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Nancy A. Roseman for the degree of Doctor of Philosophy in Microbiology presented on April 8, 1987

Title: Biology of a Constitutively-Expressed Vaccinia Virus Gene Required for DNA Replication

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Replication of vaccinia virus in the cytoplasm of the infected cell occurs under the direction of virally encoded gene products. Expression of approximately 200 viral genes follows a highly regulated temporal scheme which can be followed biochemically and morphologically during the assembly process. In order to dissect this complex genetic program conditionally lethal mutants of vaccinia have previously been generated. This report describes the utilization of a collection of temperature sensitive (ts) mutants for the study of a required vaccinia virus gene.

Previous biochemical analysis defined four biochemical phenotypes within this collection of ts mutants: DNA negative, defective-late, abortive-late, and wild-type. One mutant from each phenotype was examined at the non-permissive temperature by

electron microscopy. Each exhibited distinct morphological aberrations, but it was difficult to relate aberrant morphology to the biochemical phenotype. Therefore, one mutant, ts17, was chosen for more detailed study.

Ts17 is a member of the DNA negative biochemical class. At the non-permissive temperature no viral DNA was produced in ts17 infected cells. Early viral proteins were synthesized for up to 24 hours post-infection, and late viral proteins were not expressed. The ts17 gene was then mapped by marker rescue techniques, and the nucleotide sequence of 3.6 kilobases of DNA form that region was determined. The nucleotide sequence of a fragment from ts17 viral DNA, and from two ts17 revertants was also determined, and the nature of the ts17 mutation was identified.

Analysis of the wild-type sequence revealed three tightly spaced tandemly-oriented open reading frames. The predicted proteins encoded for by these open reading frames were confirmed by hybrid-selection in vitro translation of selected mRNAs. S1 mapping of the 5' and 3' ends of the encoded transcripts, in conjunction with a northern analysis, determined that two of the open reading frames terminate coincidentally. S1 analysis utilizing RNAs isolated over time demonstrated that the ts17 gene was transcribed throughout infection and is therefore a constitutive viral gene. Precise mapping showed the transcriptional start site of this gene to be in a proposed late regulatory element.

Biology of a Constitutively-Expressed Vaccinia Virus Gene Required for DNA Replication

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CHAPTER I

Introduction

The Family Poxviridae contains approximately fifty viruses which can infect both vertebrate and invertebrate hosts. Within the genus Orthopoxvirus are most of the poxviruses which can infect mammals. The most notorious member of this group is the causative agent of smallpox; variola virus. The best-studied member of this genus is vaccinia virus (VV). VV was ultimately identified as the immunizing agent contained within cowpox lesions used in the late 18th century by the first practitioner of vaccination, Edward Jenner (Baxby, 1981). Variola and vaccinia were found to be serologically related and ultimately VV was administered as a live vaccine in the successful campaign by the World Health Organization to eradicate smallpox (World Health Organization, 1980). VV continues to be studied as a model system to address questions central to how complex eukaryotic systems regulate and express their genetic information.

Many unique features of VV make it an attractive model system for the study of complex, highly-regulated, developmental

programs in higher organisms. Replication of poxviruses occurs in the cytoplasmic compartment of the host cell (Moss, 1985). This provides a unique system for studying essential enzymatic and structural functions, as well as possible trans-acting regulatory factors, without the interference of the host nuclear machinery. Since VV does not utilize the machinery of the host cell nucleus, the virus must package or encode many of the functions required for nucleic acid metabolism. Enzymes that have been identified as being present in the virion are: RNA polymerase, poly(A) polymerase, RNA triphosphatase, RNA guanylyltransferase, RNA (guanine-7-)methyltransferase, RNA (nucleoside-2'-)methyl-transferase, 5'-phosphate polynucleotide kinase, adenosine triphosphatase, nucleoside triphosphatase, endoribonucleases, DNA topoisomerases, protein kinase, and deoxyribonucleases (Dales and Pogo, 1981). VV is unique in that packaged within the virion are all the functions necessary for the production of mature viral mRNA (Kates and McAuslan, 1967; Munyon et al., 1967; Kates and Beeson, 1970; Wei and Moss, 1975). To date, few genes with defined enzymatic functions have been identified on the genome. These include thymidine kinase (Hruby and Ball, 1982; Weir et al., 1982); DNA polymerase (Jones and Moss, 1984; Traktman et al., 1984); the large subunit of guanylmethyl transferase (Morgan et al., 1984); RNA polymerase subunits (Broyles and Moss, 1986); and nucleoside triphosphatase (Rodriguez et al., 1986). A sequence comparison of the viral thymidine kinase (Hruby et al., 1983; Kwoh and Engler, 1984;

Bradshaw and Deininger, 1984), and VV growth factor (Blomquist et al., 1984) with their human counterparts suggests they share a significant degree of homology. The viral DNA and RNA polymerase genes also show homology with analogous genes in other eukaryotic systems (Earl, 1986). Information on basic biochemical processes derived from VV may be applicable to higher eukaryotic organisms.

In addition to addressing basic biological questions, it has recently been shown that VV can have practical applications as a eukaryotic cloning and expression vector (Panicali and Paoletti, 1982; Mackett et al., 1982). VV is well-suited for the expression of foreign genes and as a recombinant vaccine virus for a number of reasons: VV has a very broad host range, thus allowing an inserted foreign gene to be expressed in the appropriate species and tissue type (Hruby et al., 1980); the vaccinia genome has the capacity to accept up to 25,000 base pairs of foreign DNA (Smith and Moss, 1983); messenger RNA biogenesis occurs in the cytoplasm of infected cells under the direction of virally encoded enzymes, specific orientation of a gene relative to eukaryotic control elements utilized within the nucleus such as enhancers, splice junctions, polyadenylation signals and transport signals is not necessary (Moss, 1974); finally, almost 200 years of usage has proven VV to be a relatively safe vaccine strain. Already VV has proven to be a potent research tool for the study of genes which are technically difficult to study using standard approaches (Rice

et al., 1984; Dougherty et al., 1986; Thomas et al., 1986). The potential efficacy of live recombinant VV vaccine strains for human and veterinary use has already been demonstrated (Smith et al., 1983; Smith et al., 1984; Panicali et al., 1983).

The highly complex nature of the VV replication cycle in concert with the virus encoding many metabolically required functions make VV an excellent eukaryotic model system. Additionally, an understanding of VV gene expression would assist in the development of efficient viral vectors for practical use.

The viral replication cycle as it occurs within the cytoplasmic compartment of the infected cell is diagrammatically represented in Fig. I.1. The VV particle is brick-shaped and its dimensions of approximately 270 x 218 nm make it one of the largest animal viruses known. This large size allowed VV to be the first animal virus to be seen with a light microscope (von Borries et al., 1938). The virus particle has an outer membrane made up of a lipoprotein bilayer which is termed the envelope. Contained within the envelope is a complex architecture consisting of the DNA containing biconcave core and two lateral bodies which have no known function. The linear 185 kilobasepair double-stranded DNA genome is covalently cross-linked at the ends and has the capacity to encode approximately 200 polypeptides. Restriction analysis between members of the poxvirus family reveals a high degree of sequence conservation within the central portion of the genome. The

Fig. I.1. Diagrammatic representation of the vaccinia virus replication cycle. Metabolic processes which have been primarily distinguished by the use of metabolic inhibitors are shown. Distinct morphological steps associated with assembly are also shown. Listed are enzymatic functions which have been identified within the virion core or during viral infection.

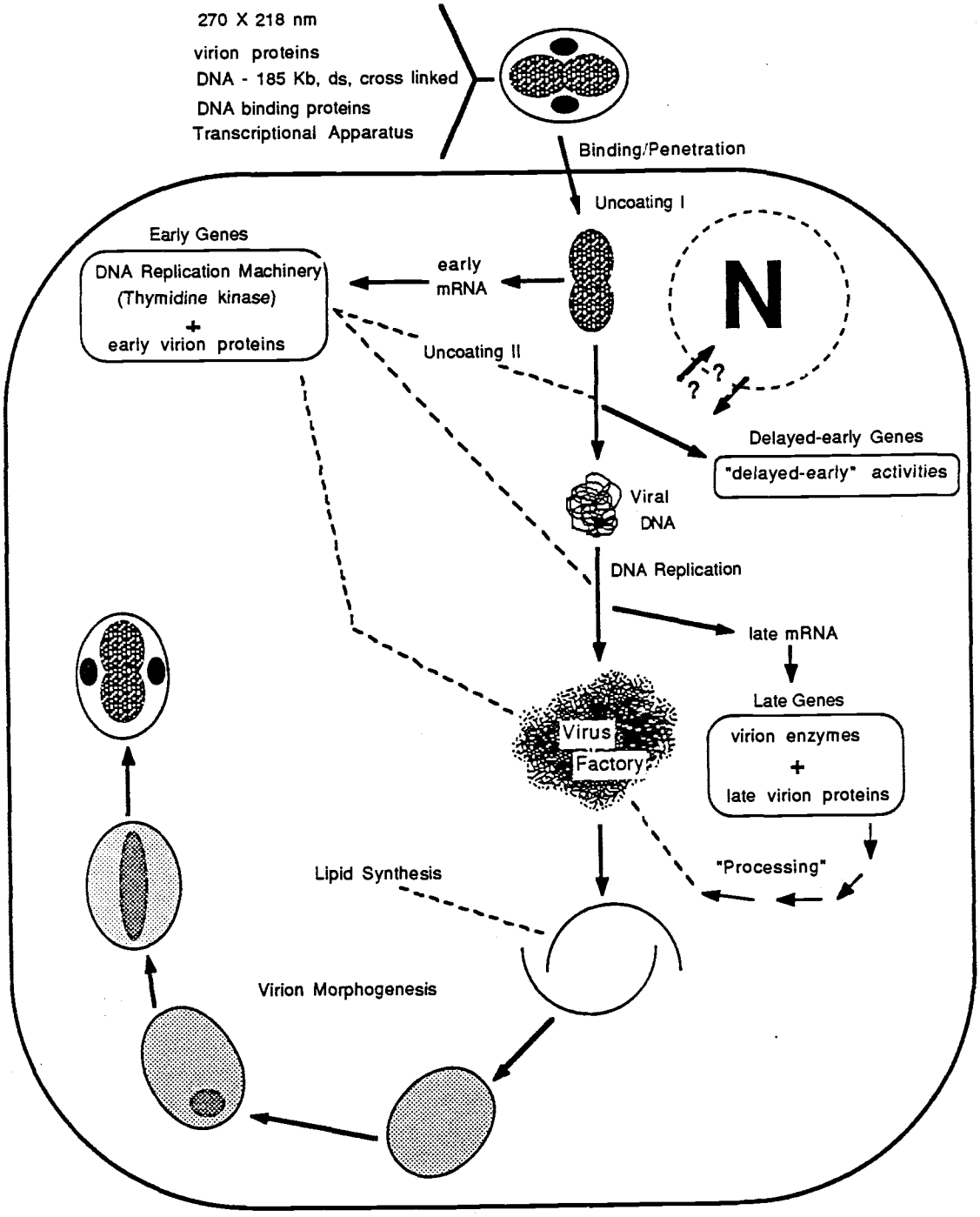

270 X 218 nm
virion proteins
DNA - 185 Kb, ds, cross linked
DNA binding proteins
Transcriptional Apparatus
Binding/Penetration
Uncoating I
Early Genes
DNA Replication Machinery
(Thymidine kinase)
+
early virion proteins
early
mRNA
N
Uncoating II
Delayed-early Genes
"delayed-early" activities
Viral
DNA
DNA Replication
late mRNA
Late Genes
virion enzymes
+
late virion proteins
Virus
Factory
"Processing"
Lipid Synthesis
Virion Morphogenesis

Fig. I.1

majority of deletions and rearrangements occur primarily at the ends which consist of inverted terminal repeats.

Upon entry into the cell by either direct penetration or membrane fusion, the viral envelope is removed by host enzymes. This process, called Uncoating I, leaves the core containing the genome intact, while the lateral bodies disappear. At this time immediate early viral genes are transcribed and the messenger RNAs are capped and polyadenylated. These enzymatic processes are all catalyzed by viral enzymes packaged within the core (Barbosa and Moss, 1978; Baroudy and Moss, 1980; Moss et al., 1975; Nevins and Joklik, 1977; Spencer et al., 1980). This has been shown by the ability of VV cores to produce accurate early transcripts in a cell-free in vitro transcription system (Pelham, 1977; Cooper and Moss, 1978). Mature early viral mRNAs are then extruded from the core and translated. An immediate early gene product is then required for Uncoating II which liberates the viral DNA from the core. An immediate early gene product is also required for the transcription of delayed early genes. These processes can be differentiated by protein synthesis inhibitors (Woodson, 1967). Concomitant with viral DNA replication is the onset of late gene expression and the repression of early genes. While early viral genes have a discrete length (Hruby et al, 1983; Mahr and Roberts 1984; Wittek et al., 1980), late transcripts are heterogeneous in length due to random 3' termination (Weir and Moss, 1984; Mahr

and Roberts, 1984b).

Mirroring this temporal scheme of gene expression is a complex series of morphogenetic events; all occuring within cytoplasmic foci termed the viroplasm or virus factories (Cairns, 1960). Assembly of the viral particle first begins with the formation of crescents made up of host-derived lipids which form around a portion of the electron dense viroplasm. The amorphous interior of the virus particle then undergoes a series of identifiable organization steps, culminating in the formation of an infectious virus particle. This entire replication scheme takes between 8 to 12 hours to complete, depending upon cell type.

Through the use of metabolic inhibitors VV genes have been classified within a temporal scheme as being either immediate-early, delayed-early or late genes. The early gene classes are defined as being prereplicative, or prior to DNA replication, while the late genes are expressed only after DNA replication and are therefore postreplicative. However, this is an oversimplification for an analysis of the kinetics of transcription (Weinrich and Hruby, 1987), and a pulse-label of infected cells with L-[^{35}S]methionine at short intervals clearly shows that there are many kinetic subclasses of VV genes (Moss and Salzman, 1968; Pennington, 1974; Weinrich and Hruby, 1985). Also, a number of genes which are transcribed both before and after DNA replication have been identified and classified as constitutive genes (Weir and Moss, 1984; Weinrich and Hruby,

1987).

The mechanisms of the temporal shift from early to late gene expression are unknown. Towards the elucidation of regulatory elements and factors a number of laboratories have sequenced various regions of the large VV genome (Venkatesan, 1981; Venkatesan, 1982; Hruby, 1983; Weir and Moss, 1983; Weir and Moss, 1984; Bertholet, 1985; Pluciennicczak, 1985; Rosel and Moss, 1985; Weinrich and Hruby, 1985; Broyles and Moss, 1986; Earl, 1986; Niles, et al, 1986). The data produced has shown VV genes to be organized in a tightly spaced, tandemly-oriented fashion. Sequences upstream of both early (Mackett, 1984; Vassef, 1985) and late genes (Weir and Moss, 1984; Bertholet, 1985) have been linked to a reporter gene, the prokaryotic chloramphenicol acetyltransferase gene; and assayed for transcriptional activity using a transient expression system (Cochran, 1985a). Although this system is preferential for late gene expression, it has shown that cis-acting regulatory sequences exist upstream of both early and late genes. Sequence analysis of upstream sequences of VV genes which have been shown to promote transcription, or are putative promoter elements, show little consensus amongst themselves or with other eukaryotic or prokaryotic promoters.

One experimental strategy to approach this highly complex system is to identify a single required gene, define the kinetics of its expression, and identify control signals required for this expression. In this way, common and unique

features of the kinetic subsets of VV genes may become apparent.

To facilitate the study of VV a number of drug resistant (Raczynski and Condit, 1983; Traktman et al., 1984; Villarreal and Hruby, 1984; Tartaglia and Paoletti, 1985) and temperature sensitive (ts) mutants (Dales et al., 1978; Drillien et al., 1978; Condit and Motyczka, 1981; Drillien et al., 1982; Ensinger, 1982; Condit et al., 1983) have been generated and isolated. One collection of temperature sensitive mutants has been roughly analyzed genetically and biochemically. This analysis suggested the organization of this collection into four biochemical phenotypes: DNA negative (DNA-), defective-late, abortive-late, and wild-type (Condit et al., 1983).

I first utilized this collection of ts mutants in an initial assay to determine if through an electron microscopic survey, morphological aberrations apparent at the non-permissive temperature may be correlated with the biochemical defect and thus provide insight into the function encoded at the mutant locus. The complexity of the assembly process without more information about the gene function involved made the resulting data ambiguous. Therefore, a single DNA- mutant, ts17, was chosen for a more detailed study. A mutant with a DNA- phenotype was chosen because: of inherent interest in functions required for DNA metabolism; DNA replication is required for the switch between early and late gene expression; and, unlike other DNA-containing animal viruses, VV DNA replication is temporally regulated (Moss, 1978). Ts17 was subjected to a biochemical

analysis to determine the defective genes impact on viral metabolism. The mutation was then mapped using marker rescue techniques. The region of the VV genome to which ts17 mapped was then sequenced, and the kinetics of expression was determined. Recent experiments have focused on dissection of putative promoter elements upstream of the transcription initiation site.

CHAPTER II

Electron Microscopy of Temperature Sensitive Mutants of Vaccinia Virus with Distinct Biochemical Phenotypes

Summary

A survey of aberrations in the morphology and assembly of vaccinia virus (VV) temperature sensitive (ts) mutants was performed using electron microscopy. This collection of ts mutants had previously been characterized as to their biochemical phenotype, and had been roughly mapped using marker rescue techniques (Condit et al, 1983). Biochemical analysis suggested the organization of this collection into four distinct biochemical phenotypes which were exhibited at the non-permissive temperature. These were DNA-, defective-late, abortive-late, and wild-type. A mutant from each biochemical class was used to infect cells at the non-permissive temperature and prepared for electron microscopy. The morphological phenotype was analyzed in reference to the specific biochemical phenotype described for the mutant.

Introduction

Vaccinia Virus (VV), the prototypical Poxvirus, contains a linear 185 kilobasepair double-stranded DNA genome. Poxviruses are typified by replicating in the cytoplasm of susceptible host cells. Upon entry into the cell, early VV genes are expressed under the control of virally encoded enzymes. At the time of DNA replication, early genes are switched off and late genes are expressed (Moss, 1978). Nothing is known about the regulation of this temporal shift, and little is known about VV gene expression in general. The VV genome has the capacity to encode approximately 200 polypeptides. Mapping of individual viral transcriptional units on such a large genome has been accomplished primarily through the utilization of cloned fragments of viral DNA in concert with hybrid-selection and cell-free translation systems (Belle Isle et al, 1981; Cooper et al, 1981; Mahr and Roberts, 1984). Locating genes of a defined enzymatic function has proven more difficult. Using specific enzymatic assays thymidine kinase (Hruby and Ball, 1982; Weir et al., 1982), and the large subunit of the guanyl methyltransferase (Morgan et al., 1984) have been mapped. Other specific genes have been mapped by the generation of drug resistant mutants as in the case of DNA polymerase by the generation of a phosphonoacetic acid resistant VV mutant (Jones and Moss, 1984). These methods although specific, are highly

laborious and have a limited chance of success.

To facilitate the study of various aspects of vaccinia virus, a number of laboratories have isolated temperature sensitive (ts) mutants (Dales et al., 1978; Condit and Motyczka, 1981; Drillien et al., 1982; Ensinger, 1982; Condit et al., 1983). Genetic and molecular techniques can then be applied to these collections to address specific questions. First, VV encodes and packages many of the enzymatic functions required for nucleic acid metabolism and presumably structural functions required for replication and assembly. If it can be assumed that many of these are essential functions, the isolation of conditionally lethal mutants in the genes encoding these functions should be possible. Second, the isolation of mutants in which the temporally regulated expression of VV genes is blocked or disrupted may provide insight into mechanisms controlling the regulation of gene expression or about the switch from early to late gene expression. Third, whether there is a functional organization or temporal organization of the genome may become apparent. Fourth, assembly of the virus is a tightly regulated and intricate process which results in the complex architecture of a mature virion particle containing a large DNA molecule and a number of specific proteins. Of particular interest is the de novo assembly of the viral lipoprotein envelope within the viral factories. This offers a unique system for the study of membrane biogenesis, as most viruses derive an envelope through a budding process (Dales and

Mosbach, 1968). Insight into this intricate assembly process has been provided by previous studies of the morphology of ts mutants of VV (Dales et al., 1978; Drillien et al., 1977). Unfortunately, these analyses were not followed by any further genetic, biochemical or mapping studies.

Here we report the morphological defects of selected ts mutants of VV that have been subjected to rough genetic, biochemical and mapping procedures (Condit and Motyczka, 1981; Condit et al., 1983). This analysis suggested the organization of this collection into four biochemical phenotypes: DNA negative (DNA-), in which early protein synthesis is prolonged and late proteins are absent; defective-late, characterized by defective late protein synthesis; abortive-late, characterized by an abrupt halt of late protein synthesis; and wild-type (wt), in which no infectious virus is produced. The biochemical and genetic analysis, in concert with marker rescue mapping techniques (Weir et al., 1982; Panicali and Paoletti, 1982), produced consistent results. Mutants which fell into the same complementation group exhibited the same biochemical phenotype. Also, marker rescue mapping using HindIII fragments of wt viral DNA confirmed the assignment of complementation groups: mutants of the same complementation group mapped to the same DNA fragment (Condit et al., 1983).

For this study, we chose one representative from each biochemical class. They were chosen on the basis of a low reversion frequency and minimal leakiness. Cells infected with

each mutant were incubated at the non-permissive temperature of 40°C. At various times post-infection the morphology was examined using the electron microscope. Defects in morphology and assembly are discussed in relation to the biochemical phenotype and the possible identity of the gene product encoded for at the mutant locus.

Methods and Materials

Cells and virus. BSC_{40} cells, a clonal derivative of BSC_1 cells selected for their ability to grow at 40°C, were grown in monolayer culture with Eagle minimum essential medium (Flow Laboratories) supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and 50 ug of gentamicin sulfate per ml. VV (strain WR) is designated as wild-type in these experiments. Isolation and characterization of temperature sensitive mutants has been described (Condit and Motyczka, 1981; Condit et al, 1983).

Electron microscopy. Confluent 100-mm plates of BSC_{40} cells were infected with wild-type or the indicated temperature sensitive mutant at a multiplicity of 10. Duplicate plates of cells were incubated at the permissive temperature of 31°C, or at the non-permissive temperature of 40°C. At 2, 7 and 9 h post-infection, infected cell pellets were collected by centrifugation and suspended in 1 ml of 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4) containing 2 mM $CaCl_2$. After 2 min at room temperature, the suspension was centrifuged, the supernatant fluid was decanted, and the undisturbed pellets were fixed in the above glutaraldehyde solution for 30 min at room temperature. The pellets were postfixed in 1% osmium tetroxide in 0.15 M cacodylate (pH 7.4) for 30 min and then stained en

bloc with 0.5% uranyl acetate buffer (pH 5.0) for 30 min, dehydrated in acetone, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with a glass knife, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Hitachi Hu-11E electron microscope at 80 kV.

Results

Selection of mutants. A representative of each biochemical class was chosen on the basis of having good genetics; each had a low reversion index and low leakiness. The ts mutants chosen along with their biochemical phenotype, genetics, and preliminary map position are shown in Table II.1. Each mutant belongs to a complementation group which contains other mutants with the same biochemical characteristics and map position.

Electron microscopy. Wild-type. For comparative purposes an electron micrograph of a wt viral factory is shown (Fig. II.1). A schematic of this replication scheme can be seen in the introduction of this thesis (Fig. I.1). Pictured in the micrograph is a virus factory in the cytoplasm of a BSC_{40} cell at 7 hours post-infection (hpi). The lipoprotein crescents which eventually form the viral envelope are apparent by approximately 3 hpi. These crescents will enclose a section of viroplasm and will ultimately form a spherical virus particle. Detail of this envelope is also shown (Fig. II.2). The viroplasm enclosed within the envelope undergoes a series of specific morphogenetic events. The viroplasm condenses and becomes highly electron dense, it then differentiates to form the DNA containing viral core and the lateral bodies. Upon maturation the virion migrates away from the viroplasm.

MUTANT NUMBER	PERCENT LEAKINESS	REVERSION INDEX	HINDIII MAP	BIOCHEMICAL PHENOTYPE
ts17	0.6	$<6x10^{-6}$	D	DNA negative
ts7	1.5	$<3x10^{-6}$	J	Defective-late
ts22	1.3	$<3x10^{-5}$	-	Abortive-late
ts5	0.25	$<5x10^{-7}$	D	Wild-type

Table II.1. Genetic and biochemical characteristics of temperature sensitive VV mutants selected for electron microscopy. The HindIII fragment which confers temperature insensitivity in a marker rescue is indicated.

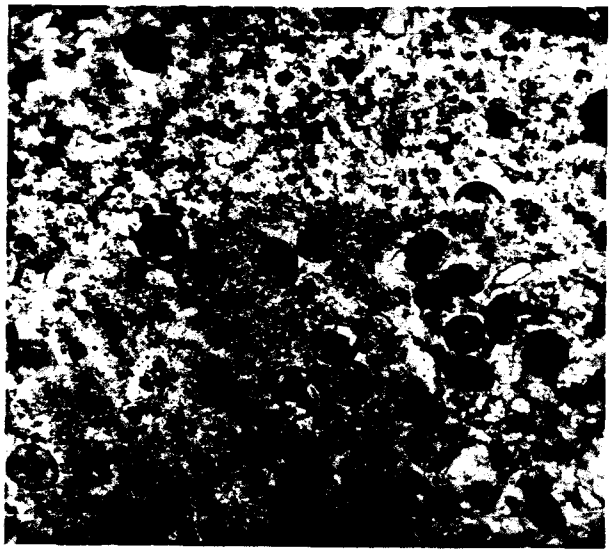


Fig. II.1. Wild-type vaccinia virus factory.

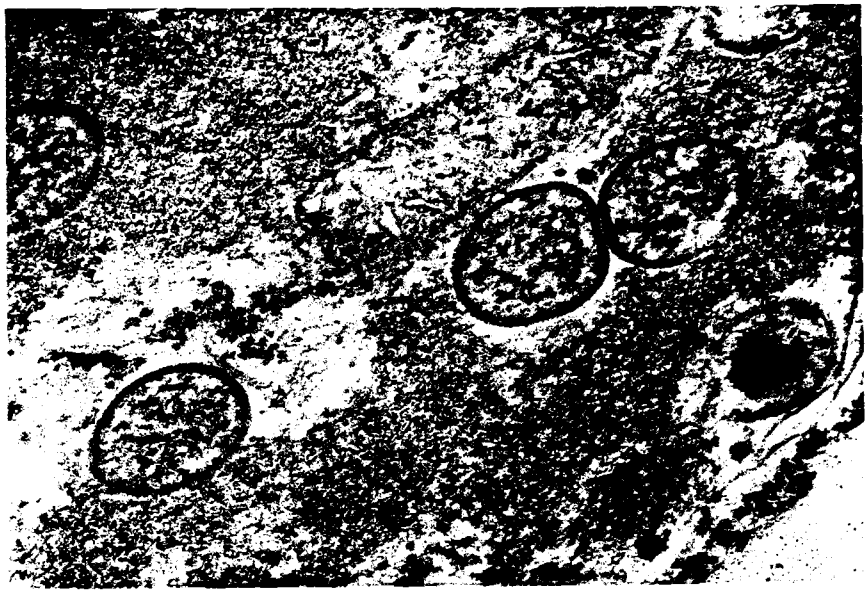


Fig. II.2. Detail of the viral lipoprotein envelope.

ts17. For the DNA- mutant ts17, no cytoplasmic structures normally seen in VV infected cells were apparent, therefore no micrograph is shown.

ts7. In an electron micrograph of the defective-late mutant, ts7, the viral envelope appears to have completely enclosed a portion of the viroplasm (Fig. II.3). Little condensation of the interior containing DNA and protein appears to have occured. Yet, the viral particles have migrated away from the viroplasm without maturation. The unusual protrusions which are apparent occur only in luscent areas of the particle (Fig. II.4).

ts22. In the case of ts22, which is from the abortive-late class, the morphology seen varied depending upon when, post-infection, the infected cells were harvested. At 7 hpi, normal looking immature particles have migrated away from any apparent viroplasmic structure within the cytoplasm (Fig. II.5). No condensation of the interior of the virus particle appears to have occured. When examined at 9 hpi, little of the electron dense material derived from the viroplasm appears within virus particles (Fig. II.6). Also, the viral envelope has lost its structural integrity.

ts5. ts5, which has a wt biochemical phenotype, exhibits a number of unusual morphological features. At 7 hpi lipoprotein crescents appear in the viroplasm (Fig II.7) and appear to have a normal structure and spicule layer which confers rigidity and curvature. However, these crescents

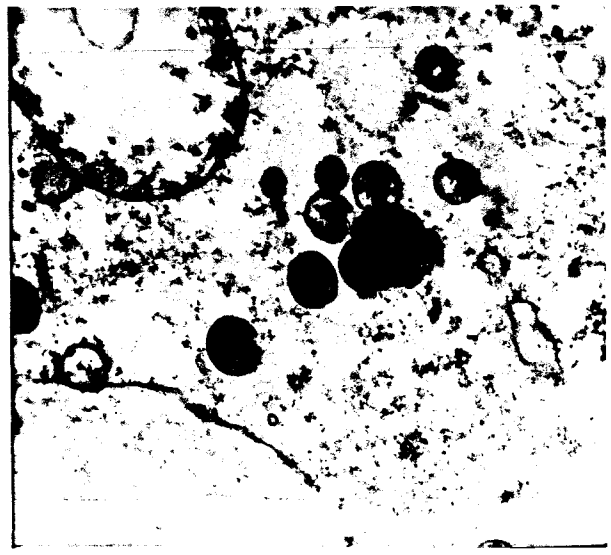


Fig. II.3. Electron micrograph of ts7 at 9 hpi.

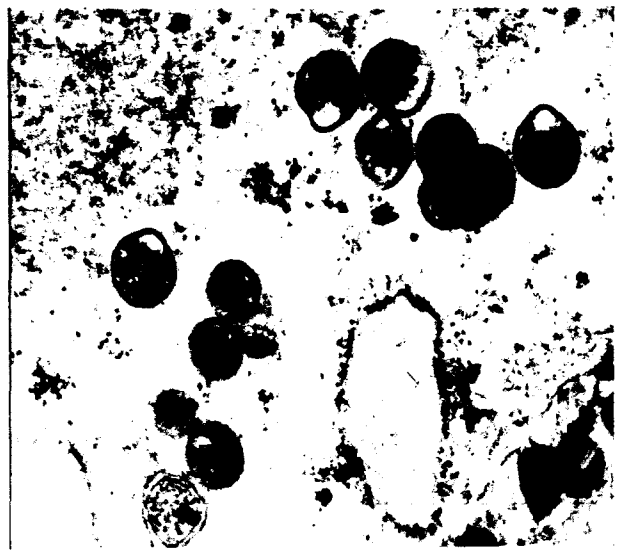


Fig. II.4. Electron micrograph of ts7 at 9 hpi.

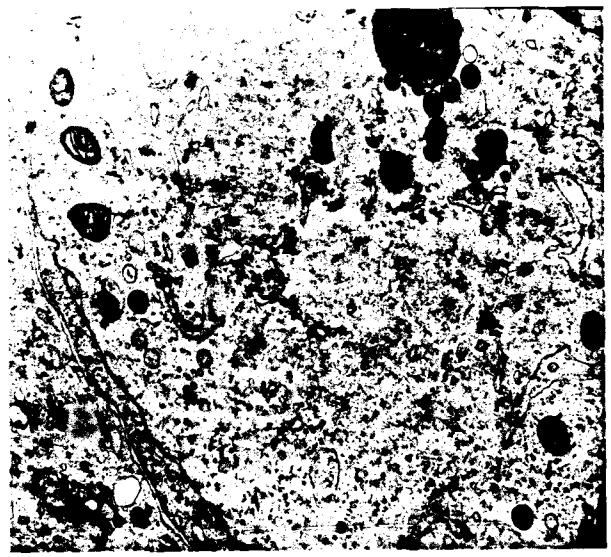


Fig. II.5. Electron micrograph of ts22 at 7 hpi.

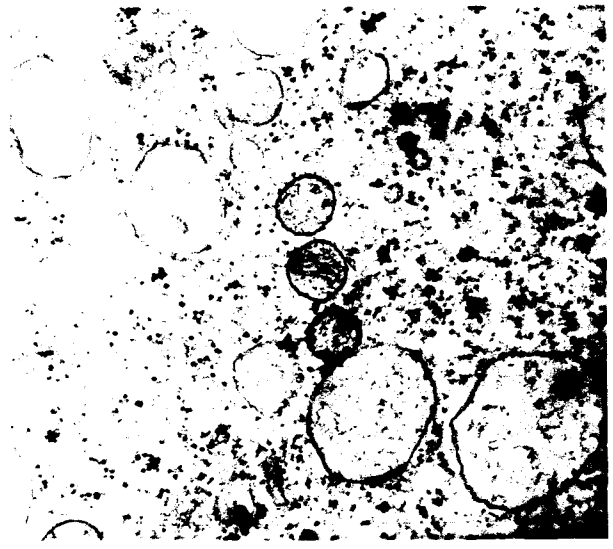


Fig. II.6. Electron micrograph of ts22 at 9 hpi.

eventually enclose little, or no viroplasm (Fig. II.8). At 9 hpi, the interior of the viral particles remained clear, and defects in envelope assembly become apparent with the formation of multilayered envelopes (Fig. II.9). Also present in and out of the viral factories are short fragments of the viral envelope organized into aggregates having a parallel arrangement (Fig. II.10).

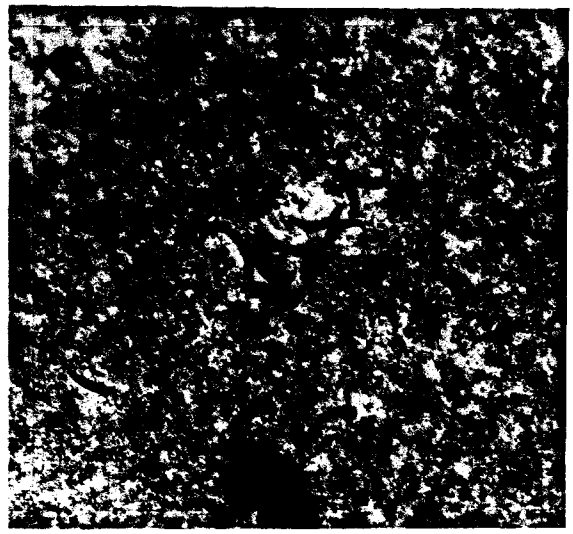


Fig. II.7. Electron micrograph of ts5 at 7 hpi.

Fig. II.8. Electron micrograph of ts5 at 7 hpi.

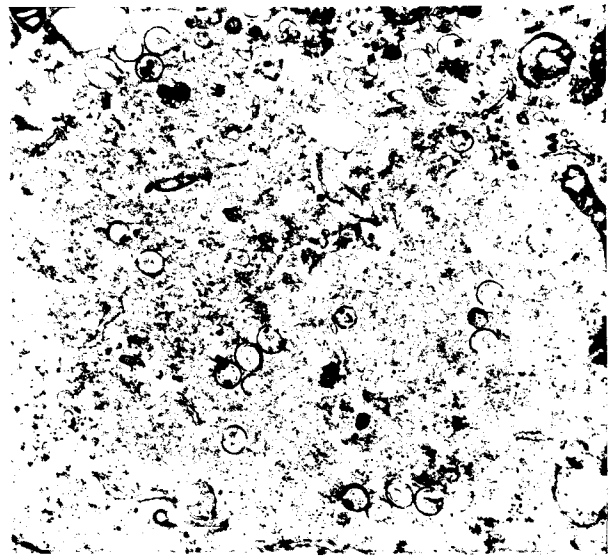


Fig. II.9. Electron micrograph of ts5 at 9 hpi.

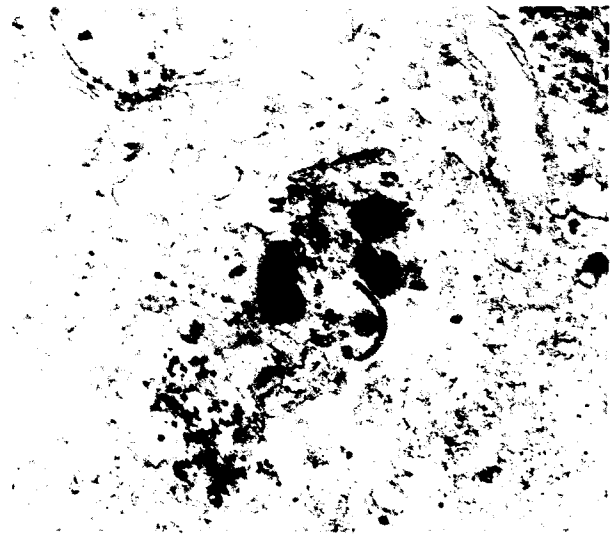


Fig. II.10. Electron micrograph of ts5 at 9 hpi.

Discussion

A previous electron microscopic survey where morphological defects were correlated with a specific mutation used a collection of ts mutants for which little or no genetic or biochemical information was obtained (Dales et al., 1978). Analysis of this assay of almost 80 ts mutants produced 17 categories of defective morphological phenotypes or blocks in assembly.

The morphological phenotypes described here are derived from representatives of four complementation groups and therefore exhibit morphological defects caused by mutations in four different required genes. The phenotypes described here are also present in the previous work by Dales. The DNA- mutant ts17 can be compared with category A, where no cytoplasmic evidence of a VV infection was present. This phenotype is consistent with results obtained when VV DNA replication is inhibited by drugs such as hydroxyurea (Pogo and Dales, 1971). The mutants ts7 and ts22, of the defective-late and abortive-late classes respectively, are similar to both categories G and I of the Dales' collection. Both G and I consist of immature particles with no differentiation of their electron dense center. Ts5, of the wild-type biochemical class, exhibited defective membrane biogenesis. It is very similar to category F of the Dales' collection. F mutant virions exhibited

multiple layers of the lipoprotein envelope. Also, aggregates of envelope fragments organized in a parallel arrangement were also common. It is of interest to note that Dales reported only two isolates in this class, suggesting that this defect may be due to a single defective function.

Many of the morphological phenotypes described here are reminiscent of phenotypes caused by specific metabolic inhibitors (Pogo and Dales, 1971; Moss et al., 1969). An advantage of using a conditionally lethal mutant instead of a drug to target a specific function is that the drug may have multiple effects on viral, as well as cellular functions. In either case, interpretation of morphological data is very difficult. To relate a specific gene function to an aberrant phenotype is not realistic, particularly in the case of as complex a system as VV. Gene expression in VV consists of overlapping layers of highly-regulated temporal classes (Weinrich and Hruby, 1987) and it would be difficult to relate a genetic defect to when it became apparent in the assembly process.

Besides ts17, which is reported on in this thesis, only ts22 has been further studied (Pacha and Condit, 1985). It was reported that in ts22, which is of the abortive-late class, transcription of late genes occurs normally, yet these transcripts are biologically inactive in vitro. It appears that at the time of aborted late protein synthesis, cleavage of rRNA and late viral transcripts occurs in vivo. It was suggested

that ts22 is defective in a function which prevents rRNA and late viral mRNA degradation. The morphological phenotype of ts22 prior to this biochemical analysis holds little information. In concert with this data, it is interesting that late in infection (9 hpi) the interior of the immature virus particle no longer contains viroplasm and the envelope loses its structural integrity. Functions that may be required to maintain these structures appear to be absent.

Since the information that can be obtained from this electron microscopy survey is limited, a single mutant, ts17, was chosen for a detailed study. Ts17 was chosen due to its excellent genetics, and because of its DNA- phenotype. A mutation in a viral gene required for DNA replication is of interest because identification of genes involved in DNA metabolism are of basic biological interest; DNA replication is required for the switch from early to late gene expression; and unlike most other DNA-containing viruses, VV DNA replication is temporally regulated (Moss, 1978). The mapping and a detailed biochemical analysis of ts17 is currently in progress.

CHAPTER III

Biochemical Characterization of a DNA Negative Temperature Sensitive Mutant of Vaccinia Virus

Summary

The biochemical phenotype of a temperature sensitive mutant of vaccinia virus, ts17, was examined. This mutant was found to exhibit characteristics typical of DNA negative (DNA-) viral mutants previously reported. Ts17 infected BSC_{40} cells incubated at the non-permissive temperature of 40°C exhibited no viral DNA synthesis when examined by hybridization methods. L-[^{35}S]methionine pulse-labelling of cells infected with ts17 at 40°C indicated that early protein synthesis was prolonged and late viral proteins were not produced. A time course of ts17 infected cells showed that early viral proteins were produced for up to 24 hours post-infection. These results are discussed in relation to the possible function encoded by the ts17 locus.

Introduction

Vaccinia virus (VV) contains a large (185 kilobasepair) double-stranded DNA genome. Viral replication, which occurs in the cytoplasm of infected cells, is characterized by the tightly regulated, temporal expression of more than 100 detectable gene products. VV apparently encodes for, and packages, all the functions necessary for the production of mature mRNA (Moss, 1974). Furthermore, a number of enzymatic activities required for viral DNA synthesis have been identified in the cytosol of VV-infected cells: thymidine kinase, DNA polymerase, deoxyribonucleases, topoisomerase, and DNA ligase (Dales and Pogo, 1981; Moss, 1974). Mapping of individual viral transcriptional units on such a large genome has been accomplished primarily through the utilization of cloned fragments of viral DNA in concert with hybrid-selection and cell-free translation techniques (Belle Isle et al., 1981; Cooper et al., 1981; Mahr and Roberts, 1984). Locating genes of a defined function has proven more difficult, with thymidine kinase (Hruby and Ball, 1984; Weir et al., 1982) and DNA polymerase (Jones and Moss, 1984; Traktman et al., 1984) being the only viral enzymes thus far mapped.

To facilitate the study of the regulatory mechanisms involved in gene expression and viral assembly, a large collection of temperature sensitive (ts) mutants has been isolated. These were subjected to genetic and biochemical

analyses which suggested their organization into four biochemical phenotypes: DNA-, defective-late, abortive-late, and wild-type (wt). Mutants which fell into the same complementation group often exhibited the same biochemical phenotype. Also, marker rescue mapping using HindIII fragments of wt DNA confirmed the assignment of complementation groups: mutants of the same complementation group mapped to the same HindIII fragment (Condit and Motyczka, 1981; Condit et al., 1983).

Utilizing this collection of mutants, we chose to identify and study a viral gene which is essential for DNA replication. An examination of functions required for DNA synthesis is of particular interest since DNA replication is required for the switch from early to late gene expression to occur (Moss, 1978). Also, the onset and cessation of DNA replication is temporally regulated. In this collection of mutants three complementation groups have been identified as having a DNA- phenotype. These are complementation groups 5, 21, and 24. Of these, group 5 has been identified as containing the DNA polymerase gene (Traktman et al., 1984).

Towards an understanding of the regulatory mechanisms involved in the extraordinarily complex replication scheme of vaccinia virus, we chose to focus on a member of complementation group 21, ts17. Ts17, together with ts24, comprise complementation group 21. Marker rescue mapping demonstrated that the 16-kb HindIII D fragment restored both members of this

complementation group to the wt phenotype (Condit et al., 1983), placing this gene in the central conserved portion of the VV genome.

Methods and Materials

Cells and virus. BSC_{40} cells were grown in monolayer cultures in Eagle minimum essential medium (MEM, Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 50 ug of gentamicin sulfate per ml. HeLa cells were maintained at a concentration of 5 x 10^5 cells per ml in suspension cultures in Eagle MEM supplemented with 5% FCS. Viral infections and plaque assay titrations were performed as previously described (Hruby et al., 1979). The isolation of temperature sensitive mutants of VV has been described (Condit and Motyczka, 1981; Condit et al., 1983). VV strain WR is designated as wild-type in these experiments.

Molecular cloning. Vaccinia virus HindIII fragments D through O cloned in pBR322 were kindly supplied by B. Moss (NIH). DNA manipulations were performed essentially as described (Maniatis et al., 1982).

Virus growth. Duplicate 60 mm plates of BSC_{40} cells were infected with VV at a multiplicity of infection (moi) of 5, then placed at either -20°C, 31°C or 40°C. After a 24 hour incubation period the infected cells were harvested and infectious virus was titered at 31°C (Hruby et al., 1979).

DNA synthesis. Confluent 60 mm plates of BSC_{40} cells were infected at a moi of 10 pfu/cell. One plate was infected in the presence of 5 mM hydroxyurea, added 1 hour prior to infection

and maintained at that concentration throughout the infection. Cells were harvested at indicated times and subjected to three rounds of freeze-thawing in 500 ul phosphate-buffered saline. 125 ul of the extract was mixed with 125 ul 0.25% trypsin and incubated at 37°C for 15 minutes, then placed on ice. The extracts were then filtered through a minifold apparatus (Schleicher & Schuell) and collected on nitrocellulose. The presence of viral DNA was then detected by southern hybridization using radioactively labelled viral DNA fragments (Maniatis et al., 1982).

Protein synthesis. 35-mm plates of BSC_{40} cells were infected with VV at a moi of 10 and placed at either 31°C or 40°C. At the indicated times post-infection, the medium was replaced with medium (minus methionine) containing 10 uCi of L-[^{35}S]methionine (New England Nuclear Corp.;1,151 Ci/mmol) per ml. Plates were then incubated for 30 minutes at either 31°C or 40°C. Cells were harvested and then pelleted by low-speed centrifugation and then resuspended in ice-cold 1 mM Tris-hydrochloride (pH 9.0).

Protein Gels. Labeled VV infected cell lysates were analyzed on 12% polyacrylamide slab gels using the method of Studier (Studier, 1973). Gels were fluorographed, dried, and Kodak XAR-5 x-ray film was exposed at -70°C.

Results

Replication of ts17. The ability of ts17 to replicate at the permissive or non-permissive temperature was examined and compared to that of wt virus. Virus growth was examined through a single cycle growth experiment. Cells synchronously infected at an moi of 10 pfu/cell with either ts17 or wt were incubated for 24 hours at either the permissive temperature of 31°C or the non-permissive temperature of 40°C. One set of infected cells were placed at -20°C at 0 hours post-infection (hpi) to quantitate ingoing virus. Following three cycles of freeze-thawing the samples were titered at 31°C (Table III.1). Ts17 exhibted no growth at 40°C, demonstrating it to indeed be a conditional lethal mutant.

DNA replication. An examination of viral DNA replication using a ^{3}H-thymidine pulse-labeling protocol confirmed the prior classification of ts17 as a DNA- mutant (data not shown). However, we chose to conclusively demonstrate that the mutant is unable to synthesize DNA, and not simply aberrant in thymidine incorporation, through a sensitive dot blot hybridization. Cells infected with either wt or ts17 were incubated at either 31°C or 40°C. Cytoplasmic extracts were made at 0 and 6 hpi. Mock infected cells and cells infected in the presence of hydroxyurea, which blocks viral DNA synthesis, were incubated at 37°C and harvested at 6 hpi. Samples were processed as

	0 hpi	24 hpi 31 C	24 hpi 40 C	% Growth
ts17	3.6×10^5	1.0×10^7	7.2×10^4	0.72
WT	1.8×10^5	2.2×10^7	1.7×10^7	77.0

TABLE III.1. Replication of ts17 as compared to wt VV at the permissive (31°C), or non-permissive (40°C) temperatures. After a 24 hour incubation at the indicated temperature, progeny virus was harvested. Titers were determined by plaque assay at 31°C.

described (methods and materials) and then filtered through a mini-fold apparatus onto nitrocellulose. The presence of de novo synthesized DNA was then determined by hybridization with nick-translated HindIII D (Fig. III.1). Little or no viral DNA is detected by the probe in the samples which were mock infected, treated with hydroxyurea, or harvested at 0 hpi. When incubated at the permissive temperature, ts17 and wt appear to synthesize comparable amounts of DNA. At the non-permissive temperature DNA synthesis by ts17 is blocked, whereas DNA synthesis by wt VV appears unimpeded.

Past work suggests that VV DNA replication is initiated at the terminus of the linear viral genome (Wittek and Moss, 1980). Therefore, to show whether DNA replication was initiated but unable to elongate, nick-translated SalI I and K fragments derived from the inverted terminal repeats were used as probes. These yielded results identical to that shown in Figure III.1.

Protein synthesis. During a VV infection, viral DNA synthesis is required for the termination of early protein production and for late proteins to be expressed. To determine the temporal expression of viral polypeptides in a non-permissive ts17 infection, cells infected with ts17 and incubated at 40°C were pulse-labeled for 30 minutes with L-[^{35}S]methionine at the indicated times. Labeled viral proteins were then analyzed by denaturing polyacrylamide gel electrophoresis (Fig. III.2). Early mRNA appears to be actively

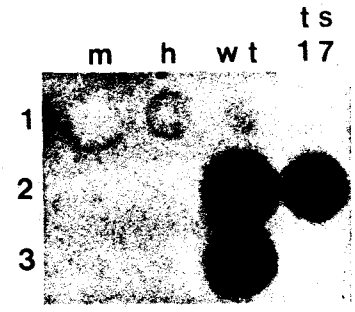


Fig. III.1. Replication of viral DNA by ts17 versus wt vaccinia virus. BSC_{40} cells infected at a multiplicity of 10 were incubated at either 31°C (lane 2), or 40°C (lane 3). Mock-infected cells (m), and cells infected with wt in the presence of hydroxyurea (h) were incubated at 37°C and harvested at 6 hpi (lane 1). Cell extracts of wt and ts17 infected cells were made at 0 (lane 1) and 6 (lanes 2 and 3) hpi. Extracts were filtered through a minifold apparatus onto nitrocellulose and hybridized with uniformly-labeled VV HindIII D DNA.

Fig. III.2. Ts17 protein synthesis at the non-permissive temperature. BSC_{40} cells infected with ts17 at a multiplicity of 10^{40} were incubated at $40^{o}C$. At the indicated times post-infection, the cells were pulse-labeled for 30 minutes with L-[^{35}S]methionine (10uCi/ml). The infected cells were then harvested, and the radioactively-labeled proteins were electrophoresed on a 12% polyacrylamide gel and visualized by autoradiography. Mock-infected pulse (lane M); labeled proteins from a reticulocyte lysate translation programmed with early viral RNA (lane E). WT VV infected cells incubated at $37^{o}C$ were similarly infected and pulse-labeled at the indicated times. Numbers at right indicate the molecular weights ($x10^{3}$) of [^{14}C]-labeled protein molecular weight standards. (Bethesda Research Laboratories).

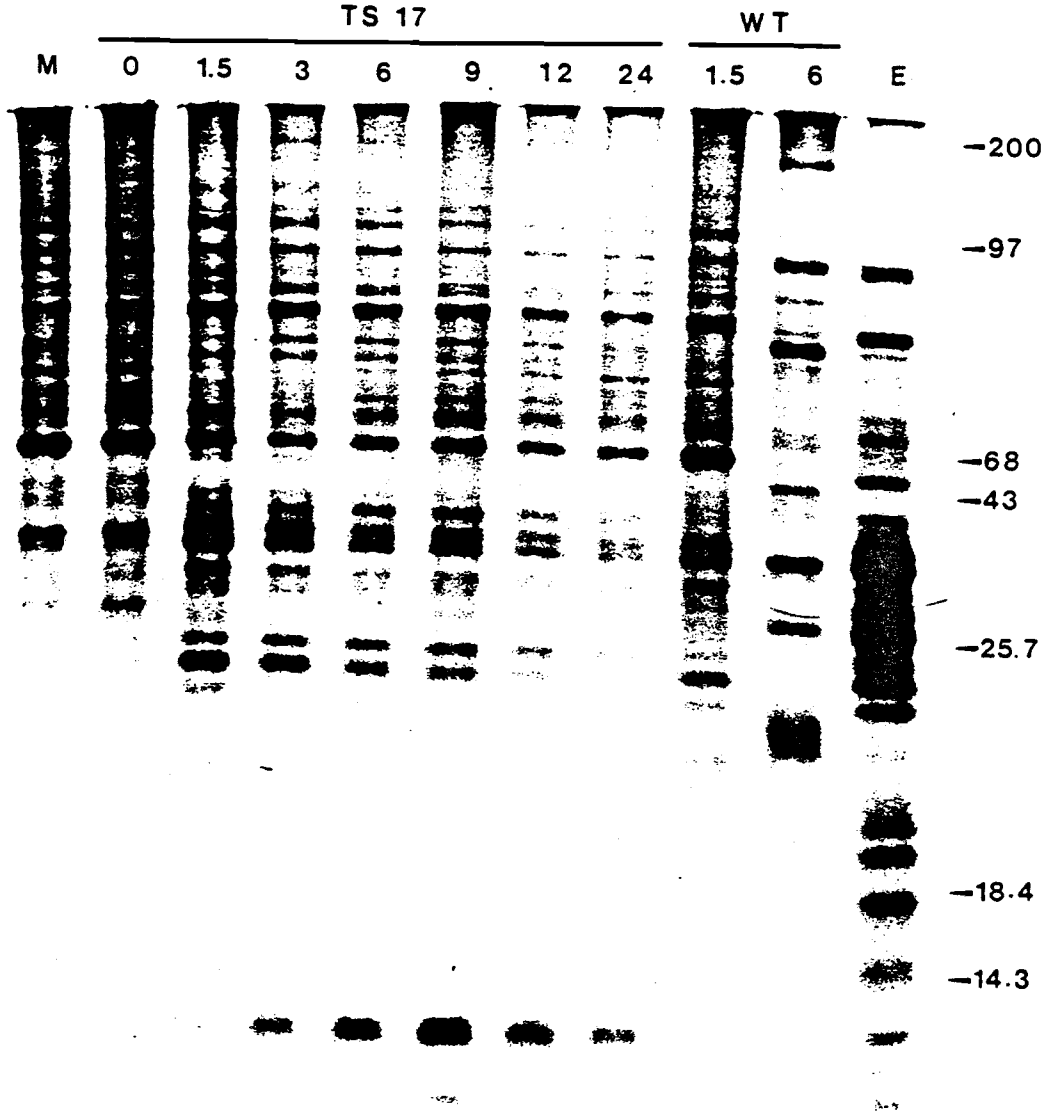


Fig. III.2

translated for at least 24 hpi at 40°C in ts17 infected cells. The production of early viral proteins for 24 hpi by ts17 appears to be consistent with the requirement of DNA synthesis for late protein expression.

Discussion

Elucidation of the regulatory mechanisms involved in the expression of VV genes requires that a number of genes from various temporal classes be specifically mapped and analyzed. Mapping of VV genes which are not assayable through enzymatic activity or which encode a protein which is in low abundance is technically very difficult. By utilizing conditionally lethal mutants in concert with marker rescue techniques, essential gene products can be mapped regardless of activity or abundance. For this study we chose a viral mutant, ts17 which is defective in DNA replication at the non-permissive temperature.

Analysis of the biochemical phenotype of ts17 at the non-permissive temperature of 40°C revealed ts17 to be defective in both DNA synthesis and late protein production. In the absence of DNA replication early proteins continue to be produced for at least 24 hours post-infection.

Of the three complementation groups which have been determined to have a DNA- phenotype, only one has been assigned a function; that being the DNA polymerase gene (Jones and Moss, 1984; Traktman et al., 1984). Other than the nonessential enzyme thymidine kinase, no other function required for DNA metabolism has been located on the viral genome. We have determined that the ts17 locus does not encode a thermolabile subunit of ribonucleotide reductase (data not shown, Slabaugh and Mathews,

1984; Slabaugh et al., 1984). The possibility that the ts17 locus encodes a topoisomerase or a deoxyribonuclease will be examined.

Examination of transcriptionally active regions of the viral genome show transcripts to be organized in tightly spaced tandem arrays (Golini and Kates, 1984; Mahr and Roberts, 1984a; Morgan and Roberts, 1984; Pluciennicczak et al., 1985; Weinrich and Hruby, 1986). Preliminary sequence analysis of the region surrounding the ts17 gene indicates that this region of the genome is similarly organized (Niles and Condit, personal communication).

Only through the detailed analysis of the regulatory sequences of specific genes can the significance of the architecture of the VV genome be understood. Therefore, a detailed study of the regulation of the ts17 gene is underway. Whether the ts17 gene product is involved in the expression or function of another gene and its product, or is directly involved in DNA replication will also be addressed.

CHAPTER IV

Nucleotide Sequence and Transcript Organization of a Region of the Vaccinia Virus Genome which Encodes a Constitutively-Expressed Gene Required for DNA Replication

Authors: Nancy A. Roseman and Dennis E. Hruby

Summary

A vaccinia virus (VV) gene required for DNA replication has been mapped to the left side of the 16 kilobase (kb) VV HindIII D DNA fragment by marker rescue of a DNA- temperature sensitive mutant, ts17, using cloned fragments of the viral genome. The region of VV DNA containing the ts17 locus (3.6 kb) was sequenced. This nucleotide sequence contains one complete open reading frame (ORF) and two incomplete ORFS reading from left to right. Analysis of this region at early times revealed that transcription from the incomplete upstream ORF terminates coincidentally with the complete ORF encoding the ts17 gene product which is directly downstream. The predicted proteins encoded by this region correlate well with polypeptides mapped by in vitro translation of hybrid-selected early mRNA. The nucleotide sequences of a 1.3 kb BglII fragment derived from ts17 and from two ts17 revertants were also determined, and the nature of the ts17 mutation was identified. S1 nuclease protection studies were carried out to determine the 5' and 3' ends of the transcripts and to examine the kinetics of expression of the ts17 gene during viral infection. The ts17 transcript is present at both early and late times post-infection indicating that this gene is constitutively expressed. Surprisingly, the transcriptional start throughout infection occurs at the proposed late regulatory element TAA

which immediately precedes the putative initiation codon ATG. Although the biological activity of the ts17 encoded polypeptide was not identified, it was noted that in ts17 infected cells, expression of a non-linked VV immediate-early gene (thymidine kinase) was deregulated at the non-permissive temperature. This result may indicate that the ts17 gene product is functionally required at an early step of the VV replicative cycle.

Introduction

Vaccinia virus is a large DNA-containing animal virus which replicates in the cytoplasm of susceptible host cells. VV is an attractive system for the study of gene regulation in higher organisms due to the temporally coordinated expression of its approximately 200 genes (Moss, 1985). At the onset of infection immediate-early viral RNAs are transcribed by the virally-encoded and packaged RNA polymerase (Munyon et al., 1967). A protein product of this temporal class is then required for the expression of the delayed early genes, as their expression is blocked by the protein inhibitor cycloheximide. Approximately two hours post-infection viral DNA replication is initiated. Concomitant with DNA replication is the repression of the expression of most early genes and the initiation of late gene expression. Thus, the expression of most VV genes can be classified as immediate-early, delayed-early or late. However, there is also an additional constitutive temporal class of genes which appear to be expressed at both early and late times during infection as typified by the well-studied 7.5 kd gene (Cochran et al., 1985b) and other VV genes (Weinrich and Hruby, 1987).

The precise mechanisms involved in the modulation of VV gene expression are unknown, although they are believed to operate at the transcriptional, post-transcriptional, translational, and postranslational levels. Through the

identification and analysis of a number of genes from specific temporal classes it should be possible to identify regulatory elements involved in gene expression. Sequence analysis of various regions of the viral genome (Bertholet et al., 1985; Hirt et al., 1986; Hruby et al., 1983; Niles et al., 1986; Pluciennicczak et al., 1985; Rosel and Moss, 1986; Venkatesan et al., 1981; Venkatesan et al., 1982; Weinrich and Hruby, 1986; Weir and Moss, 1983; Weir and Moss, 1984) has identified many open reading frames which correlate well with earlier transcriptional and translational mapping studies (Belle Isle et al., 1981; Cabrera et al., 1978; Chipchase et al., 1980). Upstream cis-acting regulatory sequences have been identified for both early and late genes, but they appear to have little obvious homology to previously identified prokaryotic or eukaryotic promoters (Bertholet et al., 1985; Hangii et al., 1986; Mackett et al., 1984; Vassef et al., 1985; Weir and Moss, 1984).

In this study we have mapped a viral gene of the constitutive temporal class by marker rescue of a temperature sensitive (ts) DNA- mutant, ts17 (Condit and Motycaka, 1981; Condit et al., 1983). The ts17 locus was mapped to within the HindIII D fragment and the nucleotide sequence of this region was determined from wild-type (wt) viral DNA. While this work was in progress the nucleotide sequence of the entire HindIII D fragment was reported (Niles et al., 1986), nomenclature for ORFS is in relation to the reported sequence and according to

the recently proposed system for VV genes (Rosel et al., 1986). Here, we independently confirm a portion of the reported sequence and show that this region is transcriptionally active *in vivo*. Comparison of sequence derived from DNA isolated from *ts*17 virus, two *ts*17 revertants (*ts*17^{r}), and from wt virus, identified the mutation responsible for the temperature sensitivity of *ts*17. Also, analysis of the sequence in conjunction with transcriptional and translational studies of this region showed that transcription of D4, which lies directly upstream of D5; to which *ts*17 maps; terminates coincidentally with the D5 transcript.

The transcriptional kinetics of the *ts*17 gene was examined by S1 nuclease protection studies of steady state mRNA levels. This showed that the *ts*17 gene is a member of the constitutive temporal class of viral genes. The putative promoter of the *ts*17 gene was compared with those of previously reported VV genes.

Methods and Materials

Cells and virus. BSC_{40} cells were grown in monolayer cultures of Eagle minimum essential medium (MEM, Flow Laboratories) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 50 ug of gentamicin sulfate per ml. VV (strain WR) is designated as wild-type in these experiments. Revertants of ts17 were generated by repeated passages of ts17 virus at 40°C. Ts17 revertants were then plaque purified at 40°C.

Marker rescue. Cloned wt VV DNA fragments were purified and sterilized by sequential extractions with phenol and ether, followed by ethanol precipitation. One to 2 ug of viral DNA was resuspended in Hepes-buffered saline and co-precipitated with salmon sperm DNA to a final concentration of 20 ug/ml using calcium phosphate (Graham and Ven Der Eb, 1973). Confluent 60-mm plates of BSC_{40} cells were infected with ts17 at a multiplicity of infection of 0.1 and incubated at 31°C for 3 hours. The media was removed, the monolayer washed with MEM(-FCS), and 15% glycerol was added for 40 seconds. The monolayer was then washed twice with MEM(-FCS) and the precipitated DNA was pipetted onto the cells. Plates were incubated at 40°C for 3 hours, washed, and MEM + 10% FCS was added. Plates were then incubated for 48 hours at 40°C, the infected cells harvested and infectious virus was titered at

31°and 40°C

Cloning and sequencing. A clone of the HindIII D fragment of VV DNA was originally obtained from B. Moss (NIH). Subfragments of viral DNA were cloned into pUC vectors by standard methodology (Maniatis et al., 1982). M13 phage vectors were used for sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977). Sequences were analyzed on an IBM PC using Microgenie software obtained from Beckman Instruments, Inc. (Queen and Korn, 1984). Chemical sequencing was performed essentially as previously described (Maxam and Gilbert, 1977). Enzymes and reagents were obtained from Bethesda Research Laboratories, Boehringer Mannheim, P-L Biochemicals, and Aldrich Chemical Company, Inc. Radioisotopes were obtained from New England Nuclear Corp.

Northern analysis. Two ug of polyadenylated viral RNA (isolated essentially as described in Weinrich et al., 1985) was electrophoresed in a formaldehyde-agarose gel as described (Lehrach, et al., 1984). Following transfer to nitrocellulose, specific transcripts were detected using the indicated nick-translated viral DNA fragments as probes.

Hybrid-selection. 20 ug of recombinant plasmid DNA was digested to completion with an appropriate restriction endonuclease and bound to 5 3-mm square nitrocellulose filters. 20 ug of polyadenylated RNA isolated from VV-infected cells in the presence of 100 ug/ml cycloheximide was used in hybridization reactions as described (Belle Isle et al., 1981).

Eluted mRNA was translated in vitro in rabbit reticulocyte lysates which contained 5 uCi of L-[^{35}S]methionine (New England Nuclear Corp., 1,085 Ci/mmol) (Hruby and Ball, 1982).

S1 nuclease mapping. 5' or 3' end-labeled DNA fragments were mixed with 20 ug of viral RNA in a total volume of 30 ul containing: 80% formamide-40mM PIPES (pH 6.4)-0.4 M NaCl-1 mM EDTA. The samples were heated to 90 degrees for 5 minutes, then incubated at 42 degrees for four hours. Hybridization reactions were then plunged into ice, 0.3 ml of ice-cold 0.28 M NaCl-0.05 M sodium acetate (pH 4,6)-4.5 mM ZnSO4-400 U/ml nuclease S1 was added. After 5 minutes on ice, the samples were incubated at 25^{o}C degrees for 1 hour, then extracted with phenol:chloroform, ether and ethanol precipitated. Protected fragments were analyzed on sequencing gels.

Thymidine kinase assay. Duplicate sets of 60-mm dishes of Ltk^{-} cells were infected at a multiplicity of 20 pfu/cell with wt VV or the indicated ts mutants. After a 30 minute adsorption at 25^{o}C, pre-warmed (31^{o}C or 40^{o}C) media was added. One set of plates was incubated at the permissive temperature of 31^{o}C and the other at the non-permissive temperature of 40^{o}C. At two hour intervals cytoplasmic extracts were prepared and frozen. Extracts were subsequently assayed for thymidine kinase activity (Hruby and Ball, 1982).

Results

Marker Rescue Mapping Previously reported marker rescue mapping using the HindIII fragments of VV located the ts17 locus within the 16 kb HindIII D fragment (Condit et al., 1983). Subclones derived by restriction enzyme digests of wt HindIII D which were used for fine mapping of the ts17 locus are shown (Fig. IV.1). Viral DNA fragments were calcium phosphate precipitated and transfected into BSC_{40} cells which had been previously infected with ts17 virus. Following a 48 hour incubation at the non-permissive temperature of 40°C, the infected cells were harvested and viral progeny were titered at the permissive temperature of 31°C and at the non-permissive temperature of 40°C (Table IV.1). At the permissive temperature of 31°C, ts17 replication was independent of the identity of the transfected DNA fragment. Whereas, at the non-permissive temperature of 40°C, replication was dependent upon the homologous recombination of a wt viral DNA fragment which contains the appropriate sequences. Marker rescue using pSW1, a 7.0 kb fragment derived from the right-hand side of HindIII D, resulted in ts17 remaining temperature sensitive. However, the 8.1 kb BamHI A fragment from the left-hand side of HindIII D was able to confer temperature insensitivity. The mutation was mapped further to an EcoRI subclone of BamHI A; the 3.3 kb EcoRI B fragment. EcoRI B was subcloned into three BglII

Fig. IV.1. Diagrammatic representation of the HindIII restriction map of the VV genome. Restriction fragments generated by digestion of the 16 kbp Hind III D fragment with BamHI (⬇) and EcoRI (⬆) are shown. Restriction fragments generated by digestion of the 3.3 kb EcoRI B fragment by BglII (▲) are also shown. The 7.0 kb fragment designated clone pSW1 is shown (▼). Those fragments used in the marker rescue study are indicated in the text and in Table 1. Fragments able to rescue ts17 to temperature insensitivity are indicated (*).

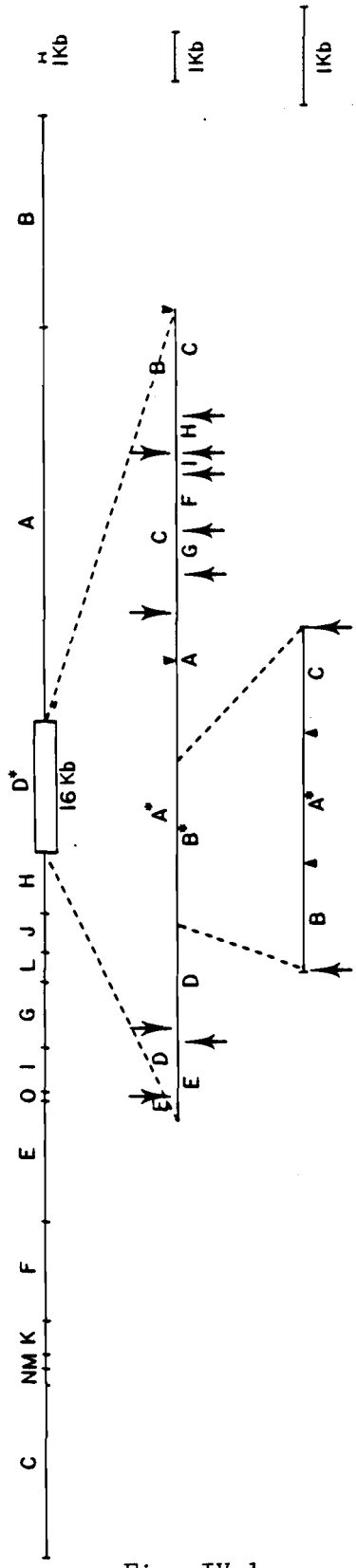


Fig. IV.1

	TITER	
DNA	31 C	40 C
HindIII D	1.7×10^5	1.9×10^5
pSW 1	1.2×10^3	0
BamHI A	6.5×10^4	1.2×10^5
EcoRI B	2.6×10^5	2.8×10^5
BglII A	8.0×10^4	1.6×10^4
BglII B	8.3×10^3	0
BglII C	2.4×10^4	0
SS	4.2×10^3	0

TABLE IV.1. Titers of progeny of marker rescue by subclones of VV HindIII D. Viral DNA was coprecipitated with salmon sperm DNA (SS) and then transfected into ts17 infected BSC_{40} cells.

fragments, and of these the 1.3 kb BglII A fragment contained the sequences required to confer temperature insensitivity to ts17.

Nucleotide Sequence and identification of the ts17 mutation. In order to facilitate study of the ts17 gene the nucleotide sequence of the 3309 bp EcoRI B fragment, and 300 bp reading rightward into the neighboring EcoRI A fragment, was determined by the dideoxynucleotide chain termination method (Fig. IV.2). This sequence was determined by cloning appropriate overlapping subfragments of viral DNA into M13 vectors in both orientations so that both strands could be sequenced. By this strategy over 80% of the sequence of both strands was determined (Fig. IV.3). The sequence reported here is in complete agreement with the recently reported sequence of this region of the genome (Niles et al., 1986).

Analysis of ORFS found within the sequence reported here show tandemly oriented ORFS which read from left to right (Fig. IV.3). In accordance with the recently proposed nomenclature for VV genes these are designated D4, D5 and D6. D4 is incomplete in this sequence, with the G at position 1 actually being the third base of the initiation ATG codon of D4 (Niles et al., 1986). Thus, D4 has the capacity to encode a 25,055 molecular weight polypeptide. The complete D5 ORF, which spans the site of the ts17 mutation, is 2297 nucleotides long and could specify a polypeptide with a predicted molecular weight of 90,367 daltons. The incomplete ORF D6 encodes 22,234 daltons of

Fig. IV.2 Nucleotide sequence reading rightward beginning at the lefthand EcoRI site of EcoRI B, and extending 300 bp rightward into the EcoRI A fragment. The RNA start sites are marked with arrowheads. The sites of the ts17 mutations are indicated (*).

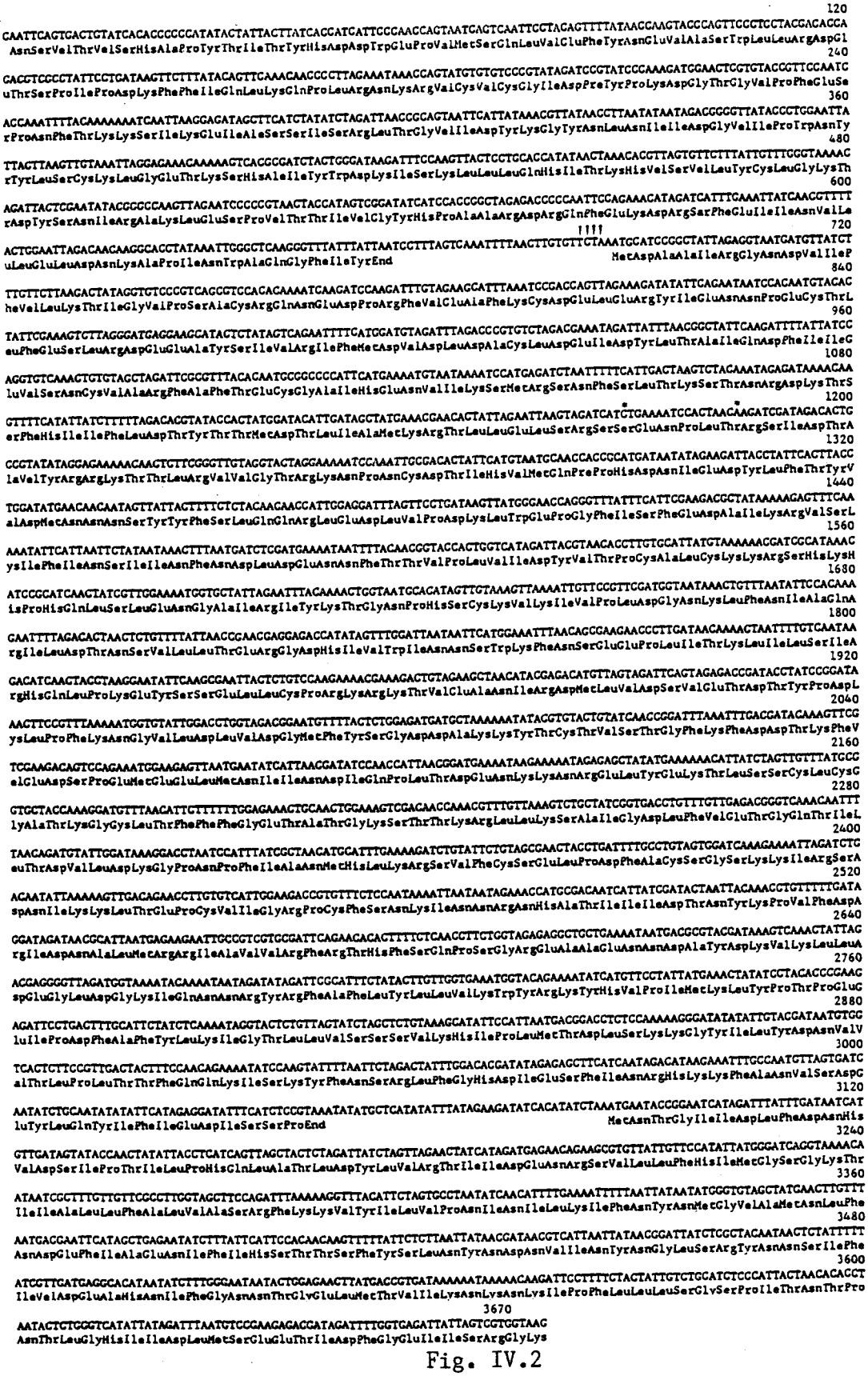


Fig. IV.2

Fig. IV.3. A restriction map of the EcoRI B fragment. The complete orf is shown with a solid line, incomplete orfs are indicated with a broken line. Predicted molecular weights of the orfs are indicated along with their numerical designations. R1, EcoRI; R, RsaI; X, XbaI; P, PstI; E, EcoRV; B, BglII; C, ClaI; K, KpnI; S, SalI; H, HinfI.

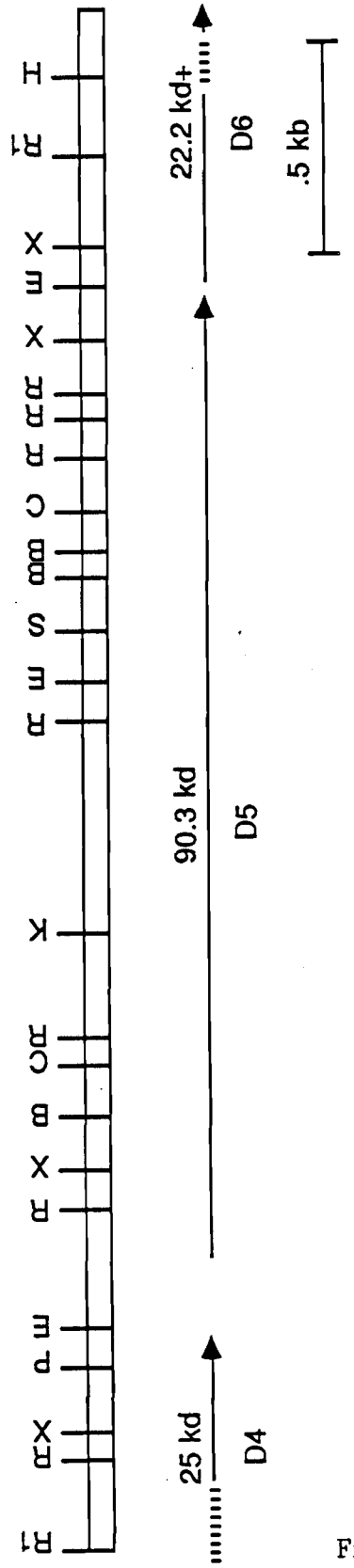

H
R1
X
E
X
RR
R
C
BB
S
E
R
K
RC
B
X
R
E
P
XR
R1
22.2 kd+
D6
.5 kb
90.3 kd
D5
25 kd
D4

Fig. IV.3

what is reported to be a 68,362 molecular weight polypeptide (Niles et al., 1986).

In order to confirm the location of the ts17 gene and to determine the mutation giving rise to it, the 1.3 kb BglII A fragment was subcloned from ts17 viral DNA and the nucleotide sequence was determined. Futhermore, a number of ts17 revertants were isolated by repeated passages of ts17 virus at 40°C. The BglII A fragment from two revertants was also cloned and sequenced (Fig. IV.4). A comparison between the wt and ts17 sequence identified two closely linked mutations. The first being a transition mutation of a C to a T at nucleotide position 1168. This causes an amino acid change of a serine to a phenylalanine. The second mutation is a transversion mutation of an A to a C at nucleotide position 1184. In this case the mutation is silent, the amino acid encoded for remains a threonine. Sequences derived from the two ts17 revertants were identical and showed both ts17^{r}1 and ts17^{r}2 to be true revertants. The nucleotide at position 1168 had reverted back to a C, returning the codon to a serine. However, the silent mutation remained unchanged, thus indicating that ts17^{r}1 and ts17^{r}2 were ts17 revertants and not wt VV. Therefore, the sequence data and the genetic evidence shows that a transition mutation at nucleotide position 1168 is responsible for the temperature sensitive phenotype of ts17 virus.

Hydrophilicity-hydrophobicity analysis. The effect of the single amino acid change caused by the transition mutation on

Fig. IV.4. Nucleotide sequence of the BglII A fragment was determined from viral DNA isolated from wt, ts17 and two ts17 revertants (ts17^r). Shown is the sequence from nucleotide positions 1158 to 1190. The amino acid encoded for is also shown. The nucleotide positions at which there is a change from wt sequence are indicated (*).

VV ISOLATE											
WT	AGT	AGA	TCA	TCT	GAA	AAT	CCA	CTA	ACA	AGA	TCG
	Ser	Arg	Ser	Ser	Glu	Asn	Pro	Leu	Thr	Arg	Ser
ts 17	AGT	AGA	TCA	T★T	GAA	AAT	CCA	CTA	AC★	AGA	TCG
	Ser	Arg	Ser	Phe	Glu	Asn	Pro	Leu	Thr	Arg	Ser
ts 17^r1	AGT	AGA	TCA	TCT	GAA	AAT	CCA	CTA	AC★	AGA	TCG
	Ser	Arg	Ser	Ser	Glu	Asn	Pro	Leu	Thr	Arg	Ser
ts 17^r2	AGT	AGA	TCA	TCT	GAA	AAT	CCA	CTA	AC★	AGA	TCG
	Ser	Arg	Ser	Ser	Glu	Asn	Pro	Leu	Thr	Arg	Ser

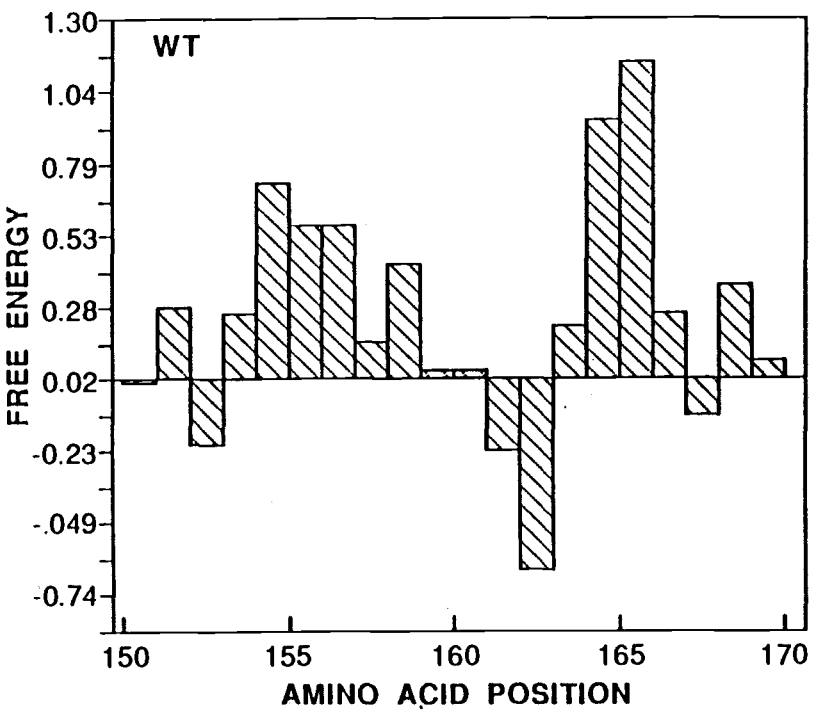
Fig. IV.4

the hydropathic index (Lawson et al., 1984; Taylor, 1986) of the ts17 protein was determined by a comparison of the hydropathy profiles of ts17 and wt (Fig. IV.5). The amino acid change clearly caused a localized change in the hydropathy profile of ts17 as compared to wt. The codons which directly preceed and follow the mutation reflect a higher degree of hydrophobicity. The hydropathy profile of the entire ts17 protein, as predicted from wt sequences was also analyzed. The ts17 protein appears to have a profile typical of a globular protein, although there is a strong hydrophobic region at the carboxy terminus of the polypeptide (data not shown).

Hybrid-selection and *in vitro* translation. To determine the translation products of the transcripts encoded for within EcoRI B, polyadenylated immediate-early viral RNA hybrid-selected by EcoRI B was translated *in vitro* in rabbit reticulocyte lysates in the presence of L-[^{35}S]methionine. Labeled protein products were then electrophoresed on a denaturing polyacrylamide gel and autoradiographed (Fig. IV.6).

The *in vitro* translation system programmed with mRNAs hybrid-selected by the EcoRI B fragment yielded two major polypeptides with molecular weights of approximately 25,500 and 88,000 daltons. This correlates well with the size of polypeptides that can be predicted from the coding capacity of D4 and D5; 25,055 and 90,367 kilodaltons respectively. There does not appear to be a translation product that correlates to D6. This may be due to D6 being of the late viral gene class

Fig. IV.5. The hydrophobicity profile of wt and ts17 in the region of the ts17 mutation. The y axis is the hydrophobicity index, the x axis is the amino acid position. The analysis shown here begins at codon 150 which corresponds with nucleotide position 1137. The ts17 mutation changes the codon at amino acid position 162.


WT
1.30
1.04
0.79
0.53
0.28
0.02
-0.23
-.049
-0.74
FREE ENERGY
150
155
160
165
170
AMINO ACID POSITION

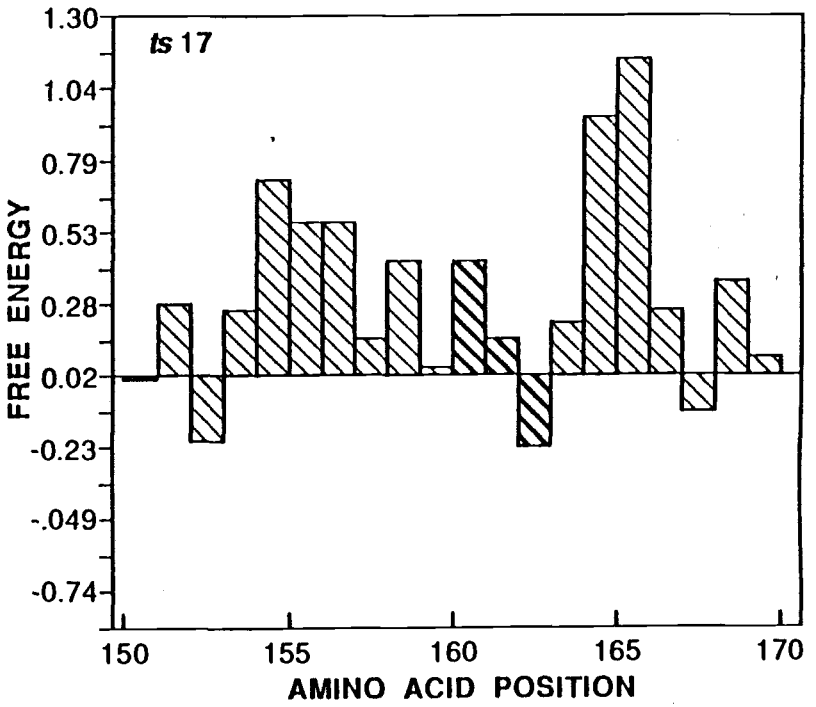

ts 17
1.30
1.04
0.79
0.53
0.28
0.02
-0.23
-.049
-0.74
FREE ENERGY
150
155
160
165
170
AMINO ACID POSITION

Fig. IV.5

Fig. IV.6. Hybrid-selected cell-free translation. Polypeptides synthesized in rabbit reticulocyte lysates were seperated by gel electrophoresis. (-), no RNA added; ET, total early viral RNA; EcoRI B, VV early RNA hybrid-selected by EcoRI B; pUC, early viral RNA hybrid-selected by pUC 18. Lane EP, polypeptides pulse-labeled at 1.5 hpi in wt VV infected cells. Numbers at left indicate molecular weights ($x10^3$) of protein molecular weight standards.

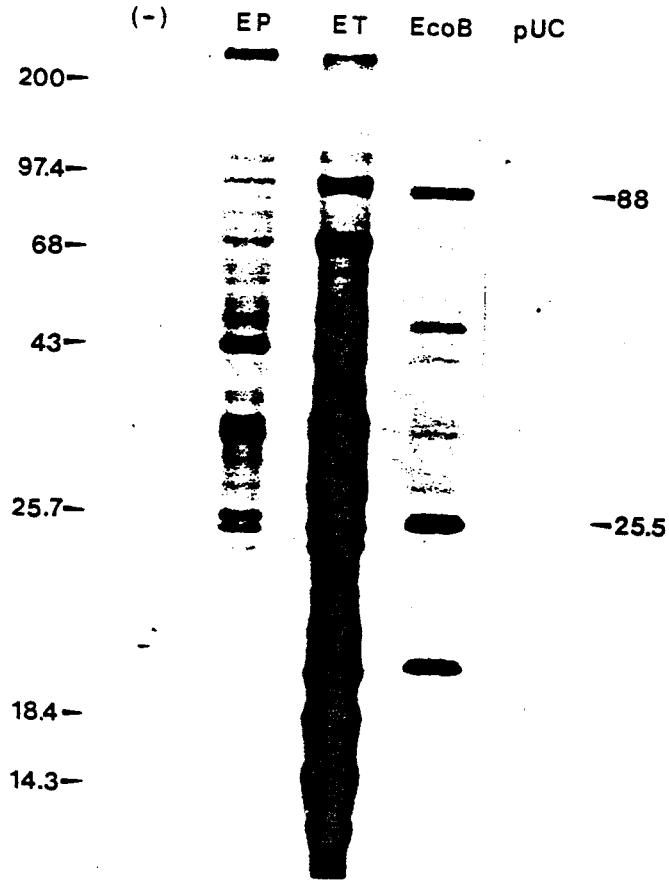


Fig. IV.6

and thus its transcript would not be present in the mRNA population used in this experiment.

The two minor polypeptides of approximately 22,000 and 45,000 molecular weight are endogenous proteins of the translation system and not of viral origin. None of the EcoRI B derived polypeptides co-migrated with any of the major early viral proteins produced either in vivo or in vitro (lanes EP and ET).

Identification of transcripts by northern analysis. To identify messenger RNAs that are transcribed within EcoRI B, polyadenylated immediate-early viral RNA was electrophoresed under denaturing conditions on a formaldehyde-agarose gel and transferred to nitrocellulose (Fig. IV.7). Nick-translated EcoRI B DNA used as a probe revealed a major transcript of 2.7 kb and a minor transcript of 3.6 kb. In order to localize these transcripts more specifically, the BglII subclones of EcoRI B were used as probes; it was found that both transcripts span the three BglII fragments (data not shown).

To further map these transcripts the EcoRI D and EcoRI A fragments which border EcoRI B were used as probes. Neither EcoRI D or EcoRI A appear to hybridize to the two transcripts which are encoded for within EcoRI B. The 2.6 kb transcript which hybridized to EcoRI D correlates well with D1 and may encode the viral guanyl transferase (Morgan et al., 1984). The 0.57 and the 0.98 kb transcripts which map to EcoRI A can possibly be assigned to D7 and D8 respectively. The absence of

Fig. IV.7. Northern blot analysis. Two ug of polyadenylated early viral RNA was electrophoresed on a formaldehyde-agarose gel, then blotted onto nitrocellulose. Nick-translated plasmids containing fragments of VV DNA were used as probes: Lane 1, EcoRI D; lane 2, EcoRI B; lane 3, BglII A; lane 4, EcoRI A. Numbers at right indicate calculated transcript sizes (kb) determined by co-electrophoresis of brome mosaic viral RNAs as molecular weight markers.

Fig. IV.7

a large transcript which can be assigned to D6 may again be due to this gene being expressed late in infection.

This analysis appears to show that the 2.7 and 3.6 kb transcripts are encoded for entirely within EcoRI B. In the case of D4, there does not appear to be a transcript of the size that could be predicted to encode a 25 kd polypeptide that would seem to be indicated by the sequence data and the results of the hybrid-selection *in vitro* translation. This result is not due to potential pleiotropic effects of the drug block (cycloheximide) used to isolate abundant early viral RNA. Only the 2.7 and 3.6 kb transcripts are hybridized by EcoRI B when a northern analysis is repeated using viral RNA isolated at 2 hpi in the absence of drug (data not shown).

S1 nuclease mapping. To determine the organization of the two early transcripts encoded for within EcoRI B, S1 nuclease protection studies were done. In these studies the indicated probe was hybridized to 20 ug of total RNA isolated from cells infected in the presence of cycloheximide. Following S1 digestion, the protected fragments were electrophoresed on a denaturing polyacrylamide gel (Fig. IV.8A).

To map the 5' end of the D4 transcript a 2080 nucleotide 5' single end-labeled XbaI probe was isolated. This probe protected a fragment of approximately 310 bases, mapping the 5' start of D4 to approximately 23 nucleotides upstream of the EcoRI D-B junction and the initiation ATG. The 5' end of D5, which encodes the ts17 gene, was determined by utilizing a 404

Fig. IV.8. (A) S1 nuclease mapping of transcripts encoded by EcoRI B. Mapping of the termini of early RNA using DNA fragments labeled at either the 5'or 3' position. Labeled DNA fragments were hybridized with early RNA, S1 nuclease treated, and protected hybrids were then analyzed on sequencing gels. Lane 1, Sau3A digest of pUC18; lane 2, full length 404 nt 5' end-labeled probe; lane 3, S1 analysis using 404 nt probe; lane 4, full length 2050 nt 5' end-labeled probe; lane 5, S1 analysis using 2080 nt probe; lane 6, S1 analysis using 3' end-labeled 3286 nt probe. The full length 3286 nt probe does not enter the gel system. Size in nt of the probes and protected fragments are indicated. (B) S1 nuclease mapping of the mRNA start site. Lane S1 is the S1 nuclease-resistant fragment (wavy line) from the hybridization of a 5' single end-labeled probe (solid line) and early mRNA. Lane A+G is a Maxam-Gilbert A+G reaction of the indicated probe. The size of the probes and the protected fragments are indicated in nucleotides. The sites of initiation are indicated with arrowheads within the context of the sequence. (C) Diagrammatic representation of the organization of early mRNAs transcribed in EcoRI B.

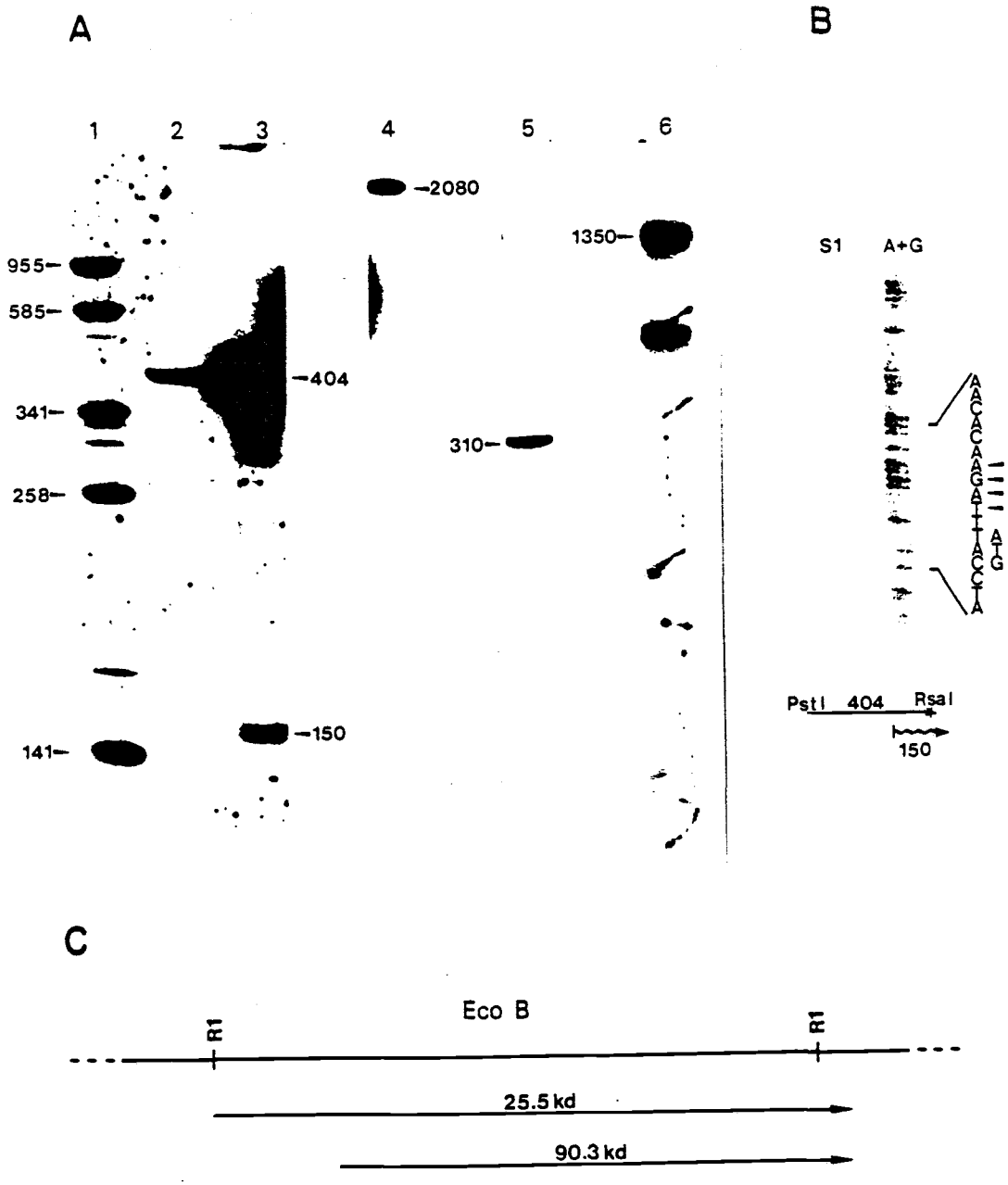

A
B
1
2
3
4
5
6
955
585
341
258
141
2080
404
310
150
1350
S1
A+G
AACACAAGAATACCTA
ATG
PstI
404
RsaI
150
C
Eco B
R1
R1
25.5 kd
90.3 kd

Fig. IV.8

nucleotide 5' single end-labeled PstI-RsaI fragment as a probe. A protected fragment of approximately 150 nucleotides maps the transcriptional start of D5 about 690 nucleotides downstream of the EcoRI D-B junction.

In order to map the transcriptional stop of the two transcripts DNA fragments which spanned the entire EcoRI B fragment were 3' end-labeled and used as probes. The results showed full length protection of these probes indicating that there is no early termination of transcription within the EcoRI B fragment (data not shown). Therefore, a 3286 nucleotide SalI fragment which spans the EcoRI B-A junction was 3' end-labeled and used as a probe. A protected fragment of 1350 nucleotides mapped a single 3' end approximately 200 nucleotides downstream of the EcoRI B-A junction. A second protected fragment seen in lane 6 does not map a 3' end within EcoRI B since a smaller overlapping probe shows only full length protection. This protection must arise from a leftward reading early transcript encoded for by EcoRI A.

This analysis identified two early transcripts reading from left to right which are approximately 3590 and 2877 nucleotides in length. This is consistent with the northern data and shows that the two transcripts terminate coincidentally (Fig. IV.8C).

To facilitate future studies of the ts17 gene and its promoter, the transcriptional start site for D5 was specifically mapped. The 404 nucleotide PstI-RsaI fragment was 5'

end-labeled at the RsaI site, gel isolated and hybridized to 20 ug of viral RNA as described above. Following S1 nuclease digestion, protected fragments were electrophoresed alongside the products of an A + G chemical sequencing reaction of the 404 nucleotide probe (Fig. IV.8B). Multiple transcription initiation sites were mapped at positions -2 to -5 nucleotides upstream of the first ATG of D5. Initiation occurs within the sequence TAA which lies directly upstream of the putative initiation codon, ATG.

Transcript kinetics. Since the sequence element TAAAT has been proposed to be a late regulatory element and has here been shown to occur in a gene the expression of which is required early, the kinetics of transcription initiation of the ts17 gene was determined. This was accomplished by S1 analysis of the steady state mRNA levels over time.

The 5' probe previously described (Fig. IV.8B), was hybridized to 20 ug of total RNA which was isolated at hourly intervals throughout infection. This analysis also included RNA isolated in the presence of cycloheximide and hydroxyurea. Cycloheximide treatment allows transcription of immediate early genes only; whereas with hydroxyurea treatment, delayed early genes are also transcribed. Both treatments block late gene expression.

Following S1 nuclease digestion a protected fragment of approximately 150 bp is detected (Fig. IV.9). This corresponds to the 5' start located in figures IV.8A and B. The ts17 gene

Fig. IV.9. Kinetics of transcription initiation. A 5' single end-labeled probe (see Fig. IV.8B) was hybridized to 20 ug of total viral RNA isolated in the presence of cycloheximide (C), hydroxyurea (H), or at the indicated times post-infection in the absence of drugs. Marker is a Sau3A digest of pUC18 (M). Full-length probe is shown (P), along with the size of the S1 nuclease protected fragment.

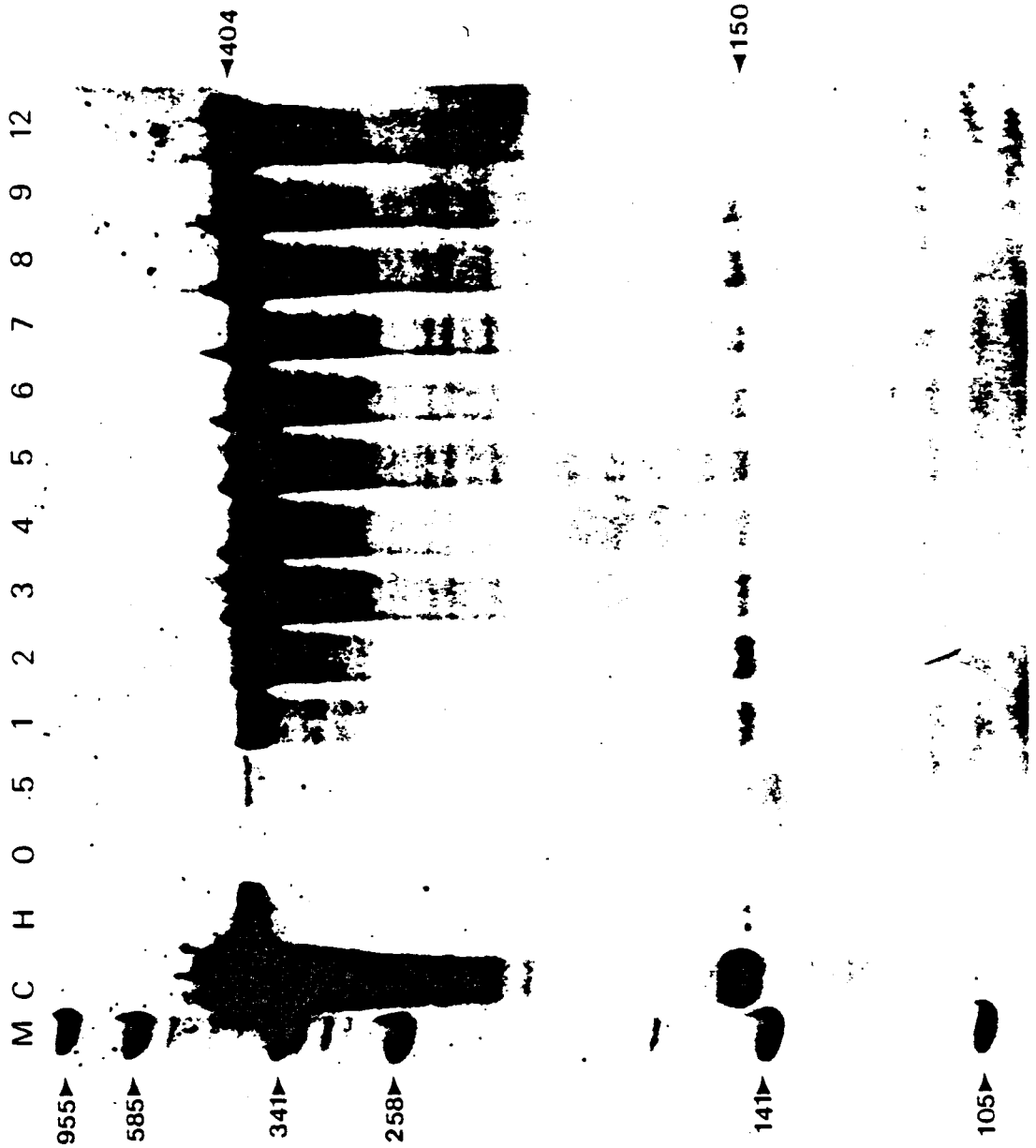


Fig. IV.9

is first transcribed at 1 hour post-infection (hpi). The level of transcription initiation remains stable until 3 hpi when it appears to decrease. The ts17 transcripts is then present for up to 12 hpi. Since the half-life of VV mRNA late in infection has been estimated to be approximately 1-2 hours (Hruby and Ball, 1981; Rice and Roberts, 1983) the presence of ts17 RNA at late times is most likely due to continued transcription.

Regulation of thymidine kinase expression by DNA- mutants. Of the three complementation groups which have been determined to have a DNA- phenotype, only one has been assigned a biological function; that being the DNA polymerase gene (Traktman, et al., 1984; Jones and Moss, 1984). It remains to be shown whether the loci of the other two DNA- complementation groups encode proteins which have a biological activity that is an integral part of DNA synthesis, or rather play an essential role not directly related to DNA metabolism. It was therefore of interest to examine the regulation of the VV thymidine kinase (tk) gene in DNA- mutant infected Ltk$^-$ cells to ascertain at which point the ordered expression of this gene might be interrupted. Expression of the VV tk gene is regulated at the transcriptional, translational, and post-translational levels by other VV gene products from both the early and late gene classes (Hruby and Ball, 1981; Hruby, 1985).

The kinetics of tk expression in cells infected with wt or with ts mutants of the three DNA- complementation groups was determined (Fig. IV.10). In wt infected cells tk is expressed

at the onset of infection, is attenuated at 4 to 6 hpi, and then remains stable. The level of expression being higher at 40°C than at 31°C is probably due to metabolic processes being increased at the higher temperature. The three DNA- complementation groups; 5, 21, and 24 are represented by ts42, ts17/ts24, and ts25 respectively. At the permissive temperature of 31°C, the regulation of tk expression for all three complementation groups is comparable to that seen for wt. However, at the non-permissive temperature of 40°C there are distinct differences in the impact of each locus and its defective gene product on tk expression. In the case of ts17 and ts24, which together comprise complementation group 21, the expression of the tk gene is not switched off. Ts42, which is the DNA polymerase gene, shows kinetics of expression very similar to that seen for wt virus. In the case of ts25, the expression of tk does not appear to be induced, the levels of tk activity are very close to that found in purified viral cores (D.E. Hruby, submitted). Thus, the derepressed phenotype seen for complementation group 21 is not the direct result of the DNA- phenotype since the other DNA- complementation groups exhibit distinctly different kinetics of tk expression.

Fig. IV.10. Thymidine kinase activity. Thymidine kinase assay was perfomed on duplicate sets of 60-mm dishes of Ltk$^-$ cells which were infected with the indicated ts mutant. The plates were incubated at either the permissive temperature of 31°C (●), or the non-permissive temperature of 40°C (■), and harvested at two hour intervals.

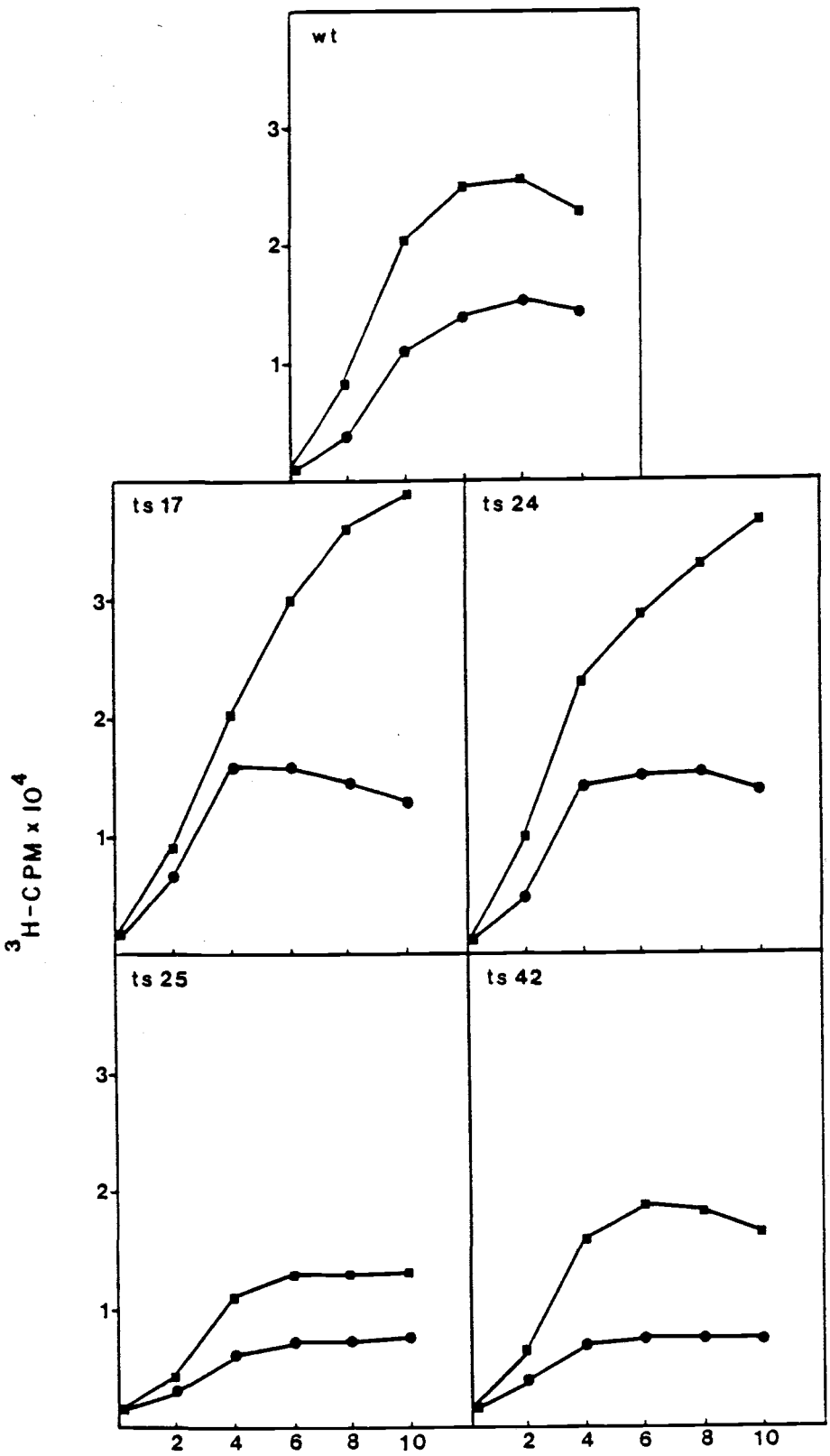


Fig. IV.10

Discussion

Transcriptional mapping and sequencing studies have shown VV genes to be organized in a tightly-clustered, tandemly-oriented fashion (Cooper et al., 1981; Mahr and Roberts, 1984a; Mahr and Roberts, 1984a; Niles et al., 1986; Pluciennicczak et al.,1985; Weinrich and Hruby, 1986; Wittek et al., 1984). Here, we independently confirm a portion of previously reported sequence and show that the predicted D4 and D5 ORFs are transcriptionally active. D4 is transcribed early in infection. Whether it is also transcriptionally active at later times is unknown. D4 has a predicted coding capacity for a 25,055 molecular weight polypeptide. However, the transcript which is apparently derived from the D4 gene is approximately 3.7 kb long; much larger than is required to encode a 25.5 kd protein. In contrast, the 3.0 kb transcript associated with D5 is of the appropriate size to encode a 90,367 molecular weight protein product, identified as the ts17 gene product. The data in Fig. IV.8 suggests that the D4 transcript terminates at the same place as the downstream D5 transcript. There does not appear to be a transcriptional stop signal proximal to the 3' end of D4. In contrast, the proposed transcriptional termination sequence TTTTTAT (Rohrmann et al., 1986) appears twice beginning at nucleotide positions 3406 and 3475 at the 3' end of the D5 transcript. This is within the coding sequence of D6. These two

signals map approximately 150 and 80 bases upstream of the shared 3' terminus of the two early transcripts mapped by the S1 analysis.

Marker rescue mapping and the comparison of sequence derived from wt, ts17, and two ts17 revertant viral stocks have conclusively demonstrated that D5 is the ts17 locus. This locus encodes a gene product which appears to be functionally required early in the viral replication cycle. Without a functional ts17 gene, the viral replication cycle is blocked prior to DNA replication. Yet, a kinetic analysis shows that the ts17 locus is trancribed continuously throughout infection, beginning at one hpi and is therefore a member of the constitutive temporal class.

The increasing amount of VV sequence data available allows for a comparative analysis of putative viral promoters and thus facilitates the identification of consensus sequences which may be regulatory elements. Transcription initiation at D5 has multiple start sites which occur between 2 to 5 nucleotides upstream of the initiation ATG. Similar findings have been reported for both early (Cochran et al., 1985b; Venkatesan et al., 1982; Weir and Moss, 1983) and late (Bertholet et al., 1985; Hirt et al., 1986; Rosel and Moss, 1985; Weir and Moss, 1984) VV genes. In the latter case, transcriptional initiation occurs within the sequence TAA which is directly upstream of the initiation ATG. It has been proposed that the sequence TAAAT is essential for late promoter function (Hanggi et al., 1986), and

may actually be part of a signal sequence for late gene expression (Rosel and Moss, 1985). However, since the sequence TAAAT serves as the transcriptional start site for the D5 locus at both early and late times post-infection, this indicates that there must be additional factors involved with specifying the temporal expression of VV genes. This finding illustrates the flexibility that must be required in order to regulate the complex expression of a variety of specific temporal classes of virally-encoded gene products.

The ts17 gene is comparable to previously sequenced VV genes in that it is 66% A-T rich, with the first 100 nucleotides upstream of the 5' start being 71% A-T rich. A close examination of the first 100 nucleotides shows an A-T rich octet at positions -20 and -40 nucleotides upstream of the first ATG. A-T octets at this position have been proposed as regulatory elements for early VV genes (Venkatesan et al., 1981; Venkatesan et al., 1982; Weir and Moss, 1983; Weir and Moss, 1984). However, the octets of D5 are not homologous to the proposed consensus sequence proposed for these regulatory elements. It appears that the D5 gene has promoter elements which have been associated with both early and late consensus sequences. This makes clear the necessity for further sequence analysis in conjunction with kinetic studies for identifying temporal subsets of viral gene expression. A directed genetics approach will then be required to determine which elements are involved in regulating gene expression. Such an approach is currently

being used to define the elements involved in the expression and regulation of the *ts*17 gene.

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