the membrane immediately placed in TTBS blocking buffer (0.1% Tween-20, 100 mM Tris-HCl, pH 7.5, 0.9% NaCl as per Ausubel et al., 1987) supplemented with 5% Carnation skim milk powder and rocked gently for 3 h to overnight. (Transfer efficiencies were determined by running prestained protein molecular weight markers (BioRad) alongside the samples). Next, the membrane was rinsed in TTBS and sealed in a plastic bag along with antisera diluted in TTBS + 1% skim milk powder. Incubation was performed overnight at 4°C with gentle rocking. The following morning, the membrane was removed from the bag and washed 3 x 30 min each in TTBS before resealing in a fresh bag. Goat-antirabbit antibody (BioRad) diluted in TTBS + 1% skim milk powder was then added and incubation performed for 1 h with gentle rocking. The membrane was removed from the bag, washed 3 x in TTBS as before, then rinsed once in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

ii) Immunodetection of Proteins with Chromogenic Substrates

Detection of transferred proteins by a secondary antibody linked to alkaline phosphatase coupled with chromogenic substrates was performed as described in Ausubel (1987). The membrane was placed in visualization solution which included: 33 µl of a 50 mg/ml nitro-blue tetrazolium solution (in 70% dimethyl formamide), 17 µl of a 10 mg/ml 5-bromo-4-chloro-3-indoll phosphate solution (in 100% dimethylformamide) and 5 ml alkaline phosphatase buffer. All components were purchased from Sigma. The reaction was performed in dim lighting conditions. Bands were detected after 2-10 minutes and once the

desired intensity was reached, the reaction was halted by immersing the blot in distilled H_2O and rinsing extensively. Blots were air-dried and stored in sealed plastic bags.

Densitometric Analysis of Autoradiograms

Appropriately exposed autoradiograms were scanned into an IBM compatible computer with a Hewlett Packard Scanjet scanner. Intensities of bands of interest were calculated using the program Scanplot (Dr. R. Shiu, Dept of Physiology, University of Manitoba).