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Isolation and Characterization of the
Tipula Iridescent Virus
Capsid Gene

by

SHAHRAJIM TAJBAKHS, B.Sc. (Hons.)

A thesis submitted to the Faculty of Graduate Studies in
partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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October 1988

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Abstract

Tipula iridescent virus is an icosahedral, dsDNA insect virus that replicates cytoplasmically in inclusion bodies called viroplasmic centers (VCs). As part of this thesis, I established that TIV is unique among the large cytoplasmically replicating dsDNA viruses in that virions contain at least 4 DNA components: L (> 176 Kbp), S1 (10.8 Kbp), S2 (~6.5 Kbp) and S3 (3 Kbp). Comparative studies using two cell lines, *Aedes albopictus* and *Estigmene acrea*, indicated that S1 DNA is TIV specific, and exists in high copy number in both cell lines, in comparison to virions. cDNA synthesized from RNA obtained from these infected cells indicated that S1 carries transcribed sequence. A 0.85 Kbp EcoRI fragment of S1 DNA was cloned in the plasmid pUC19. It was also shown that the *A. albopictus* cell line was semipermissive for TIV replication. While VC morphology, DNA synthesis and transcription were similar to the permissive *E. acrea* cells, electron microscopy revealed that *A. albopictus* VCs were devoid of progeny virions. Further, the TIV capsid protein was synthesized in both cell lines. These data suggested that a block in TIV replication in *A. albopictus* cells occurred late in infection, just prior to the assembly of progeny virions.

The main focus of this thesis was to obtain molecular information on TIV gene organization. For this purpose, the TIV capsid gene was cloned and characterized based on amino acid sequence information from the SDS-PAGE purified 46 KDa capsid protein. DNA sequencing of this gene revealed an open reading frame of 464 amino acids with a predicted molecular weight of 50 KDa. The identity was confirmed by amino acid sequencing of 6 capsid protein tryptic peptides and expression of the cloned sequence in *Escherichia coli* cells. Northern analysis, transcript mapping, and *in vivo* labelling using ³⁵S-methionine indicated that this gene was transcribed and translated in TIV-infected *E. acrea* cells. The capsid protein was routinely identified by Western analysis using an anti-capsid antiserum. The capsid protein was also found to be the most abundant in virions and remarkably stable after long term storage.

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To my family and friends

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List of Abbreviations

A	adenine
AMV	Avian myeloblastosis virus
APS	ammonium persulfate
ASFV	African swine fever virus
(K)bp	(Kilo)base pairs
BPB	bromophenol blue
C	cytosine
cDNA	DNA (complementary) made from RNA by reverse transcriptase
Ci	Curie
CIV	<i>Chilo</i> iridescent virus
cpm	counts per minute
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
(K)Da	(Kilo)daltons
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double stranded DNA
EDTA	N,N-ethylenediaminetetraacetic acid
EM	electron microscope
EtBr	ethidium bromide
FLDV	Fish lymphocystis disease virus
FV3	frog virus 3
G	guanine
GuSCN	guanidinium isothiocyanate
h	hour (s)
HPLC	high pressure liquid chromatography
¹²⁵ I	radioactive isotope of iodine
ICDV	icosahedral cytoplasmic deoxyribovirus
IPTG	isopropylthio- β -D-galactoside
IV1	iridescent virus type 1
IVT	<i>in vitro</i> translation
L	large DNA component of TIV
<i>lacZ</i>	a portion of the β -galactosidase gene (α -peptide) located on pUC19 and M13
Me	methyl
MEM	minimal essential medium

mM	millimolar
mmol	millimoles
mRNA	messenger RNA
NaOAc	sodium acetate
NPV	nuclear polyhedrosis virus
nt	nucleotide (s)
op	refers to M13 clones whose inserts are in opposite orientation (complementary) to each other
ORF	open reading frame
³² P	radioactive isotope of phosphorus
p.i.	post-inoculation
PAGE	polyacrylamide gel electrophoresis
PBS	137 mM NaCl/2.68 mM KCl/8.1 mM Na ₂ HPO ₄ pH 7.2
Pipes	1,4-piperazinediethanesulfonic acid
RF	replicative (dsDNA) form of M13 phage
RMIV	mosquito iridescent virus "R" strain
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse phase
RT	room temperature
S	small DNA component (s) of TIV
³⁵ S	radioactive isotope of sulfur
SDS	sodium dodecyl sulfate
SIV	<i>Sericesthis</i> iridescent virus
SSC	saline sodium citrate (1X: 0.15 M NaCl/0.015 M sodium citrate)
ssDNA	single stranded DNA
T	thymine
TAE	40 mM Tris-acetate/2 mM EDTA pH 8.0
TBE	89 mM Tris-borate/89 mM boric acid/2 mM EDTA pH 8.0
TC	Top Component of a virus preparation fractionated on a gradient
TCA	trichloroacetic acid
TIV	<i>Tipula</i> iridescent virus
tk	thymidine kinase
Tris	Tris (hydroxymethyl) aminomethane
VC	viroplasmic center
XC	xylene cyanol

I. Introduction

A. Background and Classification

Viruses constitute a class of infectious microorganisms. They are obligate parasites that are dependent on their host cell for supplying them with the biochemical and metabolic ingredients needed to direct their replication. It is this property of viruses that has made them so valuable in unravelling the many complexities involved in host cell development.

The study of viruses has in many instances provided a useful framework for building our understanding of the subtleties in genetics, cellular and molecular biology. In fact, many of the techniques that are currently being used in these areas were developed during the course of investigating specific viral functions.

In 1954, Xeros reported an insect pathogen isolated from the European crane fly *Tipula paludosa* (Diptera). The fat body of the diseased larvae appeared iridescent blue through the integument. The purified, pelleted virus particles also displayed the same iridescence as the infected tissue and this virus was later named *Tipula* iridescent virus (TIV; Smith and Williams, 1958). These early studies established a cytoplasmic site of replication for TIV and DNA as its genetic material.

Several characteristics of TIV have made this virus very suitable for detailed studies. These include the stability of TIV (Younghusband and Lee, 1969; Manyakov, 1977), and the ability to purify large quantities of virus from infected larvae (Williams and Smith, 1957). As a result, some of our basic understanding of the structure of icosahedral viruses came from the pioneering studies carried out with TIV (Caspar and Klug, 1962). Some of the biophysical properties of TIV are listed in Table 1.

Since the discovery of TIV, a number of other vertebrate and invertebrate viruses with similar properties have been isolated. These viruses were called icosahedral cytoplasmic deoxyriboviruses (ICDVs) and were grouped in the family *Iridoviridae* (Matthews, 1982). The best studied member of this family is frog virus 3 (FV3; Willis *et al.*,

Table 1. Biophysical properties of *Tipula* iridescent virus.

Property	Details	References
Host range	Diptera Lepidoptera Coleoptera	Smith <i>et al.</i> (1961)
Replication site	cytoplasm- all tissues of insect	Xeros (1954) Smith <i>et al.</i> (1961)
Chemical composition	80% protein 12.4-19% dsDNA 5.2% lipid	Thomas (1961) Glitz <i>et al.</i> (1968) Kalmakoff and Tremaine (1968)
Size (diameter)	130nm (dehydrated) 250nm (hydrated)	Williams and Smith (1958) Klug <i>et al.</i> (1959)
Shape	skewed icosahedron	Wrigley (1970) Stoltz (1973)
Genome	ds DNA 176-247Kbp estimated from restriction enzyme analysis; L and S DNA components	Tajbakhsh <i>et al.</i> (1986)
	190-235Kbp estimated from reassociation kinetics	Bellet and Inman (1967)

1985). Some prominent members of this group include African swine fever virus (ASFV) which causes an important disease of domestic pigs (Vinuela, 1985) and fish lymphocystis disease virus (FLDV) which is the causative agent in a chronic disease of several higher order salt and freshwater fish (Flugel, 1985). There have also been recent reports of a large (about 190 nm) ICDV that infects certain eukaryotic *Chlorella*-like green algae and has cytopathic properties similar to the other ICDVs (Van Etten *et al.*, 1983; Meints *et al.*, 1986). Further molecular studies on this virus should prove to be interesting in determining how these viruses are related.

The unifying features of the *Iridoviridae* include polyhedral symmetry, large (~170 Kbp) linear double-stranded DNA genomes and a cytoplasmic site of replication (Matthews, 1982; Willis, 1985). Table 2 lists the five genera found in this family. Insect viruses form the largest group within the *Iridoviridae* spanning the *Iridovirus* (small iridescent virus) and *Chloriridovirus* (large iridescent virus) genera. To date, over 30 insect iridescent viruses have been isolated of which TIV is Type 1 (Kelly, 1985). These viruses are referred to by their common name such as "TIV" (type 1) and "SIV" (*Sericesthis* iridescent virus, type 2) or by the proposed number IV1 (iridescent virus 1) and IV2, respectively. The insect iridescent viruses will be referred to here by their numerical assignment. Those that have appeared more commonly in the literature such as TIV, SIV, and CIV (*Chilo* iridescent virus, IV6) will be referred to by their more historical designation.

The term "iridescent" was first used to describe the turquoise coloration of systemically infected larvae and pellets of purified TIV (Williams and Smith, 1957).³ This property was investigated in detail by Klug *et al.* (1959) who showed that crystals of TIV can diffract light in a manner similar to the diffraction of X-rays by ordinary crystals. By selecting conditions of growth of TIV crystals, they established that the iridescence is due to Bragg reflections (constructive interference) where the wavelength reflected is a function of the distance between successive reflecting planes (interparticle distance) in the

Table 2. Classification of genera within *Iridoviridae*¹.

English vernacular name	International name	Type species (Other members)
Small iridescent insect virus	<i>Iridovirus</i>	<i>Tipula</i> iridescent virus (Type 1);(2,6,9,10,16-29,31,32)
Large iridescent insect virus	<i>Chloriridovirus</i>	Mosquito iridescent virus (Type 3);(4,5,7,8,11-15)
Amphibian ICDV	<i>Ranavirus</i>	Frog Virus 3 (FV3);(1,2,5-24, L2,L4 and L5,LT 1-4,T 6-20,T21)
Lymphocystis disease virus group	<i>Lymphocystivirus</i>	Fish lymphocystis disease virus(FLDV)
African swine fever virus group ²		African swine fever virus, Kenya strain

¹From: Matthews (1982) and Willis (1985).²Tentatively being reclassified in a separate group (VIth workshop on Poxvirus/Iridovirus, Sept. 24-28, 1986. Cold Spring Harbor Laboratories).

crystals. Klug *et al.* (1959) also concluded that the virus particles are packed in a face-centered array with an interparticle spacing of 250 nm, a distance nearly twice that of dehydrated virions.

B. Host range

The small iridescent viruses often have a broad host range when injected, however a narrower range is evident when entry occurs via the oral route suggesting viral breakdown by gut enzymes or resistance of gut epithelial cells to infection (Hall, 1985). TIV infects a wide range of species in at least 3 different orders: Lepidoptera, Diptera, and Coleoptera (Smith *et al.*, 1961)

C. Enzymes associated with TIV

Enzymes that have been shown to be associated with *Iridoviridae* virions include protein kinase, nucleotide phosphohydrolase, and RNA polymerase activity (Kelly, 1985). Recent studies by Franke and Hruby (1986) have shown that vaccinia virus will fortuitously package chloramphenicol acetyl transferase, a bacterial enzyme produced in the eukaryotic cell by recombinant means, into virions by way of "hitchhiking". Therefore the significance of enzymes and proteins reported to be associated with virus particles is questionable. Further, these proteins may not be viral encoded.

D. Serological relationships

In early studies on ICDVs, a popular method of comparing viruses within the *Iridoviridae* involved serological and acrylamide gel electrophoresis techniques (Kelly and Robertson, 1973). Unfortunately, these studies have not proven to be very conclusive. Comparisons using DNA hybridization techniques should prove to be a more useful approach, particularly in the classification of the numerous insect iridescent virus isolates.

E. Physical and structural properties

Iridoviridae form the largest known isometric viruses with sizes ranging from 120 nm

for some of the insect viruses (Devauchelle *et al.*, 1985) to the recently discovered frog erythrocytic virus which measures 370-450 nm in size (Gruia-Gray *et al.*, 1987). Many of the reported values of particle diameter vary due to particle orientation, degree of hydration, and the presence or absence of an outer envelope. The latter is acquired by some of the viruses through budding at the cell membrane. These viruses are not occluded in a proteinaceous matrix like other viruses such as the nuclear polyhedrosis viruses (Kelly, 1982).

Members of the *Iridoviridae* family share some common structural features. By using biophysical techniques such as negative staining and thin sectioning, freeze-etching, and neutron scattering, a number of investigators have determined that the *Iridoviridae* shell is composed of three concentric domains: a proteinaceous icosahedral capsid, an intermediate unit membrane composed of lipids and proteins, and a central core containing the viral DNA and its associated proteins (Stoltz, 1973; Cuillel *et al.*, 1979; Darcy-Tripier *et al.*, 1984). Viral cores represent the internal structure comprising the DNA and its associated proteins. Cores can be released from the shell by treatment of viral suspensions with the non-ionic detergent NP40 or chymotrypsin (Willis and Granoff, 1974; Wagner *et al.*, 1975). Klump *et al.* (1983) have demonstrated that the viral DNA within the core is attached to the coat protein to form a chromatin-like structure.

Iridescent virus particles are not sensitive to ether (Day and Mercer, 1964), suggesting that the external lipid membrane is not essential for infectivity. With FV3, the outer envelope is not required for infectivity since nonenveloped particles are also infectious (Willis and Granoff, 1974) albeit 150 fold less than enveloped virions (Braunwald *et al.*, 1979). However, Willis and Granoff (1974) showed that lipid solvents render FV3 virions non-infectious, implicating a role for certain viral phospholipids in infectivity.

Electron microscope observations have revealed an interesting structural feature of purified *Iridoviridae* virions. Stoltz (1971, 1973) and Yamamoto *et al.* (1976) showed the presence of a microfibrillar fringe located at the surface of a certain number of these

viruses such as FLDV, mosquito iridescent virus (MIV), and CIV. This fringe seems to be absent from others such as ASFV and FV3 (Devauchelle *et al.*, 1985). These microfibrillar structures can be 3-5 nm in diameter and up to 150 nm in length (Willison and Cocking, 1972). Their presence was also demonstrated with iridescent virus type 29 (IV29) as the particles became ordered in the host cell. Kelly and Robertson (1973) have suggested that these fibers might cause the interplanar spacings in crystals of these viruses.

When a crude preparation of TIV virions is centrifuged on a linear sucrose gradient, several bands are often evident (Krell and Lee, 1974). The main viral band of highest density represents intact virions corresponding to infectious virus. A less dense band called "top component" (TC; Krell and Lee, 1974) is located immediately above the main band. A band consisting of empty capsids is found nearest to the meniscus of the gradient. The three bands of virus indicated here correspond to "full", "partially filled", and "empty" virions, respectively, referring to the state of the viral core (permeability to negative stains) as visualized under the electron microscope (Lee and Brownrigg, 1982). Recently, we showed that the TC fraction corresponding to partially filled virions contained less DNA than full virions and that the DNA of TIV virions consisted of two main components L and S1 (Tajbakhsh *et al.*, 1986). This TC fraction contained the highest molar ratio of S1 DNA to the L DNA component. Structurally, TIV was found to be remarkably stable since it can be stored at 4°C for 2 months (Manyakov, 1977) or greater (present study) with breakdown of only some of the virions.

Other studies have shown that a major polypeptide, approximately 50 KDa in size, is present in the iridescent viruses. This protein constitutes up to 45% of the total proteins in the virion (Kelly and Tinsley, 1972; Willis *et al.*, 1977; Black *et al.*, 1981). Two obvious locations for such an abundant protein are the capsid of the virus or the core in association with the DNA. Tripier-Darcy *et al.* (1982) using biochemical fractionation of FV3 particles localized the major 48 KDa FV3 protein in the capsid of the virion. Moore and Kelly (1980) isolated the major structural polypeptide from three types of iridescent viruses (2, 6

and 9) by preparative polyacrylamide gel electrophoresis. Amino acid analysis of these proteins indicated a close similarity between these proteins, although their mobilities on SDS-PAGE were slightly different from each other but about 50 KDa in size. Surface labelling of IV9 with ^{125}I revealed that the capsid protein was at least partly exposed at the surface (Moore and Kelly, 1980).

F. Post-translational modifications

Studies on TIV by Krell and Lee (1974) have indicated that none of the virion proteins are glycosylated. Glycosylation was also not detectable with mosquito iridescent virus and FV3 proteins (Wagner *et al.*, 1974; Goorha and Granoff, 1979). However, Aubertin *et al.* (1980) have reported that some FV3 core proteins are phosphorylated, while the capsid (48 KDa) protein was never found to be phosphorylated *in vivo* or *in vitro*. The degree of phosphorylation may be determined by the location of the protein in the virion shell, a higher degree in those associated with the nucleocapsid core (Blair and Russel, 1978; Clinton *et al.*, 1978).

G. Icosahedral virus assembly

In constructing a virion, there are a limited number of efficient designs that can be formed from a large number of identical protein molecules. Two basic designs are icosahedral heads and helical tubes (Caspar and Klug, 1962). Most closed-shelled virus particles have been shown to have icosahedral symmetry (Harrison, 1985). The icosahedron consists of 20 equilateral triangular faces consisting of smaller morphological subunits called capsomeres. Icosadeltahedra have $20T$ facets, where T is the triangulation number (Caspar and Klug, 1962). The advantages of an icosahedral structure over that of other types of cubic structures (tetra and octahedral) in the formation of virions is that it allows for the smallest subunit repeat for shell formation as economically as possible (Finch and Klug, 1959).

With improvements in electron microscope technology by the late 1960s, more

accurate determinations of the structural features of viruses were made. Wrigley (1969), using the nasal decongestant Afrin on SIV, showed an icosahedral structure of skewed orientation that probably contained 1562 capsomeres. Subsequently, a study using TIV showed a similar structure with a revised estimate of 1472 capsomeres (Wrigley, 1970). This finding was confirmed by Manyakov (1977) who used long term storage of TIV at 4°C or treatment with chloroform to break up the virus into its structural elements. He predicted that TIV was formed by 12 pentagons and 20 equilateral triangles composed of 1472 capsomeres per virion.

Several models have been proposed for the assembly of iridescent viruses. These include: a) the formation of viral membranes or shells before development of the core (Smith, 1956); b) development of the core first followed by encapsidation with protein (Bird, 1961); and c) a parallel formation of the viral shell and DNA fibrils (Xeros, 1964). In a study by Yule and Lee (1973), an hypothesis was proposed which combined features of particle assembly suggested by Smith (1956) and Xeros (1964). By using cytological and immunological techniques, Yule and Lee (1973) demonstrated that ferritin labelled antibody was closely aligned to the inner faces of empty capsids in various stages of assembly. These data provided evidence that the viral shell is assembled prior to entry of the DNA. Similar models have been suggested for adenovirus and herpes virus assembly (Horwitz, 1985; Roizman and Batterson, 1985).

H. Pathogenesis; pathway of TIV infection

a. Symptoms of infection and gross pathology

Information on the pathogenesis of iridescent viruses is incomplete largely because little is known about how the virus gains access to the initial replication site. These viruses have a low infectivity when administered orally and are probably transmitted in the field through wounds, cannibalism, or by parasites (Fowler and Robertson, 1972; Carter, 1973a, b).

Carter (1974) found TIV to be infective to all four larval instars, to pupae, and to

adults of both sexes of *Tipula oleracea*. The most effective method of transmission of TIV appeared to be by injection and the infection was characterized by a blue iridescence of the affected organ.

The initial site of TIV multiplication is not known, however, the fat body and hemocytes have been implicated (Smith *et al.*, 1961, Kelly, 1985). The virus then produces a systemic infection by rapidly multiplying in skin, muscles, wingbuds, legs and head (Smith *et al.*, 1961). Interestingly, an anomaly of TIV infection of the silkworm *Bombyx mori* is the production of epidermal "tumors" which result from the proliferation of epidermal cells to form a multilayered epidermis (Hukuhara, 1966). However, these tumors were not demonstrated to contain virus. Tumor formation has not been reported from other insect species, although some iridescent viruses of vertebrates and of the octopus are associated with tumors (Runnger *et al.*, 1971; Hall, 1985)

Massive amounts of virus can result from a productive infection of larvae. TIV was reported to constitute at least 25% of the body weight of a dead larva (Williams and Smith, 1957), the highest concentration often being found in the epidermis and fat body. The fat organ in insects often parallels the vertebrate liver in function. This organ, which is most extensively developed in larvae, serves as a storage organ for lipids, protein, and glycogen as well as playing a crucial role in metamorphosis (Wigglesworth, 1984). The resulting infection renders larvae flaccid within 7-10 days of infection, although death may take longer (Kelly, 1985).

b. Cytopathology and replication

Much of our understanding of insect iridescent virus cell pathology comes from infection studies carried out on cells *in vitro*. The major features of replication are common among the iridescent viruses with some variations in the timing of events (Hall, 1985). The focal point of viral replication and assembly is the cytoplasm (Younghusband and Lee, 1969; Kelly, 1985), although it is not clear if nuclear involvement occurs, as happens with FV3 (Goorha *et al.*, 1977, 1978). Following inhibition of host DNA synthesis

with heat-inactivated FV3, Goorha *et al.* (1978) observed by electron microscope autoradiography that 30% of the viral DNA was synthesized in the nucleus. These authors showed by DNA-DNA hybridizations that this labelled viral DNA was associated with extensively purified nuclei. Nuclear involvement in ASF DNA synthesis is less clear. Studies by Ortin and Vinuela (1977) revealed that ASF virus failed to replicate in enucleated VERO cells. Further, experiments involving pulse labelling of cells and separation into nuclear and cytoplasmic fractions suggested that the viral DNA is synthesized in the nucleus and subsequently transported to the cytoplasm (Tabares and Sanchez-Botija, 1979). However, these latter results may have been prone to experimental artifact due to incomplete fractionation of the nuclei since Vigario *et al.* (1967) have reported that the ASFV inclusion bodies are formed near the nucleus. Further evidence for a cytoplasmic site of ASFV DNA synthesis was provided by Pan *et al.* (1980) using pulse-chase experiments.

The pathway of TIV infection in *Estigmene acrea* hemocytes in suspension culture has been extensively studied by light and electron microscopy (Mathieson and Lee, 1981; Lee and Brownrigg, 1982; Kloc *et al.*, 1983; Kloc *et al.*, 1984). Electron microscope studies by Mathieson and Lee (1981) determined that the virus inoculum adsorbed to the plasma membrane of the cultured cells is internalized by phagocytic engulfment into membrane bound vesicles within 1.5 hours post-inoculation (p.i.). A general breakdown of the virus occurs within the vesicles and as early as 4 hours p.i., cytoplasmic lesions called viroplasmic centers (VCs) appear in the cytoplasm. These increase in size and number over the course of infection.

Viroplasmic centers appear as membrane-free isolated pockets of cell cytoplasm containing ribosomes, engulfed by a homogeneous "dense matrix" resembling chromatin in appearance and devoid of ribosomes. Electron microscope autoradiography has localized viral DNA exclusively in the dense matrix zones whereas progeny virions are assembled within the cytoplasmic component of the VC (Mathieson and Lee, 1981). These studies

also revealed that viral DNA synthesis occurs as early as 6 hours p.i. and progeny virions can be seen at 16 hours p.i. Viral release occurs by exocytosis or lysis, giving enveloped and non-enveloped virions, respectively, although the majority of virus particles remains cell associated and therefore non-enveloped (Kelly and Robertson, 1973).

Studies on the formation and composition of TIV VCs have revealed that there may be differences in the assembly sites of some members of the *Iridoviridae*. For example, investigations of FV3 assembly sites by electron microscopy and the use of cytoskeletal disruptive agents, implicated a functional role for intermediate filaments in the formation of FV3 assembly sites (Murti and Goorha, 1983; Murti *et al.*, 1984; Murti *et al.*, 1988). In contrast, similar studies carried out on TIV VCs in *E. acraea* and *Aedes albopictus* cells revealed that microtubules and microfilaments are not involved in VC formation or maintenance (Seagull *et al.*, 1985; Bertin *et al.*, 1987). More recently, immunofluorescent studies by Bladon *et al.*, (1986) showed that *in situ* VCs and fractionated VCs reacted with monoclonal antibodies raised against lymphocyte nuclear matrix proteins. The possibility that highly conserved mammalian nuclear scaffolding proteins are involved in TIV VC formation warrants further study.

Some of the cytopathic effects caused by TIV infection include a profound decline in cellular DNA, RNA, and protein synthesis and the formation of multinucleate cells due to cell fusion (Mathieson and Lee, 1981). The inhibition of host macromolecular synthesis is a common feature of invertebrate and vertebrate viruses and with a few exceptions, is poorly understood (Wagner, 1984).

Recent studies by Chinchar and Caughman (1986) suggest that heat inactivated FV3 selectively inhibited equine herpesvirus type 1 protein synthesis at the level of translation initiation. Although no such detail is available on the other iridescent viruses, Lee and Brownrigg (1982) showed that nuclei of cells inoculated with UV-inactivated virus and empty viral capsids failed to incorporate [^3H]-thymidine whereas virus treated with the protein inactivator B-propiolactone had no effect. These findings suggest that a protein

component of the invading virus subverts nuclear nucleic acid metabolism. Similar observations have been made with FV3 and poxvirus inhibition of host DNA synthesis (Willis and Granoff, 1976a; Pogo and Dales, 1973).

I. Molecular information available on other viruses

a. Genome organization

Willis and Granoff (1980) have reported that FV3 DNA is methylated by analyzing this DNA using various isoschizomeric restriction enzymes which differentiate between methylated and non-methylated sequences. For example, DNA cleavage by HpaII (C/CGG) does not occur in the presence of a 5-methyl group at the internal cytosine residue, while MspI (C/CGG; C/^{Me}CGG) cleaves at the same residue of the recognition sequence irrespective of the presence of a methyl group at this position (Waalwijk and Flavell, 1978).

In a recent study of the CIV genome, Delius *et al.* (1984) concluded that the DNA is linear and about 158 million daltons in size, is circularly permuted and has a terminal redundancy of about 12%. Two other *Iridoviridae* genomes, FV3 and FLDV, have also been shown to be circularly permuted and terminally redundant (Goorha and Murti, 1982; Darai *et al.*, 1983). These physical features are common to some viruses like bacteriophages T4 and lambda (Miller, 1975; Skalka, 1977) and have not been demonstrated for any other eukaryotic viruses.

As mentioned earlier, Goorha *et al.* (1978) showed that the FV3 genome enters the nucleus where it is transcribed during the early stages of replication, probably by a modified host RNA polymerase II (Goorha, 1981). Goorha (1982) later showed that FV3 DNA replicated in two stages which were distinguishable by the genome size and the site of replication. The first stage was confined to the nucleus where the DNA ranged from genome to twice genome size. The second stage took place exclusively in the cytoplasm, and the replicating DNA was concatemeric. Results of pulse-chase experiments showed that this concatemeric DNA served as the precursor for the production of mature FV3

DNA. This concatemeric arrangement is consistent with the finding that FV3 DNA is circularly permuted and terminally redundant and provides additional evidence for a specific pathway of virus assembly, namely packaging of DNA into preformed heads.

b. Transcription

Although cytological data are available on iridescent virus replication, including FV3, FLDV and ASF, information is lacking on many of the molecular aspects of the replication of these viruses. Studies with the cytoplasmic viruses vaccinia and FV3 and nuclear viruses such as herpes and the insect nuclear polyhedrosis virus (NPV) have shown a temporal regulation of mRNA synthesis which can be generally subdivided into three classes: immediate-early, early and late depending on whether synthesis occurs before or after DNA replication (Moss, 1978; Willis *et al.*, 1977; Honess and Roizman, 1974a; Wood, 1980). The division into three classes is based on differences in the order of gene expression and in the viral gene products that are necessary for their expression.

The immediate-early class of RNA can be distinguished from subsequent classes by its ability to be transcribed in the presence of the protein inhibitor cycloheximide, while early and late classes are dependent on *de novo* protein synthesis (Honess and Roizman, 1974b; Willis and Granoff, 1978). Early genes generally code for many enzymes, some involved in DNA replication, whereas late genes code predominantly for structural proteins (Moss, 1978; Horwitz, 1985; Roizman and Batterson, 1985).

Studies on vaccinia virus and FV3 have provided much information on the fine structure and regulatory sequences of viral genes of cytoplasmic viruses. Vaccinia virus, a member of the Poxvirus family, contains double-stranded DNA approximately 187 Kbp in size and replicates in the cytoplasm of infected cells. This virus encodes many of the processive enzymes required for DNA replication and the transcription of early and late genes (Moss, 1985). Recent studies have indicated that certain vaccinia virus genes are clustered along the DNA molecule, occasionally overlapping at either the 5' or 3' ends (Plucienniczak *et al.*, 1985; Weinrich and Hruby, 1986; Miner *et al.*, 1988; Lee-Chen and

Niles, 1988). Transcriptional maps of selected regions of the vaccinia virus genome and DNA sequencing data have revealed that the putative promoter regions of vaccinia viral early and late genes do not resemble its prokaryotic or eukaryotic counterparts (Weaver and Weissman, 1979; Venkatesan *et al.*, 1982; Hanggi *et al.*, 1988). Early vaccinia virus transcripts are initiated 10 to 50 base pairs upstream of their AUG start codon, and termination occurs at a discrete site (consensus: TTTTNT) downstream of the coding sequences (Mars and Beaud, 1987; Yuen and Moss, 1987). Late genes of vaccinia virus appear to have a much shorter 5' untranslated sequences which are located very close to the highly conserved TAAATG encompassing the translation initiation codon (Weir and Moss, 1984; Hirt *et al.*, 1986; Weinrich and Hruby, 1986). Peculiar features of these transcripts include a poly(A) leader sequence at the 5' end and a variable 3' terminal sequence (Bertholet *et al.*, 1987; Schwer *et al.*, 1987; Lee-Chen and Niles, 1988).

The study of FV3 genes has revealed that, like vaccinia virus, there is a lack of consensus with prokaryotic or eukaryotic transcription regulatory sequences. DNA sequencing of two immediate-early FV3 genes revealed the lack of a classical TATA box and no significant identity in DNA sequence between their promoters (Willis *et al.*, 1984; Beckman *et al.*, 1988). Further, these regulatory sequences did not resemble those of known vaccinia viral gene sequences (Willis *et al.*, 1984).

c. Polyadenylation

Unlike other animal DNA viruses, the majority of FV3 transcripts do not appear to undergo processing and polyadenylation at their 3' ends (Willis and Granoff, 1976b). This feature is common to prokaryotes and their viruses which provides further evidence for evolutionary links between prokaryotic and eukaryotic viruses. Further studies on the invertebrate TIV should elaborate on these observations.

J. TIV genome

The TIV genome is complex consisting of four DNA components. Two of these, L (~170 Kbp) and S1 (10.8 Kbp) are seen consistently with ethidium bromide staining of

agarose gels after electrophoresis of total TIV DNA. The other components (S2 and S3) may be deletion products of L and/or S1 DNA (Tajbakhsh *et al.*, 1986). TIV L DNA is linear and double-stranded with an estimated size of 176-247 Kbp, as predicted from summations of DNA fragment sizes produced by 7 restriction endonucleases (Tajbakhsh *et al.*, 1986). The S1 DNA component is double-stranded and appears to be circular (S. Tajbakhsh, B. Luck, and V.L. Seligy, unpublished EM observations). To date, no other *Iridoviridae* or large DNA containing viruses have been reported to contain multiple DNA components, although an earlier study with an iridescent virus from mosquitoes (RMIV) suggested that this virus may contain two identical DNA molecules. Size estimates for the other *Iridoviridae* genomes range from 100-250 $\times 10^6$ Da or 150-280 Kbp (Matthews, 1988).

K. Research Objectives

As previously mentioned, a large amount of information has accumulated on the gross structural properties of TIV in the last three decades. These studies have increased our understanding of viral architecture and have helped in the creation of models to explain the icosahedral design (Caspar and Klug, 1962). However, aside from these pioneering studies, little or no molecular information has been produced to explain the mode of replication, pathogenesis, and the regulation of gene expression of this and other iridescent insect viruses. Recent investigations of the TIV genome (Tajbakhsh *et al.*, 1986) have indicated that this virus has some novel features that have not been described for other *Iridoviridae* members or other large double-stranded DNA viruses. In addition to these features, the ability to purify large quantities of this virus from infected larvae make TIV a good candidate for experimentation to answer some of the questions raised in this section. Therefore, the main objective of this thesis was to provide molecular information on genes coded by this virus. For this purpose, the TIV capsid gene was chosen as the main candidate for detailed analysis. This gene was selected for a number of reasons. First, this gene is interesting insofar as an immediate and significant biological function can be ascribed to its protein product. A number of studies with some of the other *Iridoviridae*

have indicated that this protein is expressed at very high levels and is the major component in the morphological subunits that constitute the virion. Second, the isolation of this gene and the study of its protein product with respect to self-assembly properties should complement and advance some of the earlier structural studies that were carried out with TIV. Third, the purification of the capsid protein, by virtue of its abundance in virions, should facilitate the production of capsid-specific antisera for the identification of this gene and the study of its biosynthetic properties. Fourth, very little is known about late genes of large cytoplasmically replicating viruses such as vaccinia virus or FV3. The extensive symmetrical transcription seen late in infection with vaccinia virus and the enormous heterogeneity in the 5' and 3' regions of vaccinia late RNA transcripts have rendered late genes less accessible to analysis than immediate-early or early genes (Wittek, 1982; Lee-Chen and Niles, 1988). Therefore, a study of the late genes of TIV can contribute towards our understanding of gene organization, regulation and the evolution of these large double-stranded DNA viruses.

II. Materials and Methods

A. Virus purification

TIV was propagated and purified from *Galleria mellonella* larvae essentially as described by Yule and Lee (1973) with some modifications. Larvae were macerated in liquid nitrogen with pestal and mortar, resuspended in 10 mM sodium phosphate buffer pH 7.2 and subjected to two cycles of low speed (2000 g for 5 min) and high speed (55,000 g for 1 h, SW 25.1) differential centrifugation. Viral pellets were resuspended in buffer, layered on to 10-40% linear sucrose gradients, and centrifuged at 25,000 g for 30 min. The main viral band was removed, diluted with buffer, pelleted, and layered on to a second sucrose gradient. The final viral pellet was resuspended in buffer containing 50 µg/ml garamycin. Viral concentration was determined as described by Younghusband (1969) by measuring absorbance at 260 nm.

To fractionate TIV on cesium chloride (CsCl) gradients, sucrose gradient purified virus was layered onto CsCl step gradients (1 ml each of 1.3, 1.4, 1.5, and 1.6 g/cm³) and centrifuged using a SW 50.1 rotor, at 30 Krpm for 1 h. Viral bands were then removed and viewed by electron microscopy.

B. Insect cell maintenance and infection

Cell lines were grown at 28°C with 2.5% CO₂. *Estigmene acrea* (BTI-EAA) cells were obtained from Dr. R. Granados (Boyce Thompson Research Institute, Ithaca, N.Y., USA) and propagated in GTC-100 medium containing Grace's insect medium, 10% Fetal Bovine Serum (FBS), 0.26% bactotryptose broth and 50 µg/ml garamycin at pH 6.25. *Aedes albopictus* C6/36 cells were obtained from Bonnie M. King (US Department of Agriculture, Denver, CO., USA) in 1984 and grown in minimal essential medium (MEM) pH 7.2, supplemented with 10% FBS, non-essential amino acids and penicillin (5000 units/ml)/streptomycin (5000 µg/ml).

Cells were infected with TIV at a viral concentration of 80 µg/ml for 1-2 h at 28°C.

After this period, cells were centrifuged for 5 min. at 200 g, washed twice with medium, resuspended in fresh medium, and incubated at 28°C until harvesting.

C. Light microscope autoradiography and Microscopy

For light microscope autoradiography, *E. acraea* and *A. albopictus* cells were infected for 1-2 h, viral inoculum was removed by washing cells with medium and cells were further incubated at 28°C until 10 h p.i. Cells were then labelled with [methyl-³H] thymidine at 15 µCi/ml (total 30 µCi) for 2 h, washed twice with medium containing 1 mM cold thymidine, and incubated until 16 h p.i. Cells then were prepared for light microscope autoradiography and electron microscopy as described previously (Mathieson and Lee, 1981).

a. Assay for infection

For the detection of VCs in cells, TIV-infected cells were fixed in 1% glutaraldehyde for 5 min., washed in phosphate buffered saline pH 7.2 (PBS), permeabilized in 0.2% TritonX-100 in PBS (Chaly *et al.*, 1984), or alternatively, methanol fixed (see section C,b), washed in PBS and stained with the fluorescent DNA dye Hoechst 33258 (1 µg/ml in PBS, 2 min.; American Hoechst Corp., San Diego, CA., USA). Following staining, cells were washed (2 X 5 min.) with PBS and mounted in 0.1% p-phenylene diamine in 50% glycerol/PBS and examined with a Zeiss photomicroscope equipped with epifluorescence. This assay was routinely used to determine the percentage of infected cells.

b. Immunofluorescence

Antiserum (primary antibody) was tested for its ability to detect capsid protein intracellularly by indirect immunofluorescence (Chaly *et al.*, 1984; Bladon *et al.*, 1986) with a fluorescein-conjugated goat-antirabbit antibody (secondary, 1:400 dilution; Cappel Laboratories, Inc.). Various approaches were tried, including using 1% gelatin as blocking agent, or preincubating antisera with paraformaldehyde fixed uninfected cell lysates. Typically, TIV-infected *E. acraea* or *A. albopictus* cells were fixed (16 to 22 h p.i.) in paraformaldehyde and sometimes in combination with glutaraldehyde (Bladon *et al.*, 1986)

then processed for indirect immunofluorescence as previously described (Chaly *et al.*, 1984). Anti-capsid antiserum obtained from several rabbits were tested (see section M). Antiserum #610 was used routinely at dilutions ranging from 1:100 to 1:1000. Controls consisted of substituting preimmune serum or PBS for the primary antibody. Poly-D-lysine coated coverslips (0.1 mg/ml, 30,000-70,000 MW, Sigma; Lynn and Hink, 1980) were used for the attachment of the *E. acraea* cells during cell growth; and poly-L-lysine after fixation of cells (Simmonds, *et al.*, 1983). Methanol fixation was also attempted to avoid the possibility that aldehyde fixatives rendered capsid protein antigenic sites inaccessible to antibody. To do this, cells were resuspended in 2 ml methanol (absolute methanol was diluted to 95% and chilled at -20°C before use) and incubated at -20°C for 8 min. Aliquots of the fixed cells were applied to coverslips and allowed to air dry for 20 min. Cells were then assayed by indirect immunofluorescence.

D. Plaque assay

Several attempts were made at developing a plaque assay (Hink and Vail, 1973; Brown and Faulkner, 1978) for TIV using *A. albopictus* cells. These cells were chosen because of their ability to grow attached to the culture dish. Additionally, IV22 successfully replicated in these cells (Kelly, 1976). Typically, *A. albopictus* cells in logarithmic phase of growth were seeded at a cell density of about 1×10^6 cells/3.5 cm petri dish and incubated for 2 days at 28°C. The medium was removed by suction, and cells were infected with TIV (100-300 μ l) for 1-2 h at 28°C, at various concentrations ranging from 200 μ g/ml to a 10^{-10} dilution of the stock, with gently rocking at least three times over this period. After removal of the inoculum by suction, a 2 ml overlay was gently added to each plate, followed by addition of 1 ml medium (Brown and Faulkner, 1978), and plates were incubated at 28°C for 2-10 days. After this period, the 1 ml of medium was substituted with overlay containing 0.02% (w/v) neutral red and the dishes incubated for a further 1-4 days. The overlay was prepared by mixing 2X medium (35-40°C) with liquid Seakem agarose (0.75 or 1% final and cooled to 50°C; FMC, Mandel Scientific Co.).

E. Bacterial transformations

Competent cells for plasmid and M13 transformations were prepared as described by Hanahan (1985). The protocol for M13 transformation is outlined in Messing (1983).

F. Bacterial growth and media

Throughout these studies, several *Escherichia coli* bacterial strains were used for different purposes. The JM101 strain was used for M13 transfections and pUC transformations, however other strains were also used: JM103, JM109, HB101, DH5 α , and DH5 α F'. JM strains were maintained on M9 plates (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 2 mM MgSO₄, pH 7.4) at 4°C or stored frozen in 2YT/20% glycerol. Media used were LB (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2), 2YT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl), TB (1.2% Bacto tryptone, 2.4% yeast extract 0.4% glycerol and 100 ml of 0.17 M KH₂PO₄/0.72 M K₂HPO₄ per 900 ml TB; BRL Focus 9.2)

G. Molecular manipulations

DNA from different sources was digested with restriction endonucleases using low, medium, or high salt buffers, and fractionated on horizontal agarose (Seakem) as described in Maniatis *et al.* (1982). Digests were terminated by addition of 1/10th volume of stop buffer (100 mM EDTA, 0.5% SDS, 25% glycerol, and 0.1% BPB) and heated at 65°C for 3-5 min. before loading, or stored at 4°C. DNA ligations and recombinant DNA manipulations such as preparation of labelled markers were also carried out as described in Maniatis *et al.* (1982) unless otherwise indicated in various sections of this thesis.

H. DNA purifications

a. DNA from TIV

TIV DNA was isolated as previously described (Tajbakhsh *et al.*, 1986) from virus obtained from experimentally infected *G. mellonella* larvae. Purified TIV was resuspended in TE (1 mg/ml), and SDS (3% final) and proteinase K (100 μ g/ml final) were added.

After incubation at 37-42°C for 18 h, NaCl was added to the clear solution to a final concentration of 1 M. The aqueous phase was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) then reextracted with chloroform: isoamyl alcohol (24:1). The aqueous phase was then dialysed against several changes of 15 mM NaCl/1.5 mM EDTA for 24-48 h. The DNA was recovered by ethanol precipitation and resuspended in TE at a concentration of about 0.5 mg/ml.

b. S1 DNA component

1. Isolation from virions

In initial studies with the S1 DNA component, the source of this DNA was from virions. Total DNA was extracted from virions and electrophoresed in agarose gels (0.7%). S1 DNA was then purified by one of the methods described in Section J.

To demonstrate that S1 DNA is packaged within virions, TIV particles (~ 1 mg/ml) were DNase I (5 µg/ml) and RNase A (100 µg/ml) treated for 45 min. at 37°C to remove any contaminating host nucleic acid. To establish that these conditions were effective in removing nucleic acid, lambda phage DNA and poly(A)⁺ RNA were added to virions, treated as described, and the resulting mixture assayed by agarose gel electrophoresis. These conditions effectively removed the exogenously added nucleic acid.

For isolation of S1 DNA from CsCl fractionated virions (see section A), 100 µl viral suspension was combined with 100 µl proteinase K (1 µg/ml final), and 25 µl 20% SDS in a total volume of 500 µl. After incubation for 30 min at 37°C, the mixture was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), once with chloroform:isoamyl alcohol (24:1) and precipitated overnight at -20°C with 2.5 volumes ethanol. The nucleic acid was recovered after centrifugation and electrophoresed in agarose. This protocol was used by Storey *et al.* (1984) to isolate nucleic acid from the RNA virus, human parainfluenza virus 3, and was used here to obtain total nucleic acid from TIV. This protocol was also used to isolate total nucleic acid from the small virus particles (see Results, F).

2. Isolation from insect cells

TIV-infected *E. acraea* cells were harvested at 22 h p.i. and washed twice in PBS. The final cell pellet ($\sim 2.5 \times 10^7$ cells) was resuspended in 800 μ l PBS containing 0.5% SDS/10 mM EDTA. To this cell lysate, a solution containing 0.5% SDS, 0.5 M EDTA and 100 μ g/ml proteinase K was added in a final volume of 8 ml and the mixture incubated at 42°C overnight. The lysate was then extracted twice with an equal volume of phenol (Maniatis *et al.*, 1982), once with phenol: chloroform: isoamyl alcohol (25:24:1) and ethanol precipitated twice. S1 DNA was then purified after agarose gel electrophoresis of the total DNA and electroelution into NA-45 paper (see section J).

c. Plasmid, M13 RF

Both miniprep and bulk preparations of plasmid and M13 RF DNA were isolated by the alkaline lysis procedures outlined in Maniatis (1982). Plasmids were isolated from *E. coli* grown in 2YT, TB or LB media whereas M13 RF was isolated from *E. coli* grown in 2YT medium.

d. ssDNA template

To prepare M13 template DNA, a 1/50 dilution of an overnight culture of *E. coli* JM101 cells was used to inoculate 2YT media. After 30 min of growth at 37°C the cultures (1.5 ml or 10 ml) were inoculated with a plaque or 50 μ l of phage supernatant and grown for 7-12 h. The cultures were centrifuged at 10,000 rpm for 15 min and phage was precipitated from the supernatant by addition of 1/5 volume of 20% PEG/2.5 M NaOAc pH 7.0 at RT or 4°C for 20-30 min. After centrifugation at 10,000 rpm for 20 min (or 13 K rpm for 15 min in a micro-centrifuge), the tubes were inverted for 1-3 h and excess PEG was wiped from the sides of the tube with a sterile cotton swab. The phage pellets were resuspended in 600 μ l STE and extracted once with an equal volume of phenol, twice with phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) before ethanol precipitation. DNA pellets were washed once with 70% ethanol and resuspended in TE⁺ (10 mM Tris-HCl pH 7.5/0.1 mM EDTA).

1. Thymidine kinases

The vaccinia virus thymidine kinase gene was kindly provided by Dr. J.P. Weir (Department of Health & Human Services, NIH, Bethesda, Maryland, USA; Weir and Moss, 1983) in plasmid pJ5. The 531 bp tk ORF is located on an ~ 2 kbp HindIII/HincII fragment in pBR322. A 65 bp HinfI/EcoRI fragment containing sequences that are highly conserved among several thymidine kinases (Kit, 1985) was cloned into M13 mp19 for the preparation of a hybridization probe. M13 mp19 (140 ng), digested with SmaI/EcoRI was ligated with the 65 bp HinfI/EcoRI fragment of pJ5 (15 ng) in a total volume of 10 µl containing 1X ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 10 mM DTT, 100 µg/ml BSA), 1 mM ATP and 0.1 unit T4 DNA ligase. After incubation at RT for 25 min., to ligate EcoRI compatible ends, the ligase was heat inactivated at 68°C for 10 min. The HinfI end of the pJ5 fragment was made blunt by a fill in reaction involving the addition of 1 µl 10X nick translation buffer (1X: 50 mM Tris-HCl pH 7.2, 10 mM MgSO₄, 100 µM DTT, 50 µg/ml BSA), 0.5 µl 25 mM dNTPs and 1 unit Klenow enzyme in a total volume of 20 µl. The reaction was then incubated at RT for 25 min., heated to 68°C for 10 min., diluted to 50 µl with TE, extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) and subjected to a spin column pre-equilibrated with 1X ligation buffer. The blunt ends of SmaI and HinfI (filled in) were then ligated overnight at 4°C in a total volume of 60 µl containing 1 mM ATP, 1X ligation buffer, and 3 units ligase. After transfection of competent JM101 cells, recombinant clones were isolated and verified by DNA sequencing (M.J. Dove). One such clone, called TKC was used for preparation of a hybridization probe. The thymidine kinase gene of herpes simplex virus type 1 (Wagner *et al.*, 1981) was kindly supplied by Dr. M. McBurney (U. of Ottawa, Health Sciences, Ottawa, Canada) in plasmid pX-1. The 1128 bp ORF is located on a 3.5 Kbp BamHI fragment in pBR322. This 3.5 Kbp fragment was purified from the plasmid and used as a hybridization probe.

J. DNA electrophoresis and purification from agarose gels

Agarose gel electrophoresis of DNA was carried out either in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA pH 8.0) or TAE (40 mM Tris-acetate, 2 mM EDTA pH 8.0) buffers (Maniatis *et al.*, 1982) in the presence or absence of the fluorescent intercalating dye ethidium bromide (EtBr, Sigma) at a final concentration of 0.5 µg/ml. Gel fractionated DNA was visualized by short wave ultraviolet light (254 nm) and recorded by photography using Polaroid type 52 and 55 film with a red filter. Polaroid negatives were developed in a solution of 22% NaSO₃ for 30 sec. and washed in running tap water for 15 min. Typical DNA molecular weight markers included lambda wild type DNA digested with HindIII or HindIII/EcoRI, and pBR322 digested with HinfI. Radiolabelled DNA markers were prepared by 3' end filling with [α -³²P]dATP and the Klenow fragment of *E. coli* DNA polymerase I (Maniatis *et al.*, 1982). To detect labelled DNA, the gel was fixed in 7% trichloroacetic acid (TCA) for 30 min., rinsed in H₂O, and vacuum dried on Whatman 3 MM at 60°C. Autoradiography was performed by exposure of the gel to X-Ray film at room temperature, or at -70°C with an enhancing screen.

Three methods were used for DNA purification from agarose gels. The first method made use of a commercial electroeluter (IBI). The DNA band was visualized by long ultraviolet light, excised, and electroeluted from the gel slice in 0.5X TBE into a 7.5 M NH₄OAc cushion containing 0.01% bromophenol blue. After electroelution, the cushion containing the DNA was removed and ethanol precipitated. The DNA pellet was washed with 70% ethanol and resuspended in TE. A second method involved electroelution into dialysis bags as described by Maniatis *et al.* (1982). The third method involved direct electroelution of the DNA fragment into NA-45 (Schleicher & Schuell) paper (Dretzen *et al.*, 1981; Lizardi *et al.*, 1984) at right angles to the direction of electrophoresis. DNA was eluted from the paper at 65°C for 1 h in 20 mM Tris-HCl pH 7.5, 1 mM EDTA, and 2.5 M NaOAc, ethanol precipitated, and resuspended in TE.

K. RNA isolation and analysis

RNA was extracted from insect tissue culture cells using the protein denaturant guanidine isothiocyanate (GuSCN) by the procedure of Chirgwin *et al.* (1979) and further purified using a CsCl cushion (Glisin *et al.*, 1974). All solutions and materials were sterilized by autoclaving. For RNA time course experiments, cells were infected with TIV as described above in several 250 ml Falcon flasks, washed with medium and aliquoted into ten 250 ml Falcon flasks each representing a time point (4, 8, 10, 12, 14, 16, 18, 20, and 24 h p.i.) plus one mock-infected (buffer without virus) control flask (14 h p.i.). At the designated time, cells were chilled on ice for 5-10 min., scraped from the dish with a rubber policeman and centrifuged as described above. Cell pellets were washed once with ice cold PBS and either frozen on dry ice for the time course samples and collectively processed, or processed immediately. Cells were resuspended, while vortexing, in at least 4 volumes of GuSCN buffer prepared as follows: to 30 ml of a GuSCN stock solution (4 M GuSCN, 25 mM sodium citrate pH 7.0, 1 mM EDTA), 150 μ l Na N-laurly sarcosine (0.5% final), 300 μ l 2-mercaptoethanol (1% final) and 100 μ l Sigma Antifoam A were added just before use and the solution filtered (0.2 μ m). Cell extracts were then clarified by centrifugation at 25,000 g for 20 min and layered onto a 1.3 ml bed of 5.7 M CsCl/0.1 M EDTA, pH 7.0 in a Beckman ultracentrifuge tube. The RNA was pelleted by centrifugation at 30,000 rpm for 16 h at 18°C in a SW50.1 rotor. Supernatants were removed by aspiration and each tube quickly inverted to avoid contamination of the RNA pellet by residual supernatant. The pellet was rinsed quickly in 70% ethanol, resuspended directly in sterile H₂O, and transferred to a 1.5 ml Eppendorff tube. NH₄OAc was added to 2.5 M and the RNA precipitated by addition of 2.5 volumes ethanol. This precipitation was repeated and the RNA was either stored in this fashion or frozen (-70°C) in aliquots in H₂O.

RNA concentrations were measured by absorbance at 260 nm. RNA integrity was determined by electrophoresis in 1% agarose gels containing 25 mM citrate pH 3.5 followed by staining with 0.5 μ g/ml ethidium bromide (EtBr), and subsequently by

hybridization. RNA samples were resuspended in loading solution (dimethylsulfoxide [DMSO], 0.1% BPB, 0.1% xylene cyanol [XC]) and heated briefly at 65°C prior to loading.

Formaldehyde gels were used for electrophoretic separation of RNA as described by Davis *et al.* (1986). After electrophoresis was complete, the gel was rinsed in H₂O for several hours to remove the formaldehyde, and a portion containing a BRL RNA ladder and yeast poly(A)⁺ RNA provided by Dr. K. Prakash (Molecular Genetics Section, Division of Biological Sciences, NRC), as markers was stained in 5 µg/ml EtBr for 5 min and destained overnight in H₂O.

L. Immobilization of nucleic acids and probe preparation

DNA fractionated on agarose gels was transferred to nylon membranes (Biotrans, ICN; Hybond, Amersham) by alkaline blotting according to Reed and Mann (1985). The DNA was depurinated in 0.25 N HCl for 7-10 min. (Wahl *et al.*, 1979), denatured in 0.3 M NaOH/1.5 M NaCl for 30 min. and transferred to nylon in same for 2 h. After the transfer was completed, the filter was briefly rinsed in 2X SSC (1X saline sodium citrate: 150 mM NaCl, 15 mM sodium citrate pH 7.0), baked for 1 h at 80°C and stored dry until use.

RNA was immobilized on nylon or nitrocellulose filters using a variety of methods. The first method involved an electrophoretic transfer of RNA from agarose to Hybond membrane in 12 mM Tris-HCl pH 7.5, 6 mM Na acetate, 3 mM EDTA at 5°C for 1 h at 10 volts followed by 2 h at 40 volts (Gene Screen Plus, Du Pont Co.). Other methods involved soaking the gel for 10 min and transferring to nylon membrane in 3 mM NaOH for 4 h (Bio-Rad Laboratories Molecular biology reports [1987], 1(2): 3) or transferring the RNA directly to nitrocellulose filter by capillary action in 20X SSC overnight (Maniatis *et al.*, 1982). After RNA transfer was complete, the filter was rinsed briefly in 2X SSC and baked at 80°C for 1 h (for nitrocellulose, 2 h *in vacuo*). Filters were stored dry until use.

DNA from recombinant colonies or plaques was transferred to nylon membrane for identification by hybridization. "Lifts" were prepared by layering individual nylon filters

marked for orientation onto plates containing the recombinant colonies or plaques. After 2 min, the filters were gently lifted with forceps, placed cell side up onto B plates and incubated at 37°C for 4-6 h. DNA was released by placing lifts onto drops of 0.3 M NaOH/1.5 M NaCl for 5 min at room temperature (RT) on Saran wrap (enough solution to be barely visible at edge of filter). Lifts were then transferred to drops of 0.5 M Tris-HCl pH 8.0/3 M NaCl for 2 min and subsequently placed in 2X SSC for 5-10 min. The lifts were baked at 80°C for 30 min and placed in 2X SSC/0.1% SDS up to 24 h. During this period, excess cellular debris was wiped from the filter surface with gloved hands to reduce hybridization background. Filters were then transferred to prehybridization solution or stored dry.

Nucleic acids were also immobilized on filters by direct application called "slot" or "dot" blotting. Slot blotting of RNA was performed according to the manufacturer's protocol (Schleicher & Schuell) using the Minifold II slot-blotter assembly. The time course RNA samples were initially calibrated by concentration using A_{260} readings where $1 A_{260} = 37 \mu\text{g/ml}$. Sample concentrations were verified and adjusted by multiple runs on minigels to visually normalize ribosomal RNA concentrations. RNA samples were then made up to 100 μl with H_2O and a 300 μl solution of 6.15 M formaldehyde/10X SSC was added. After incubation at 65°C for 15 min., the RNA samples were applied to a Hybond filter using the manifold. Sample wells were then washed with 400 μl of 10X SSC and the filter baked at 80°C for 1 h.

A partial bank of TIV DNA digested with BamHI was made using pBR322 digested with the same enzyme (Tajbakhsh *et al.*, 1986). Individual TIV DNA containing plasmid clones were dot blotted to nitrocellulose using a Hybridot manifold (Schleicher & Schuell). Plasmid DNA (15 μl or less) was denatured by addition of 400 μl 0.3 N NaOH. After a 20 min incubation at RT the samples were mixed with 400 μl 0.3 N HCl and 600 μl 0.4 M Tris-HCl pH 8.0/3 M NaCl and applied to the manifold. Sample wells were then washed with 100 μl SSC and the filter baked at 80°C for 2 h *in vacuo*.

a. Nucleic acid hybridizations

Filters containing DNA or RNA were hybridized in plastic bags (Seal-a-Meal) at different stringency conditions. Generally, DNA-filters were prehybridized in hybridization solution (1 M NaCl, 1% SDS, 100 μ g/ml denatured salmon sperm DNA; Gene Screen Plus Manual) for at least 1 h before addition of boiled (10 min.) probe to the bag containing fresh buffer. Probe concentration ranged from 10^5 to 2×10^6 cpm/ml. After hybridization (18-24 h), the filters were washed at high stringency in 2X SSC/0.1% SDS 3 times at RT, followed by 2 times at 65°C for 30 min. each, and finally in 0.1X SSC for 15 min. at RT. For low stringency hybridizations, the 30 min. washes were done at 42-50°C, followed by a 5 min. wash in 2X SSC at RT. The filters were kept damp by placing in between Saran wrap and exposed to X-ray film with or without the use of intensifying screens. If a higher stringency wash was needed, the damp filter was rewashed by using a combination of lowered SSC concentration and/or higher temperature, then re-exposed to X-ray film.

Hybridization with radiolabelled mixed oligonucleotides was carried out in buffer (1 M NaCl, 1% SDS, 50 mM Tris-HCl pH 7.5, 1X Denhardt's, 100 μ g/ml denatured salmon sperm DNA) at 42°C with a probe concentration of 2×10^6 cpm/ml. Filters were washed in 6X SSC/1% SDS for 30 min. at RT, autoradiographed, rewashed at 50°C, and re-exposed.

DNA-RNA hybridizations were done in a similar manner to DNA-DNA hybridizations. Hybridization temperature was typically 50°C and washes were done as follows: three times at RT for 5 min. in 2X SSC/0.1% SDS, followed by 2 X 20 min., and finally for 10 min. at RT in 2X SSC. Filters were exposed to X-ray film as previously described.

M. Protein purification and analysis

The procedures for trypsin digestion, reverse phase high pressure liquid chromatography (RP-HPLC), and microsequencing of the capsid protein were carried out

at the National Research Council (NRC), Division of Biological Sciences by Dave Watson in the section of Molecular Genetics.

a. Immunization of rabbits

G. mellonella-TIV structural proteins were separated by SDS-PAGE and the viral proteins were visualized by staining with ice cold 0.25 M KCl for 5 min. (Hager and Burgess, 1980). The main viral band (46 KDa) was excised from the gel and macerated by passing through a 21 gauge syringe. The protein was electroeluted using a home-made apparatus consisting of a 10 ml syringe barrel plugged with agarose (to 0.5 ml mark) and fitted with a 0.5 cm diameter glass tube. The other end of the tube was covered by a 3500 MW exclusion dialysis membrane (Pierce). Electroelution was monitored by the migration of bromophenol blue dye which migrated approximately half as fast as the capsid protein. After the elution was completed, the current was reversed briefly. The apparatus was then dismantled and the protein recovered by overnight precipitation with 5 volumes of acetone at -20°C.

The protein was taken up in H₂O and, one tenth volume of 10X PBS was added along with an equal volume of Freund's adjuvant (Difco labs). Several rabbits (New Zealand Reds) were injected intramuscularly (by Jack Scott, Division of Biological Sciences, NRC) with 15-30 µg protein in Freund's complete adjuvant and boosts (2-3) were administered in Freund's incomplete adjuvant at multiple subcutaneous sites. The first boost was given after 4 weeks and subsequent boosts were given at 2-3 week intervals. Rabbits were bled by heart puncture about 3 weeks after the last boost. Several preparations of anti-capsid antiserum were obtained (numbered 599, 604, 605, 610, 611) and tested against purified TIV capsid protein, applied directly to nitrocellulose filters, using the radioimmunoassay procedure detailed in section M.e. Antibody #610 appeared the most specific (most intense signal) and was chosen for all future experiments.

b. Trypsin digestion and HPLC

Trypsin digestion of the 50 KDa TIV capsid protein was carried out as follows.

Trypsin (bovine pancreas, twice crystallized) treated with TPCK (L-1-tosylamide-2-phenylethylchloromethyl ketone) to remove residual chymotrypsin activity, was obtained from Sigma and was further purified by RP-HPLC (Liquid chromatograph Varian 5000) prior to use according to the method of Titani *et al.* (1982). Fifty μ g purified capsid protein were dissolved in 50 mM NH_4HCO_3 pH 8.0 and trypsin was added in a 1:50 w/w ratio (trypsin:capsid). After incubating for 3 h at 37°C a second trypsin aliquot was added and the incubation continued for another 3 h. The reaction mixture was then lyophilized. The resulting tryptic peptides were fractionated by elution with a linear acetonitrile gradient (1%/min.) at a flow rate of 1 ml/min from a Synchropak RP-8 column (4.1 X 250 mm) (Synchrom Inc., Linden, Ind., USA) previously equilibrated in 0.05% trifluoroacetic acid. The effluent was monitored at 220 nm, and individual peptides were collected and lyophilized. Those peptides displaying signs of heterogeneity were re-fractionated in a similar fashion at pH 7.0 in 10 mM ammonium acetate.

c. Automated gas-phase sequence analysis

Automated gas-phase sequencing was performed on an Applied Biosystems 470A sequencer (Foster City, CA., USA). Reverse phase column and all chemicals used for sequencing were obtained from Applied Biosystems Inc. The samples were dissolved in 30 μ l of Milli-Q water (Millipore) and applied to a glass fibre disc containing 1.5 mg of precycled polybrene (Applied Biosystems Inc.). The PTH (phenylthiohydantoin) derivatised amino acids were identified using an on-line 120A PTH HPLC analyzer (Applied Biosystems Inc.) equipped with a C18 reverse phase column maintained at 55°C in 120 mM sodium acetate/5% tetrahydrofuran pH 4.5 with a non-linear gradient elution using acetonitrile (0-60%).

d. Electrophoresis of proteins

At various times p.i., insect cells were harvested by centrifugation, resuspended in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and stored frozen at

-20°C. Just before analysis, an aliquot was removed and mixed with an equal volume of 2X gel loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.01% BPB). For analysis of bacterial proteins, bacterial cell pellets were resuspended in TE, mixed with an equal volume of 2X gel loading buffer, and stored at -20°C until use.

Samples were boiled, loaded onto a 4% stacking/10% or 12% separating polyacrylamide gel and electrophoresed in gel electrophoresis buffer (25 mM Tris-HCl pH 8.3, 0.1% SDS, 192 mM glycine) for 16 h at 45 volts as described by Laemmli (1970). The separating and stacking gels were prepared from an acrylamide stock solution of 29.2% w/v acrylamide (BDH)/0.8% N,N'-methylenebisacrylamide (BDH). Separating gels contained 375 mM Tris-HCl pH 8.3, 0.1% SDS, and 0.04% w/v ammonium persulfate (APS), while stacking gels contained 120 mM Tris-HCl pH 6.8, 0.1% SDS, and 0.06% APS. Gel polymerization was initiated by addition of N,N,N',N'-tetramethylethylenediamine to a final concentration of 0.15% v/v.

After electrophoresis, proteins were detected in the gels in the following manner depending on the experiment: a) silver stained according to Morrissey *et al.* (1981); b) stained with 0.1% Coomassie Blue R-250 (Sigma) in 40% methanol/10% acetic acid for 1 h and destained in 40% methanol/10% acetic acid; c) fluorography. Gels to be fluorographed were fixed in 30% methanol/10% acetic acid for 30 min, treated with Amplify (Amersham) for 30 min, vacuum dried onto Whatman 3 MM at 60°C, and exposed to Kodak X-Omat AR film at -70°C with enhancing screen; d) "western blotted" by the method of Towbin *et al.* (1979). For western blotting, proteins were transferred from gels to nitrocellulose by electrophoresis (Schleicher & Schuell) for 2-3 h at 250 mA in western transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, and 20% methanol) and filters were stored in fresh buffer until immunodetection was performed, typically the same day.

e. Immunodetection of proteins

Proteins were electrophoretically transferred to nitrocellulose as described in section

M, d and probed with anti-capsid antiserum using two methods. The first immunodetection method was a modification of the procedure described by Towbin *et al.* (1979) using ^{125}I -protein A. All reactions were performed at RT. Filters were washed for 10 min. in TBS buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% v/v Tween 20) containing 20% fetal calf serum (FCS, Flow Laboratories) or 1% gelatin as the blocking agent. The filters were then rinsed briefly with TBS prior to the addition of anticapsid antiserum (100 μl /10 ml) and gently rocked for 2 h. The filters were washed with 25 ml TBS/blocking agent for 5 min. followed by 3 X 5 min. washes in 20 ml TBS. Filters were then placed in a solution containing ^{125}I -protein A (ICN; McConahey and Dixon, 1980) in TBS/blocking agent at 1×10^6 cpm/filter for 20-30 min. with gentle rocking, followed by a 5 min. wash in TBS/blocking agent and 3 X 5 min. washes in TBS. Filters were air dried and exposed to Kodak X-Omat AR film with intensifying screens overnight at -70°C .

The second method made use of an alkaline phosphatase immunodetection assay as detailed by the manufacturer (Protoplot Immunoscreening System, Promega Biotech). All reactions were performed at RT. Non-specific binding was blocked by soaking the Western blotted filter in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) with 1% BSA for 30 min. The blocking solution was replaced with anti-capsid antiserum (primary) in TBST (1:100 dilution) that had been pre-incubated with uninfected *E. acraea* cells as follows: 250 μl antiserum was combined with $\sim 5 \times 10^6$ cells lysed in 400 μl RIPA buffer, in a 1.5 ml Eppendorff tube and gently rocked at 4°C for 2.5 h. The suspension was centrifuged in a Beckman B microfuge for 2 min. and the supernatant added to 25 ml TBST. After incubation with primary antibody, the filter was washed 3 times in TBST for 5-10 min. each to remove unbound antibody, and incubated for 30 min. in TBST containing a 1:7500 dilution of anti-IgG alkaline phosphatase conjugate (secondary). The filter was washed 3 times for 5-10 min. each to remove unbound secondary antibody, then transferred to the color development solution containing 66 μl NBT substrate (nitro blue tetrazolium) and 33 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate) per 10 ml AP buffer

(100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The color reaction was stopped by rinsing in deionized water for several minutes. The filter was photographed while still moist.

N. Biosynthesis of TIV proteins

a. *In vivo* labelling

The *in vivo* labeling time course experiments were carried out in parallel with RNA time course experiments. Additional time points of 30 and 36 h p.i. for TIV-infected cells were included as well as control samples labelled at 8, 14, and 20 h p.i. The designated times correspond to the time at which the radiolabel was added. TIV-infected cells (2.5 ml) were aliquoted into 6 cm petri dishes and incubated at 28°C until processed. Cells were then centrifuged, washed with 5 ml medium containing no methionine, serum or bactotryptose broth (MST⁻ medium), and resuspended in 2 ml MST⁻ medium. After incubation at 28°C for 1 h, 40 μ Ci 35 S-methionine (800 Ci/mmol, 15 μ Ci/ μ l, Amersham) was added and the dish incubated at 28°C for 1 h. At the end of this period, the cells were harvested by microcentrifugation in Eppendorff tubes for 3 min., washed with ice cold PBS, resuspended in 100 μ l RIPA buffer and stored at -70°C until SDS-PAGE analysis.

b. *In vitro* translations and immunoprecipitations

In vitro translations (IVTs) were done using a rabbit reticulocyte lysate system from BRL. Eight μ g aliquots of total RNA were precipitated in 2.5 M NH_4OAc and 2.5 volumes ethanol. The final reaction mixture (30 μ l) contained RNA (8 μ g), 1.3 μ l 2 M $KOAc$ pH 7.2, 15 μ Ci 35 S-methionine (1205 Ci/mmol, 15 μ Ci/ μ l), 3 μ l reaction mixture (10X: 250 mM Hepes pH 7.2, 400 mM KCl, 100 mM creatine phosphate, 19 amino acids, 500 μ M each), and 10 μ l rabbit reticulocyte lysate. After incubation for 60 min at 30°C, 3 μ l RNase A (1 μ g/ μ l) were added and the mixture incubated for a further 15 min. The reaction was stopped by placing on ice and ~3/4 of the sample was used for immunoprecipitation.

For the immunoprecipitation of IVT products, 22-25 μ l of the IVT reactions were combined with 20 μ l anticapsid antiserum and 150 μ l Buffer B (50 mM Tris pH 7.5, 5 mM

EDTA 150 mM NaCl, 0.05% Nonidet P-40; Willick *et al.*, 1984), and the mixtures gently rocked for 2-22 h at 4°C. Fifty μ l of a Protein A-Sepharose (Pharmacia) slurry were added and the mixtures were gently rocked for 30 min. to 4 h at 4°C. The mixtures were then microcentrifuged (13 Krpm, 3 min.) and the pellets washed 5 times with buffer. The final pellet was resuspended in 30 μ l buffer and 30 μ l 2X loading buffer (see above). Prior to loading, the samples were boiled, repelleted, and the supernatants loaded.

Immunoprecipitation of *in vivo* 35 S-labelled proteins was done in a similar manner. Anticapsid antiserum was pre-incubated with uninfected cell extracts as described in section M, e using RIPA buffer.

For immunoprecipitation of denatured *in vivo* labelled capsid protein, *in vivo* labelled material was boiled in Laemmli loading buffer and dialysed extensively against RIPA buffer. The immunoprecipitation procedure described in this section was then followed.

O. Preparation of radiolabelled DNA probes

Double stranded DNA was radiolabelled with [α - 32 P]dATP by nick translation (Rigby *et al.*, 1977; Maniatis *et al.*, 1982) or by the method of "random primed" DNA labelling developed by Feinberg and Vogelstein (1983). For nick translation, DNA (100-500 ng) was combined with 60 μ M each of dGTP, dCTP and dTTP, 50-100 μ Ci [α - 32 P]dATP (800 Ci/mmol, 10 μ Ci/ μ l) in 50 mM Tris-HCl, 10 mM MgSO₄, 100 μ M DTT, 50 μ g/ml BSA. One μ l DNaseI (0.1 μ g/ml) and 7-10 units of *E. coli* DNA polymerase I were added and the reaction incubated at 16°C for 1 h. The reaction was terminated by heating at 65°C for 10 min. and addition of 2 μ l 250 mM EDTA. Fifty μ l STE (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) was added and the unincorporated nucleotides removed by spin-column chromatography in Sephadex G-50 (Maniatis *et al.*, 1982). To calculate the percent incorporation of label into DNA, 1 μ l of pre- and post-spin column solutions were spotted on Whatman glass microfiber filters and total cpm were calculated by scintillation counting using a Beckman LS 7000 liquid scintillation counter (Berger, 1984). Typically, probes were obtained with specific activities up to 2×10^8 dpm/ μ g with approximately

20-30% of the label being incorporated.

Random priming was performed using a random hexaoligomer obtained from a DNA labelling kit (Boehringer Mannheim). DNA was denatured by boiling for 10 min. in the presence of 2 μ l of the hexanucleotide mixture in 10X reaction buffer, and subsequently cooled on ice. To the primer-annealed DNA, 1 μ l each of dGTP, dCTP, and dTTP (0.5 mmol/l), 50 μ Ci [α - 32 P]dATP (800 Ci/mmol), 2 units pol I Klenow were added and the reaction made to 20 μ l with H₂O. After incubation for 30 min. at 37°C, the reaction was terminated and unincorporated nucleotides removed by use of a spin column. Typical specific activities using this method were up to 8×10^8 dpm/ μ g with approximately 30% of the label being incorporated.

To make single stranded radiolabelled cDNA, first strand cDNA was synthesized from total RNA template essentially as described by Gubler and Hoffman (1983) with modifications (Rutledge *et al.*, 1988). Total RNA (5-20 μ g) in 10 μ l was denatured in 1 μ l methyl mercury for 10 min at RT followed by addition of 2 μ l 700 mM β -MeOH (Maniatis *et al.*, 1982). The following ingredients were then added in a total volume of 45 μ l: oligo dT(5 μ g), 1 mM final each of dGTP, dTTP, dCTP, 50-100 μ Ci [α - 32 P]dATP (800 Ci/mmol), 10 μ l 5X cDNA buffer (500 mM Tris-HCl pH 8.3, 50 mM MgCl₂, 250 mM KCl) and 40 units reverse transcriptase (Life Sciences). The reaction mixture was incubated for 1 h at 37°C then dATP was added to 1 mM final and the reaction was incubated for an additional 15-20 min. The reaction was stopped by addition of 2 μ l 250 mM EDTA and the volume adjusted to 100 μ l with H₂O. Unincorporated nucleotides were removed as described above. Incorporation of label into double-stranded cDNA ranged from 15-30%.

Labelling of oligonucleotides was done as described by Maniatis *et al.* (1982). Typically, 100-200 ng oligonucleotide were 5' end-labelled with 100 μ Ci [γ - 32 P]dATP (3000 Ci/mmol, 10 μ Ci/ μ l) in 15 μ l containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, 100 μ M spermidine, 100 μ M EDTA and 10 units T4 polynucleotide kinase

(Pharmacia). After incubation for 30 min. at 37°C, the reaction was terminated and unincorporated nucleotides were removed using P-6 Bio-Gel spin columns (Bio-Rad). Single stranded primer extended probes using M13 were prepared as described by Burke (1984).

P. Construction of cDNA bank

To construct a cDNA bank, double stranded cDNA was made from TIV-infected *E. acraea* total RNA (20 µg) isolated 8 h after TIV infection as described in section O. In this case 8 µCi [α -³²P]dATP was added to monitor the integrity of the cDNA by electrophoresis. Deoxynucleotide concentrations were 1 mM each (reaction volume of 50 µl). At the end of first strand synthesis, the following were added to a final volume of 100 µl: 10 µl 5X cDNA buffer, 20 units *E. coli* DNA polymerase I, 2.5 units RNase H (BRL). After incubation for 2 h at 16°C, 2 µl of 250 mM EDTA were added and the mixture extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Unincorporated nucleotides were removed by use of a spin column and the ds-cDNA was ethanol precipitated. The amount of cDNA synthesized was calculated by estimating the percent incorporation of radiolabel into the first and the second strands as described in section O. An aliquot of the double stranded products was electrophoresed in 1.2% agarose in TAE buffer and autoradiographed as described in section J. Typical incorporation of label was approximately 15%. The size of the cDNA product is shown in Figure 6A.

Ten ng of the blunt ended cDNA were ligated with 250 ng SmaI digested M13 mp19 treated with calf intestinal phosphatase (CIP, Boehringer Mannheim; 0.22 units/3 µg, added 20 min. prior to termination of the digest) in the presence of 1 mM ATP, ligation buffer (Maniatis *et al.*, 1982) and 3 units T4 DNA ligase (Pharmacia). After incubation at 12°C overnight, one-half of the ligation was used to transfect JM109 cells.

Q. Transcript mapping

Mapping of the 5' end of the capsid transcript was carried out by primer extension analysis of mRNA (Calzone *et al.*, 1987) using reverse transcriptase (AMV). Two separate primers were made (Dr. W. Sung, Molecular Genetics Section, Division of Biological Sciences, NRC). The first (STM1F: 3'-GTAC AGA TAC AGG AGT AGC TTA TAT TGG-5') was designed to begin priming at the first nucleotide (C) upstream of the methionine start codon of the capsid ORF. The second (PE2: 3'-CCG CCG GGT TGT CGT TGT CGT ATG AAAC-5') spanned nucleotides #688 to #715. For primer extension, the primer was labelled at the 5' end by phosphorylation with polynucleotide kinase and [γ - 32 P]ATP (see section O) and 400 fmole was combined with 20 μ g of total TIV-infected *E. acraea* RNA (16 h p.i.) or total yeast RNA provided by Dr. K. Prakash (NRC). Annealing was carried out by resuspending the RNA and primer in 4 μ l, adding 1 μ l 5X hybridization buffer (1X: 40 mM Na Pipes pH 6.4, 400 mM NaCl, 1 mM EDTA), heating to 68°C for 2 min and cooling slowly to about 45°C over a 30 min period. To this mixture, the following were added to a final volume of 50 μ l: 10 μ l 5X cDNA buffer, dNTPs (1 mM final of each), 40 units of AMV reverse transcriptase. The reaction was then incubated at 37°C for 1 h and the RNA removed by alkali hydrolysis (50 mM NaOH for 1 h at 68°C). After neutralization with HCl and 20 μ l 1 M Tris-HCl pH 8.0 the mixture was passed through a P6 spin column. The primer extended product was analyzed on an 8% polyacrylamide sequencing gel along side a sequencing ladder made from the same primer using clone MH26 as template (see section S).

R. Characterization of M13 clones

a. Phage lysis

M13 recombinant clones were sized on 0.7% agarose gels by direct phage lysis of the culture supernatant (Messing, 1983). To 20 μ l of culture supernatant, 5 μ l lysis buffer (restriction enzyme stop buffer containing 2% SDS) were added and the solution incubated at least 1 h at RT prior to electrophoresis in TBE buffer. Samples were stained with EtBr

after electrophoresis.

b. C tests

The identity of recombinant clones was often verified by the C-test method (Messing, 1983). Ten μ l of each M13 phage DNA preparation was mixed with 1 μ l 2.5M NaCl and 5 μ l C-buffer (60% deionized formamide, 1% SDS, 0.1% BPB) and incubated at 65°C for 1 h. The samples were electrophoresed in 0.7% agarose with TBE buffer until the BPB migrated approximately 5 cm. Potential positives (hybrids of two clones) were judged against known positive and negative control M13 DNA preparations.

c. S1 nuclease digestion

To verify M13 insert sizes or to prepare double stranded insert DNA from the template DNA, clones containing inserts in opposite orientations were hybridized and digested with S1 nuclease (Seligy *et al.*, 1987). Typically, 1 μ l of each template was combined with 6 μ l H₂O and 1 μ l 10X S1 buffer (Maniatis *et al.*, 1982). After incubation at 65°C for 20 min., 1 μ l S1 nuclease (1 unit/ μ l) was added and the mixture incubated at 37°C for 20 min. The reaction was stopped by adding 1/10th volume of endonuclease stop buffer.

S. DNA sequencing

DNA sequencing was carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) using either the *E. coli* DNA polymerase I "Klenow" fragment or modified T7 DNA polymerase (Tabor and Richardson, 1987). The latter referred to as "Sequenase" was provided as a kit by the manufacturer (USB). All sequencing was done using [α -³⁵S]dATP (Biggin *et al.*, 1983). For sequencing using the Klenow enzyme, dNTP stocks were prepared according to Messing (1983). Sequencing with Sequenase was done as described by the manufacturer. In a typical reaction, template DNA (up to 500 ng) was mixed with 2 μ l 5X sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl), 2 μ l universal 17-mer primer, and made up to 10 μ l with H₂O. The mixture was heated to 65°C for 2 min., then allowed to cool slowly to RT over 30 min. To the annealed template-

primer, the following were added: 1 μ l 100 mM dithiothreitol (DTT), 2 μ l diluted (1:5) labelling mix (5X dGTP: 7.5 μ M dGTP, 7.5 μ M dCTP, 7.5 μ M dTTP), 1 μ l [α - 35 S]dATP (~650 Ci/mmol, 10 μ Ci/ μ l; Amersham), and 2 μ l of Sequenase (~110 units/ μ l, diluted 1:8 in TE'). After incubation at RT for 5 min., 3.5 μ l of this reaction were added to 2.5 μ l of each of the prewarmed termination mixes and the reaction incubated at 37°C for 5 min. (Termination mixes: 80 μ M of each dNTP, 50 mM NaCl plus: 8 μ M ddGTP (ddGTP); 8 μ M ddATP (ddATP); 8 μ M ddTTP (ddTTP); 8 μ M ddCTP (ddCTP)).

Double-stranded supercoiled DNA sequencing was carried out on plasmid DNA (DNA sequencing instruction manual, Stratagene, San Diego, USA) isolated by the mini-prep method (section H,c). One μ l plasmid DNA (200-500 ng) was denatured for 5 min. at RT by addition of 4 μ l NaOH in a 20 μ l volume containing 0.5 mM EDTA. After placing the tube on ice, 2 μ l 2 M NH_4OAc pH 5.4 were added and the DNA was ethanol precipitated. The DNA was immediately centrifuged for 15 min. at 13 Krpm at 4°C. The DNA pellet was then rinsed with 70% ethanol, dried in a speed vac concentrator (Savant) and resuspended in 2 μ l reverse sequencing primer (Pharmacia), 2 μ l 5X sequenase buffer and 6 μ l H_2O . The primer was annealed for 15 min. at 37°C and the DNA sequenced with Sequenase as described above.

All sequencing reactions were terminated by heat inactivation at 68°C for 10 min. Reactions were then vacuum dried, resuspended in 4 μ l stop solution (95% formamide, 20 mM EDTA, 0.05% BPB, 0.05% xylene cyanol FF), and prepared for loading or frozen at -20°C.

a. Polyacrylamide gel electrophoresis

BRL sequencing units (S0 and S2) with 43 cm long glass plates were used for all sequencing runs. Wedge spacers (0.4-1.2 cm) and regular spacers (0.4 cm) were used for wedge and stretch gels, respectively. A 5% acrylamide concentration (19:1 acrylamide/bis), in 0.5X TBE with 8 M urea was used for wedge gels (Carlson and Messing, 1984). Stretch gels were 6% acrylamide (19:1 acrylamide/bis) in 0.5X TBE with 8 M urea and were used

for reading deeper sequences. Gels were pre-electrophoresed in 1X TBE at 55-60 watts for 30 min (constant wattage; voltage upper limit of 1850 volts). Wedge gels were run until the BPB dye reached the bottom of the glass plate. Samples were boiled for 2.5-3 min and typically one-half of the reaction (2 μ l) was applied to a sharktooth comb (5 mm).

After electrophoresis, the gels were fixed (Ornstein and Kashdan, 1985) in 5% methanol/5% acetic acid for 30 min. (wedge gel) or 10 min. (stretch gel), transferred to Whatman 3 MM paper, covered with Saran wrap and dried at 80°C on a slab gel drier. X-ray film was placed in direct contact with the gel and exposure was carried out at RT for at least 18 h.

b. Cloning and sequencing of capsid gene

The cloning of HindIII #26b into M13 mp19 and pUC19 vectors was done according to the protocol described by Zabarovsky and Allikmets (1986). Briefly, XbaI digested vector was partially filled-in with dCTP and dTTP, while the HindIII insert was partially filled-in with dGTP and dATP using Klenow enzyme. The resulting XbaI/HindIII sites are complementary and can be ligated together while no recircularization of either vector or insert occurs. This cloned fragment in pUC19 was called pH26b.

The following strategy for sequencing was followed: a) Dale deletions (Dale *et al.*, 1985) were produced and sized by electrophoresis; b) template DNA was prepared from deletion clones that differed by ~200-300 bp; c) the insert size of the Dale clones was verified by S1 nuclease digestion of the clone hybridized with the complementary sequence of its full length parent (a clone in the opposite orientation; see section R, c for details); d) sequencing of Dale deletions on wedge and stretch gels. Step c was found to be very useful particularly with clones containing large insert sizes.

DNA sequencing was carried out by creating Dale deletions (Dale *et al.*, 1985) in M13 mp19 or by subcloning into M13 vectors. The vector M13 mp19 was chosen for the creation of all the Dale deletions because it was found that digestion of the RD20 primer/template with EcoRI was more efficient and reliable than the HindIII digestion of

RD29 primer/template.

Following the production of deletion clones (see below) and transfection, individual plaques were recovered, sized, and then amplified in 1.5 ml cultures of JM101 (1/50 dilution of an overnight culture inoculated into 2YT and grown at 37°C for 30-60 min.) and grown at 37°C for 7-12 h. Dale clones were sized, by supernatant lysis of recombinant phage (see section R, a), on 0.7% agarose gels in TBE buffer without EtBr during electrophoresis. Clones were then selected and template DNA was prepared from 1 ml of the culture supernatant as described above.

For sequencing of the capsid gene, Dale deletion clones were created, in both orientations, from TIV Sall #33 (MS33 and MS33op) and TIV HindIII #26b (MH26 and MH26op) each cloned in M13 mp19. A 1050 bp Sall/BamHI and a 400 bp BamHI/Sall from HindIII #26b were also subcloned into M13 mp19 to complete the sequencing.

T. Capsid protein expression in *E. coli*

To attempt expression of the capsid gene in *E. coli* using TIV regulatory sequences, HindIII fragment #26b was cloned into the XbaI site of plasmid pUC19 as described in section S, b. The resulting plasmid called pH26b was transformed into *E. coli* JM101 cells and expression studied without induction of the *lacZ* promoter with IPTG.

To produce an α -peptide/capsid fusion protein in *E. coli*, the 1852 bp ClaI/EcoRI fragment from pH26b (300 ng) was ligated (vector to insert ratio of ~1:3) with BamHI/EcoRI pUC19 (150 ng) in a 25 μ l reaction containing 1X ligation buffer, 1 mM ATP and 0.5 units ligase (BRL) for 2 h at RT. Both vector and insert DNAs were electrophoresed in agarose (1.2%) and purified using the IBI electroeluter (see section J). To produce an in frame fusion between the two proteins, it was necessary to fill in the BamHI site of pUC 19 with dGMP, delete the remaining overhanging bases of the resulting BamHI site as well as the ClaI site from the capsid gene sequence, and blunt end ligate (see Figure 31). Therefore, after inactivation of the ligase at 68°C for 10 min., the following were added to a final reaction volume of 50 μ l: 2.5 μ l 10X nick translation buffer,

0.5 μ l 10 mM dGTP, and 2 units Klenow enzyme. After incubation for 25 min. at RT, 1 μ l *E. coli* DNA polymerase I was added for blunt end formation of the BamHI and ClaI sites and incubation continued for a further 25 min. The reaction mixture was extracted once with an equal volume of chloroform: isoamyl alcohol (24:1), precipitated with two volumes of ethanol, and the pellet rinsed with 70% ethanol to remove residual salt that might inhibit the ligation. The purified DNA was then blunt end ligated for 4.5 h at RT in 10 μ l containing 1 mM ATP, 1X ligation buffer, and 3 units T4 DNA ligase. This mixture was used to transform competent JM101 cells. Initial screening of transformants was done by digestion of recombinant plasmids with XbaI to release an internal 125 bp fragment. Two positive clones were selected for sequencing using the reverse primer and one of these, pLC26, containing the in frame fusion gene was used for further studies.

Stationary cells were prepared by growing overnight cultures in 2YT of JM101 cells in the absence of antibiotic, and pUC19 and pLC26 recombinants in the presence of ampicillin (35 μ g/ml). Each of these cultures was then used to inoculate (1/50) 1.5 ml 2YT cultures containing no inducer, or IPTG (0.5 mM final; Slocombe *et al.*, 1982) as an inducer. The cultures were grown for 18 h at 37°C, chilled on ice for 10 min., and bacterial cells washed twice with ice cold TE. The final cell pellets were resuspended in 200 μ l TE and 200 μ l 2X Laemmli loading buffer added. Cells were disrupted by 3 cycles of freezing on dry ice (3 min.) followed by sonication for 1 min. at 50°C. Samples were then boiled for 5 min. prior to analysis by SDS-PAGE.

III. Results

A. Characterization of the TIV genome

a. Estimation of genome size

In order to study TIV at the molecular level, it was necessary to analyze the genome of this virus by restriction endonuclease digestion and agarose gel electrophoresis. For such an analysis, total TIV DNA isolated from virions was subjected to limit endonuclease digestion and electrophoresis in agarose. The cleavage patterns obtained with the endonucleases BamHI, HindIII, EcoRI, PstI, Sall, XbaI, and XhoI shown in Figure 1 and Table 3 are very reproducible. The complexity of this genome is revealed by the presence of several submolar fragments, and variability in estimated genome size obtained from summation of the restriction enzyme fragments (176-247 Kbp).

b. Multiple DNA components of TIV

To understand the TIV genome in greater detail, total TIV DNA was isolated from particles and examined by fractionation in agarose gels. As part of the preliminary work I did on my thesis, it was shown that total DNA purified from TIV particles contains 4 DNA components that are TIV specific (Tajbakhsh *et al.*, 1986). These components were called L (large), S (small) 1, 2, and 3 in order of diminishing size. The L (> 176 Kbp) and the S1 (0.8 Kbp) components are detected consistently in ethidium bromide stained agarose gels of total TIV DNA (see Figure 2A) whereas the S2 and S3 DNA components are made visible only after hybridization of S1 DNA to Southern blots of total TIV DNA. Since the S2 and S3 DNA components may be deletions of L and/or S1 (Tajbakhsh *et al.*, 1986), the L and S1 DNA components were the only ones chosen for more detailed analysis in this thesis. The S1 DNA has four EcoRI sites yielding 3 fragments (8.5, 1.5 and 0.85 Kbp; Tajbakhsh *et al.*, 1986) for potential cloning. However, after numerous repeated attempts only the 0.85 Kbp segment was successfully cloned into the plasmid vector pUC19. This clone was called pE3 and was subsequently used as a probe for the detection of S1

Figure 1. Agarose gel electrophoresis of TIV DNA digested with restriction endonucleases.

Two micrograms of total TIV DNA were subjected to limit digestion with 8-10 units of enzyme prior to agarose (0.7%) gel electrophoresis for 95 h at 0.6 V/cm. After electrophoresis, fragments were stained with 0.5 $\mu\text{g/ml}$ of ethidium bromide. Molecular size markers (Kbp) were lambda/HindIII, pBR322/TaqI and YEP-*ADE1* (Dimock *et al.*, 1983). Band numbers are indicated to the left of the lanes. See Table 3 for summary of the size estimates of TIV DNA restriction fragments.

Table 3. Size estimates of TIV DNA restriction fragments¹.

Band No.	Fragment Size (Kbp)						
	BamHI	EcoRI	HindIII	PstI	Sall	XbaI	XhoI
1	19.0	>25	(14.0)	32.0	(23.0)	21.5	26.0
2	18.0	(22.0)	(12.2)	26.0	19.5	(20.0)	22.0
3	17.0	14.0	(10.2)	23.0	18.0	17.0	19.0
4	15.2	(13.5)	8.5	(21.0)	(17.0)	(15.3)	(18.0)
5	14.9	(13.0)	8.0	(19.0)	16.0	(13.7)	13.5
6	14.3	12.5	(7.3)	18.0	15.0	12.8	(13.0)
7	13.5	11.8	(7.1)	(14.0)	14.5a,b	12.2	12.5
8	(12.5)	9.0	6.7	13.0	13.4	9.8a,b	11.9
9	(11.8)	8.7	6.0	11.0	(13.0)	(9.5)	10.2
10	10.8a,b	8.5	5.9	10.2	(11.0)	9.0	9.7
11	(10.2)	(8.0)	5.5a,b	(10.0)	10.2	8.7	9.4
12	(10.0)	(7.4)	5.4	8.7	8.6	8.4a,b	8.5
13	(9.8)	(7.1)	5.2	(8.6)	(8.3)	(8.2)	7.9
14	(9.5)	(6.9)	4.6	(8.4)	8.1	7.5	(7.4)
15	(8.7)	5.8	4.4	8.0	7.6a,b	7.4	6.6
16	6.7a,b	4.9	4.1a,b	5.5	(7.0)	7.1	6.2
17	5.2	4.5a,b	3.8a,b	(5.4)	6.6	6.3a,b	5.1
18	4.4	4.4	3.7	4.8	(6.2)	5.7	3.7a,b
19	3.3	4.3	3.5	4.4	5.9	5.4a,b	2.95
20	0.75	4.0	3.3	2.10	5.6a,b	4.4	(2.35)
21	0.65	3.9	3.2a,b	1.50	5.2	3.7	(1.82)
22	0.60	3.6a,b	3.0	1.40	(5.0)	3.0	(1.65)
23		3.5a,b	2.9		4.8	2.80	
24		3.4	2.75		4.4a,b	2.65	
25		3.3	2.60a,b		(4.0)	2.50	
26		3.0	2.47a,b		3.7	2.3	
27		2.70	2.25		3.4	1.9	
28		2.50	2.15		(3.1)	1.8	
29		2.35	1.87		2.55	1.45	
30		2.1	1.82a,b		2.3	1.3	
31		1.74	1.70a,b		2.2	0.85	
32		1.40	1.6		2.0	0.75	
33		1.36	1.25		1.95a,b		
34		1.30	1.15		1.94		
35		1.20	1.05		1.45		
36		0.95	0.87a,b		1.1		
37		0.70	0.80a,b		0.3		
38		0.40	0.60				
39			0.3a,b				
Total	234.3	242.8	190.9	256.0	317.9	264.8	223.1
Total of submolar fragments	161.8	164.9	140.1	169.6	220.3	198.1	178.9

Average size of genome = 176 ± 10⁶ to 247 ± 17 Kbp¹Revised from Tajbakhsh *et al.* (1986). Values in parentheses represent submolar fragments.²Value determined by elimination of submolar fragments.

Numbers highlighted in bold are of relevance to this thesis.

Figure 2. Analysis of total TIV DNA by agarose gel electrophoresis and investigation of the possible methylation of the TIV genome.

(A) Purified TIV particles were DNase I and RNase A treated and DNA was purified (see Materials and Methods, section H,b1). Sall digested (lane 1) and undigested TIV DNA (lane 2) were electrophoresed on a 0.7% agarose gel and stained with EtBr. Note the presence of both L and S1 DNA components in the total uncut TIV DNA sample (lane 2).

(B) TIV DNA digested with restriction endonucleases HpaII and MspI. Markers (M, Kbp) represent lambda phage digested with HindIII and EcoRI.

DNA. This sequence is also present in the L DNA component.

Since methylation of FV3 DNA is extensive (Willis and Granoff, 1980), similar modifications of TIV DNA could potentially obscure interpretation of TIV restriction fragment analysis. To determine if TIV DNA is modified by methylation, total TIV DNA was digested with the isochizomeric nucleases HpaII and MspI. This data shown in Figure 2B shows similar restriction profiles for TIV DNA using these restriction endonucleases. Therefore, methylation of CpG does not seem to occur to any significant extent in TIV DNA.

c. S1 DNA is present in virions and infected cells

Since the presence of a smaller DNA component is a novel finding among the large dsDNA viruses, it was necessary to have further evidence that S1 DNA is indeed packaged into virions and is TIV specific. A number of approaches were chosen. First, purified virions were subjected to DNase I and RNase A digestion to remove any contaminating host nucleic acids and virion DNA was subsequently purified (see Materials and Methods, section H, b2). These results shown in Figure 2A indicated that L and S1 DNA components were still present after these treatments.

The second approach was basically an extension of our earlier observation (Tajbakhsh *et al.*, 1986) which indicated that the L to S1 ratio was approximately 1 in "full" virions, but "partially filled" virions (which migrated above the main ["full"] viral band in sucrose gradients) contained less L than S1 DNA, giving an S1 to L ratio greater than 5. To further verify those observations, pooled TIV from sucrose gradients was fractionated on a CsCl step gradient (see M & M, section A) and viral bands identified by EM to correspond to empty, partially filled, and full capsids were removed and DNA was extracted. Figure 3 shows that empty capsids, as expected, contained no detectable DNA (lane 1). However, as seen before, partially filled virions (lane 2) contained more S1 than L in comparison to that contained by the main viral fraction (lane 3).

The third approach was directed at determining if the S1 DNA component of TIV is

Figure 3. Agarose gel electrophoresis of DNA extracted from CsCl fractionated TIV.

Viral bands corresponding to empty (lane 1), partially filled (lane 2) and full (lane 3) capsids were removed from CsCl step gradients and the DNA was electrophoresed on an agarose gel (0.7%). Lane 4 contains TIV DNA from conventionally purified virions. L and S1 DNA components are indicated. Markers (M, Kbp) represent lambda phage DNA digested with HindIII.

present in infected cells. These experiments involved extracting total DNA from uninfected and TIV-infected *E. acraea* and *A. albopictus* cells. As seen in Figure 4A, a DNA fragment 11 Kbp in size is present in both infected cell lines (lanes 2 and 4). This band comigrates with S1 DNA found in purified virions (lane 5) and yields the same EcoRI digestion pattern as S1 DNA (not shown). Cellular and TIV L DNA were not separated from each other under these electrophoretic conditions. In order to estimate the levels of S1 DNA relative to L in infected cells and in purified virions, the gel in Figure 4A was blotted and hybridized with the radiolabelled 0.85 Kbp EcoRI fragment of S1 DNA (see Figure 4B). The blot was then rewashed and hybridized with radiolabelled pTB17-6 containing the 5.2 Kbp BamHI TIV DNA fragment #6 that is specific only for the L DNA component (Figure 4C). The results seen in Figure 4B show that S1 DNA is present in both cell lines. The higher concentration of S1 DNA in infected *E. acraea* cells was expected due to a higher percentage (>70%) of TIV-infection of this cell line compared to *A. albopictus* (10-40%). By comparing the EtBr stained gel (Figure 4A) with the hybridization results (Figure 4B, C), it is evident that the intracellular ratio of S1 DNA to L DNA is at least 10 fold higher than that found in purified virions.

B. Semipermissive replication of TIV

In attempting to clarify some of the complexity in the TIV genome, plaque purification of TIV was undertaken. Since *E. acraea* cells grow only as a suspension culture, it was necessary to select a cell line that would grow attached to the culture dish. The Dipteran *A. albopictus* C6/36 cells were chosen for these studies. These mosquito cells are epidermal in origin and were used by Kelly (1976) to replicate IV22. Initial attempts at viral production in the *A. albopictus* cells under a variety of pH and temperature conditions (M. Frosch, N. Olson, P.E. Lee, unpublished results) were unsuccessful. Plaque assay studies were then carried out. These studies showed a general increase in the amount of cell death as the viral inoculum concentration was increased. However, repeated attempts at reinfesting *A. albopictus* cells with "plaque-like" regions that were generated during this

Figure 4. Presence of S1 DNA component in infected cells.

Total DNA was harvested from uninfected (lanes 1 and 3) and TIV-infected (lanes 2 and 4) *A. albopictus* and *E. acraea* cells, respectively, electrophoresed in 0.7% agarose, and stained with EtBr (A). Lane 5 contains TIV DNA extracted from virions. The DNA was transferred to a Biotrans nylon filter and hybridized with the nick translated insert of pSE3 containing an 0.85 Kbp cloned fragment of S1 DNA (B). This blot was then rewashed and hybridized with nick translated insert of pTB17-6 containing sequence specific to the L DNA component (C) (see text).

A

1 2 3 4 5

B

1 2 3 4 5

C

1 2 3 4 5

-L

-S1

procedure, were unsuccessful. A more detailed analysis at the molecular and microscopic level was then undertaken to determine the extent of TIV replication in these cells.

a. Viral DNA and VC synthesis

To determine the extent of TIV replication in *A. albopictus* cells, comparisons were made with the permissive *E. acraea* cells. We first looked at VC formation and ^3H -thymidine uptake into these VCs. TIV infection of *A. albopictus* cells varied from 10-40%, as determined by VC formation (Figure 5A). In contrast, TIV infection of *E. acraea* cells was often observed to be 70% or greater. An increase in the virus concentration resulted in no significant increase in the numbers of infected cells. VCs of both infected cell lines looked similar as visualized by Hoechst staining (*E. acraea* not shown).

TIV-infected *A. albopictus* cells showed ^3H -thymidine uptake into VCs, similar to that seen with the *E. acraea* cells, as determined by the presence of silver grains over these regions (Figure 5B and 5C). Further, the inhibition of host DNA synthesis was seen with both infected cell lines to the extent that was reported earlier with *E. acraea* cells (Mathieson and Lee, 1981). As a control, uninfected *A. albopictus* cells treated in the same manner as infected cells, incorporated large amounts of label into nuclei (Figure 5D).

b. TIV RNA synthesis

Since TIV-infected *A. albopictus* cells displayed ^3H -thymidine incorporation into viral DNA in cytoplasmic VCs, we compared the major viral transcripts produced in infected *A. albopictus* and *E. acraea* cells. To do this, a method that does not require direct labelling of the RNA was employed. Total RNA was extracted from TIV-infected *A. albopictus* and *E. acraea* cells at 4, 8, 12, 16, and 24 h p.i. Radiolabelled first strand cDNA was made from each time point RNA using oligo dT as a primer and AMV reverse transcriptase (see Figure 6A). These single stranded cDNA probes were then hybridized to replica blots of TIV DNA digests (Figure 6B). A method similar to this was used successfully to isolate the abundant transcript of the polyhedrin gene of *Bombyx mori* nuclear polyhedrosis virus

Figure 5. Light microscope autoradiography of TIV-infected cells.

(A) Hoechst 33258 staining of infected *A. albopictus* cells showing differential staining of VCs (v) and nuclei (n). (B) and (C) TIV-infected *A. albopictus* and *E. acraea* cells, respectively. Cells were infected, labelled with ^3H -thymidine (10-12 h p.i.), chased with cold thymidine, and fixed (16 h p.i.) for light microscope autoradiography (see Materials and Methods, section C for details). (D) Uninfected *A. albopictus* cells labelled and fixed as above. Bar represents 10 μm .

Figure 6. Hybridization analysis of major transcripts in infected cells.

Total RNA was extracted from TIV-infected *A. albopictus* and *E. acraea* cells at 4, 8, 12, 16, and 24 h p.i. and each was used to make ^{32}P -labelled cDNA. (A) A profile (1.4% agarose) of radiolabelled cDNA made from one such RNA sample using AMV reverse transcriptase, and oligo dT as primer. These time course cDNA probes were then used for hybridization to replica blots of EcoRI (E), HindIII (H), and Sall (S) digests of TIV DNA (B). Radiolabelled markers (M) are pBR322/HinfI (see Materials and Methods, section J). (C) Radiolabelled cDNA made from uninfected *E. acraea* (Ea) and *A. albopictus* (Aa) cell RNA showed no hybridization to TIV DNA. The insect DNAs were digested with EcoRI. (D) and (E) show hybridization results using cDNA prepared from RNA of infected *E. acraea* and *A. albopictus* cells, respectively. Undigested TIV DNA (T) showing L and S1 DNA components are indicated. Marker sizes for lambda phage digested with HindIII/EcoRI are indicated.

(Maeda *et al.*, 1985). These results shown in Figure 6D and 6E indicate that the major transcripts produced in both of the cell lines, are located on similar sized TIV DNA fragments. Such a qualitative analysis could not determine if 2 or more different transcripts were encoded within the same DNA fragment, making it difficult to distinguish individual transcripts. This phenomena of gene clustering is not uncommon with other viruses (Rankin *et al.*, 1986; Weinrich and Hruby, 1986). By comparing the smaller hybridizing fragments between the two cell lines, the possibility of gene clustering should be reduced.

Two fragments of interest shown in Figure 6D, E are Sall fragments #24a (4.4 Kbp) and #36 (1.1 Kbp). Both of these fragments code for transcripts which appear as early as 4h p.i. and remain transcriptionally active throughout the infection. With this type of analysis, peak transcriptional activity was found to be at 12 h p.i. for both *E. acraea* and *A. albopictus* cells (Figure 6D, E). This transcription analysis also revealed that the S1 DNA component of TIV contains sequences that are transcribed. This data is shown for the 12 h samples of both cell lines (Figure 6D, E). This DNA component hybridized with radiolabelled cDNA throughout the assay period (not shown).

Control hybridization experiments were also done with 12 h and 24 h RNA from uninfected *A. albopictus* and *E. acraea* cells. The hybridization results for the 12 h RNA from both *E. acraea* and *A. albopictus* cells is shown in Figure 6C. These control experiments showed no hybridization to TIV DNA, and hybridization only to the respective genome. Similar results were obtained using 24 h RNA. These transcription studies indicate that TIV infection of *A. albopictus* cells results in temporal changes in transcription in a manner similar to that observed in TIV-infected *E. acraea* cells.

c. Assembly of progeny virions

After determining that infected *A. albopictus* cells synthesize TIV DNA, RNA and VCs, an electron microscopic comparison between TIV-infected *E. acraea* and *A. albopictus* cells was carried out. A time course EM analysis was made of TIV-infected *A. albopictus* at

10min, 1, 4, 8, 12, 16, 24, 48 and 96 h p.i. From such an analysis, shown in Figures 7 and 8, we determined that even by 96 h p.i., over 80% of the VCs in *A. albopictus* cells viewed under the EM were devoid of progeny virions or partially assembled capsids whereas progeny virions were seen as early as 16 h p.i. in over 80% of the infected *E. acraea* cells (see Figure 7A; also Mathieson [1978]). Membrane bound viral particles representing viral inoculum were seen up to at least 4 h p.i. (see Figure 8A). VCs were seen as early as 4 h p.i. and these increased in size and number as the infection progressed (data not shown). VCs in *A. albopictus* cells appeared like those in *E. acraea* both by light microscopy and EM. Figure 7A shows a typical *E. acraea* VC consisting of dense matrix enclosing cell cytoplasm. The easily distinguishable progeny virions in the *E. acraea* VC are notably absent in the *A. albopictus* VC as shown in Figure 7A and 7B.

d. TIV capsid protein produced during infection

From the data presented thus far, it appears that the block in TIV replication in *A. albopictus* cells most likely occurs during or prior to assembly of progeny virions. It also appears that overall RNA synthesis is comparable to that of infected *E. acraea* cells. It was therefore of interest to determine if at least one viral protein, the major TIV structural or putative "capsid" protein, is synthesized in these semipermissive cells. This protein had a measured molecular mass of 46 KDa on SDS-PAGE gels, however it will be referred to in this thesis as the 50 KDa protein (calculated from the protein size predicted from DNA sequence analysis).

To do these experiments, it was necessary to produce an antibody directed against the TIV capsid protein. This was done by preparing purified capsid protein from mature *G. mellonella*-TIV by electrophoresis, and immunizing rabbits with the purified protein. The resulting antiserum detected at least 1 ng of purified protein by radioimmunoassay using ¹²⁵I-protein A or alkaline phosphatase linked antirabbit antibody (data not shown). By western analysis the antibody detected a protein of expected size (46 KDa) and one that is approximately 64 KDa, both intracellularly and in purified virions. This latter band may

Figure 7. Transmission electron microscope analysis of TIV-infected cells.

Cells were infected with TIV, fixed at the designated times and processed for EM analysis.

(A) High magnification of a TIV-infected *E. acraea* cell harvested at 16 h p.i. Virions are found within the cytoplasmic (Cy) component of the VC which is surrounded by a dense matrix (D). Virions are indicated by arrows. (B) High magnification of an *A. albopictus* VC (96 h p.i.). Note the absence of progeny virions or viral capsids. Bar represents 0.5 μ m. N = nucleus.

A

Figure 8. A time course EM analysis of *A. albopictus* cells infected with TIV.

(A), (B), (C) and (D) show TIV-infected *A. albopictus* cells fixed at 1, 8, 24 and 48 h p.i. respectively. Note the absence of progeny virions or viral capsids. Bar represents 1 μ m.

N = nucleus

V = viroplasmic center

i = viral inoculum.

represent a precursor or modified form of the capsid protein. It may also represent a binding with another protein(s), forming a complex that cannot be dissociated under the conditions employed.

A time course western blot analysis of the synthesis of this protein was made from *E. acraea* cells and *A. albopictus* cells at 4, 8, 12, 16, 24, and 48 h p.i. using the anticapsid antibody. These results shown in Figure 9 indicate that the major TIV structural protein is detectable in both *E. acraea* and *A. albopictus* cells. Further, the protein is detectable as early as 4 h p.i. in both cell lines with a peak synthesis occurring about 24 h p.i. in *E. acraea* cells (Figure 9B, D). In contrast, no such distinguishable peak was seen with *A. albopictus* cells over the same assay period. Although the amount of 48 h sample loaded in Figure 9C was less than the other corresponding lanes there appeared to be a slight decline in the amount of capsid protein in infected *A. albopictus* cells. A duplicate experiment where ^{125}I protein A was substituted for the secondary antibody verified this result. In both cell lines, TIV structural proteins were not readily detectable by silver staining. The most notable difference between the cell lines occurred at 8 h p.i. where a protein was detected in TIV-infected *E. acraea* cells (about 70 KDa) that was not detected in *A. albopictus* samples (Figure 9A, C). It is not known if this protein is also synthesized in the *A. albopictus* cells at times that were not assayed. The identity or function of this protein has yet to be determined.

C. Isolation of capsid gene: strategy based on cloning of most abundant transcripts

It is evident that more molecular information is needed, specifically on TIV genes to elucidate the mechanisms that are involved in TIV gene expression. The semipermissive studies indicated that the block in TIV replication in *A. albopictus* cells likely occurs prior to assembly of virions. If this is indeed the case, the most useful gene for analysis would be the TIV capsid gene (see also Introduction for other rationale).

In order to isolate the capsid gene, several strategies were considered. Based on other examples (Wittek *et al.*, 1984; Tham *et al.*, 1986; Heine *et al.*, 1974; Horwitz *et al.*, 1969)

Figure 9. Synthesis of the TIV capsid protein in infected cells.

A. albopictus and *E. acraea* cells were TIV-infected and harvested at 4, 8, 12, 16, 24 and 48 h p.i. Intracellular proteins were separated by SDS-PAGE (4% stack/10% base) and silver stained. Duplicate gels were western blotted to nitrocellulose filters and the capsid protein was detected by incubating the filters with anti-capsid antiserum (primary) followed by alkaline phosphatase conjugated antirabbit IgG (secondary). (A) and (C) show silver stains, and (B) and (D) show western blots for *E. acraea* and *A. albopictus*, respectively. The capsid (50 KDa) protein is included. U = uninfected cells. M = markers. Dots represent the locations of the capsid and capsid-related (64 KDa) proteins.

E. acrea

A M U 4 8 12 16 24 48

Silver Stains

B U 4 8 12 16 24 48

Western Blots

A. albopictus

C M U 4 8 12 16 24 48

D U 4 8 12 16 24 48

one might expect that the TIV capsid gene will be expressed in relatively abundant amounts late in infection. This would mean that the most intensely hybridizing fragments in Figure 6D and 6E could be possible candidates for containing the capsid gene. The two most abundant transcripts appeared to be located on two unlinked fragments, Sall #24a and #36. These two transcripts were called AB1 and AB2 respectively.

a. Characterization of the AB1 gene

An insert containing sequences for the putative AB1 gene was isolated from a pBR322 plasmid, L13, shown in Figure 10C. This plasmid was selected from several such recombinant clones by hybridization of radiolabelled cDNA (see section B, b) to a partial TIV DNA bank in pBR322 (data not shown). More detailed mapping revealed that this plasmid contains BamHI fragment #4 (15.2 Kbp) which harbors a 2.2 Kbp Sall/BamHI fragment of Sall fragment #24a. This 2.2 Kbp Sall/BamHI fragment was subsequently cloned into M13 mp19 and called SL13-1A. Hybridization of insert DNA from SL13-1A back to digests of TIV DNA (results shown in Figure 10A, B) confirmed that this fragment is a subset of Sall fragment #24a and is present as a single copy in the genome. Hybridization of ³²P-labelled cDNA made from 16 h total TIV-infected *E. acraea* RNA revealed that the 1600 bp HindIII/BamHI of SL13-1A contains the AB1 gene (Figure 11A, B). In addition, single stranded template prepared from SL13-1A failed to hybridize with the radiolabelled cDNA (Figure 11A, B; lane 3) suggesting that this template (plus strand) contains the DNA coding strand for the AB1 gene.

Insert DNA from SL13-1A was used to hybridize to a Northern blot of total *E. acraea* RNA and a slot blot containing equivalent amounts of total RNA extracted at various times after infection. These data shown in Figure 12A, B, indicate that the AB1 transcript is present throughout infection. Further, the transcript size was estimated to be 1.1 Kb. Since the capsid transcript was expected to contain at least 1.4 Kb of coding information, the AB1 gene may not code for the capsid protein. Nevertheless, experiments were done to clone the AB1 mRNA (cDNA) sequences.

Figure 10. Hybridization of the AB1 gene to TIV DNA digests.

TIV DNA was digested with various restriction endonucleases, electrophoresed in 0.7% agarose (A) and alkaline transferred to nylon (Hybond) membrane. (B) X-ray film of a hybridization using insert DNA from SL13-1A radiolabelled with ^{32}P by nucleotide chain extension using random primers. The first lane contains L13 insert DNA. The adjacent lane contains a mixture of Sall fragments 24a,b digested with BamHI. (C) Restriction endonuclease map of plasmid L13 from which clone SL13-1A was obtained.

M = markers; lambda digested with HindIII/EcoRI

U = Undigested TIV DNA

B = BamHI

E = EcoRI

H = HindIII

P = PstI

S = Sall

Xb = XbaI

Xh = XhoI

A L13 S24a,b/B MUB EHP SXbXhM

B L13 S24a,b/B MUB EHP SXbXh M

C

LEGEND

B : BamHI
E : EcoRI
S : SalI

L13

Figure 11. Mapping of the AB1 gene on SL13-1A.

SL13-1A RF DNA was restriction endonuclease digested, electrophoresed in 1% agarose (A) and ~~alkaline~~ transferred to nylon (Biotrans) membrane. Radiolabelled cDNA, made by oligo dT priming of *E. acraea* RNA isolated 16 h after TIV infection, was hybridized to this blot (B). Marker sizes for lambda DNA digested with HindIII are indicated (see also Figure 3). (C) Restriction map of SL13-1A.

Lane 1 = total TIV DNA

Lane 2 = Sall digested TIV DNA

Lane 3 = single stranded DNA from SL13-1A

Lane 4 = SL13-1A digested with Sall/BamHI

Lane 5 = SL13-1A digested with HindIII

Arrow = 600 bp HindIII fragment

SL13-1A

LEGEND

S = *Sac*HI
 E = *Eco*RI
 H = *Hind*III
 B = *Bcl*I
 mcs = Multiple
 Cloning
 Site

Figure 12. AB1 transcript size and expression in *E. acraea* cells.

(A) Uninfected (U) and infected (I) *E. acraea* total RNA (4 h p.i.; 4 μ g) were electrophoresed in a 1% agarose formaldehyde gel, transferred to a nitrocellulose filter in 20x SSC, and hybridized with nick translated SL13-1A insert DNA (2.2 Kbp) in 50% formamide at 42°C. (B) A slot blot of uninfected (U) and infected *E. acraea* total RNA harvested at various times (hours) p.i., was hybridized at 50°C with SL13-1A insert DNA, radiolabelled with 32 P using random primers, and washed at low stringency (50°C; see Materials and Methods, L, a). Exposure to Kodak X-AR5 film was done after sensitization of the film by preflashing with a Toshiba 312 flash unit at a distance of 2 meters (see also Laskey and Mills, 1977).

A U I

-1.1Kb

B

U
4
8
10
12
14
16
18
20
24

A cDNA bank made from total TIV-infected *E. acrea* RNA (8 h p.i.) was constructed in the vector M13 mp19. This bank was screened with radiolabelled SL13-1A. As a result of this screening, 53 cDNA clones were identified by hybridization from an estimated total of 3000 clones. The SL13-1A specific cDNA clones were first plaque purified, and sized by electrophoresis of single stranded recombinant M13 template DNA on agarose gels. RF DNA was also prepared from 13 of the largest clones and from these, 4 clones were selected for sequencing. These were pC33 (900 bp) and pC50 (240 bp), which were in opposite orientation to SL13-1A, and pC32 (700 bp) and pC45 (270 bp), which were in the same orientation as SL13-1A. SL13-1A is currently being sequenced (M. J. Dove, Division of Biological Sciences, NRC) by use of deletions produced by the Dale procedure (Dale, 1985) and the cDNA clones already discussed.

b. Characterization of the AB2 gene

To isolate the AB2 gene, Sall fragment #36 was subcloned into M13 mp19 in both orientations giving clones MS36 and MS36op. The "op" designation indicates that the two inserts are in opposite orientation to each other relative to the direction of transcription of the α -peptide of *lacZ* in M13. Hybridization of radiolabelled Sall #36 back to TIV DNA digests (Figure 13A, B) revealed that the AB2 gene is also present as a single copy gene in the TIV genome. Hybridization of the Sall #36 fragment to Northern blots of TIV-infected *E. acrea* RNA revealed a single transcript with an estimated size of 0.75 Kb (see Figure 14A), again smaller than the expected capsid transcript size. This same DNA was hybridized to a slot blot containing total TIV-infected *E. acrea* RNA extracted at various time intervals after infection. These results revealed that the AB2 transcript is detectable as early as 4 h p.i. and is present throughout infection. This data is in general agreement with the cDNA hybridization data (Figure 6D, E) that showed AB2 is detected throughout infection. However, the drop in the AB2 transcript level at 16h p.i. seen in Figure 6D and 6E is not in agreement with the more accurate slot blot experiment. Hybridization of radiolabelled cDNA to MS36 and MS36op templates produced a signal with MS36op and

Figure 13. Hybridization of the AB2 gene to TIV DNA digests.

(A) TIV DNA was digested, electrophoresed and transferred to nylon as described in the legend to Figure 11. (B) Insert DNA from MS36 (Sall #36) was radiolabelled by nick translation and hybridized to an alkaline transfer of restriction digests of TIV DNA. (C) Restriction map of pE7, a pUC 19 clone of EcoRI fragment #7 (11.8 Kbp) containing Sall fragment #36 (1.1 Kbp).

B = BamHI

E = EcoRI

H = HindIII

P = PstI

S = Sall

Xb = XbaI

Xh = XhoI

M = markers; lambda digested with HindIII/EcoRI

A B E H P S Xb Xh M

B B E H P S Xb Xh M

C

LEGEND

B = BamHI
 Bg = BglII
 CE = ClaI
 H = HindIII
 K = KpnI
 P = PstI
 S = SalI
 X = XbaI
 ac = Multiple Cloning Site

pE7

Figure 14. AB2 transcript size and expression in *E. acrea* cells.

(A) Uninfected (U) and infected (I) *E. acrea* total RNA (12 h p.i.; 8 μ g) were electrophoresed in a 1% agarose formaldehyde gel, Northern blotted to Biotrans (nylon) filter in 20X SSC, hybridized with nick translated MS36 insert DNA at 50°C, and washed at low stringency (50°C; see Materials and Methods, L, a). The transcript size was calculated from three separate Northern hybridizations. (B) A slot-blot of uninfected (U) and infected total *E. acrea* RNA was hybridized with MS36 insert DNA, radiolabelled with 32 P using random primers, as described in the legend to Figure 12.

A U I

← 0.75Kb

B

U

4

8

10

12

14

16

18

20

24

not MS36 indicating that MS36 contains the DNA strand coding for the AB2 transcript (Steve Bottega, experiments conducted as part of a 4th year honors project, Carleton U.).

The results in Figure 13A, B indicated that EcoRI fragment #7 contains Sall fragment #36. EcoRI fragment #7 was subsequently cloned into pUC 19 and called pE7. Figure 13C reveals a restriction map of pE7 showing Sall #36 situated on a 3.1 Kbp EcoRI/ClaI fragment at one end of pE7.

The cDNA hybridization strategy employed above to isolate the AB1 and AB2 genes was not sufficient to identify the TIV DNA fragment coding for the capsid transcript. Perhaps extensive secondary structure of late TIV transcripts, as seen with vaccinia virus (Duesberg and Colby, 1969; Bertholet *et al.*, 1987), make identification of the capsid transcript on the basis of abundance less amenable by this method. Further, oligo dT may not have been a suitable primer if TIV transcripts are not polyadenylated. An alternate strategy was then devised that involved amino acid sequencing of the tryptic peptides derived from the capsid protein and synthesizing mixed DNA probes by reverse-translation of the amino acid sequences.

D. Isolation and DNA sequencing of capsid gene: strategy based on capsid amino acid sequence

a. Purification of the capsid protein

Capsid protein was purified in the same manner as for the production of polyclonal antiserum (see Materials and Methods, section M). Initial attempts to sequence the N-terminus of the capsid protein were unsuccessful suggesting that the N-terminus of this protein was blocked. Purified capsid protein (Figure 15A) was subsequently digested to completion with trypsin and the resulting peptides fractionated by reverse phase HPLC. This profile shown in Figure 15B reveals a total of 28 peaks. Several peaks were isolated for microsequencing. The first peaks to give clean amino acid sequence were #11-4 and #16; the former was isolated by a repurification of peak #11 (Figure 15C). Figure 16 shows the nucleotide sequence obtained from back-translation of the amino acid sequences.

Figure 15. Separation of capsid protein tryptic peptides by reverse phase HPLC.

(A) Purified TTV virions (b) and capsid protein (c) were fractionated on an SDS-PAGE gel (see legend to Figure 9) and silver stained. High molecular weight markers (M; BRL) are indicated.

(B) Purified capsid protein was trypsin digested and applied to a reverse phase-HPLC column as described in Materials and Methods section M. (C) A refractionation of peak 11 in ammonium acetate by RP-HPLC.

A

PEPTIDE #11-4

TYR MET TYR GLY GLY TRP
CYS THR ALA THR ALA TYR PHE

OLIGO #11-4

5' TAC^C_T ATG TAC^C_T GG^C_G GG^C_G TGG^C_T ACC^C_G GC^C_G ACC^C_G GC^C_G TAC^C_T TT^C_T 3'

PROBE SEQUENCE

3' AT^G_A TAC AT^G_A CC^G_C CC^G_C ACC^G_A TG 5'

B

PEPTIDE #16

GLY GLY ASP TYR LEU LEU GLN THR TRP LEU
ALA

OLIGO #16

5' GCC^A_T GG^C_T GAC^C_T TAC^C_T CTC^C_T CTC^C_G CAG^A_G ACC^C_G TGG CTC^C_T 3'

Figure 16. Conversion of peptides #11-4 (A) and #16 (B) to DNA sequence for the construction of oligonucleotide probes.

of peptides #11-4 and #16. Because of the degeneracy of the genetic code, these peptides yield DNA sequences with 192 and 512 possible combinations, respectively. A 20-mer mixed oligonucleotide was then made from peptide #11-4 for hybridization with the TIV genome.

b. Oligonucleotide hybridization to TIV DNA

The mixed oligonucleotide probe was end labelled with [γ - 32 P]ATP, hybridized against digested TIV DNA (Figure 17A), followed by washes at low (Figure 17B) and high (Figure 17C) stringency (see legend of Figure 17 for definition of stringency). These results indicated several candidate fragments, the most notable being Sall #33 (see legend to Figure 17 for the other fragments). Hybridization was also carried out against a partial TIV DNA bank constructed in the BamHI site of pBR322 (Figure 17D). The latter hybridization results revealed two positive plasmids: L1 (15.8 Kbp) and L10 (16.1 Kbp) containing BamHI fragments 11.5 and 11.8 Kbp in size, respectively. Further hybridizations carried out with these plasmids using the mixed oligonucleotide probe revealed that L10 corresponded to the TIV genomic bands that no longer hybridized after a high stringency wash (Figure 17C, E). The hybridizations to L1 were found to be non-specific.

c. Characterization of subclones of L10

Plasmid L10 was mapped by restriction endonuclease analysis (Figure 17E) and appropriate fragments were subcloned into M13 mp19. Hybridization with oligonucleotide #11-4 localized this sequence to a 1.05 Kbp Sall/HindIII fragment of L10 (see Figure 17E). Sequencing of this region revealed that 18 out of the 20 bases of oligo #11-4 matched, accounting for some minor gaps (Figure 17E). Further, the mixed oligonucleotide acted as a sequencing primer during the sequencing of this region, however, the amino acid sequence in this region does not match that of peptide #11-4. Sequencing of the 1.05 Kbp Sall/HindIII fragment revealed an open reading frame corresponding to the entire length of this fragment. When this fragment was radiolabelled

Figure 17. Identification of the capsid gene on the TIV genome by oligonucleotide hybridization.

TIV DNA was restriction endonuclease digested, electrophoresed in 0.7% agarose (A) and alkaline transferred to a Biotrans nylon membrane. End labelled 11-4 oligonucleotide was hybridized to this blot and the blot washed under low (B) and high (C) stringency conditions (RT and 50°C, respectively). BamHI fragments #10 (10.8 Kbp) and #6 (14.3 Kbp), EcoRI fragments #10 (8.5 Kbp) and #1 (~25 Kbp), and Sall fragments #33 (2.0 Kbp), #23 (4.8 Kbp) and #11 (10.2 Kbp) fragments hybridized with the probe with low stringency washes only. BamHI #10 (10.8 Kbp), EcoRI #1 (~25 Kbp), and Sall #33 (2.0 Kbp) hybridized after the high stringency wash. (D) hybridization of the oligonucleotide to a dot blot of pBR322 clones containing BamHI fragments of TIV DNA (low stringency wash). (E) DNA sequence alignment of oligonucleotide #11-4 with the corresponding region on plasmid L10. (F) uninfected (12 h p.i.) and infected *E. acrea* total RNA (6 µg) were electrophoresed and Northern blotted as described in the legend to Figure 14 and hybridized with a nick translated 1.05 Kbp Sall/HindIII cloned fragment from L10.

B = BamHI

E = EcoRI

H = HindIII

S = Sall

E

$$\begin{array}{ccccccc}
 & T & & T & & T & \\
 T & A & T & G & T & A & C \\
 C & & & & & & \\
 & T & & G & G & & C \\
 & C & & A & & G & G \\
 & & & G & & G &
 \end{array}$$

ttatTACATGT TGGAGGTgGTggACaa

B = BamHI
 E = EcoRI
 H = HindIII
 K = KpnI
 S = SalI
 ~ = pBR322

F

and hybridized against a Northern blot containing total TIV-infected *E. acrea* RNA, a 3.6 Kb transcript was identified (Figure 17F). Northern blot experiments often showed considerable heterogeneity in the size of this transcript, ranging from a smear below the 3.6 Kb band to multiple bands smaller than the apparent full length transcript.

Electrophoresing the RNA sample immediately after heating helped marginally in reducing this observed size heterogeneity. This gene called LW1, has now been completely sequenced by W.A. Home (MSc student) and its expression is currently being studied.

d. Characterization of S33 and DNA sequencing of the capsid gene

The 2.0 Kbp Sall fragment (#33; located on BamHI #10 and EcoRI #1) identified by oligonucleotide hybridization to TIV DNA in Figure 17B, and C was cloned in both orientations in M13 mp19 to give MS33 and MS33op. Re-hybridization of the radiolabelled oligonucleotide to digests of these clones indicated its location to be on a 770 bp HindIII/Sall fragment located on the right-hand end of MS33. Dale deletions were made from these clones and sequencing revealed the identity of the #11-4 peptide.

However, the peptide sequence was not completely identical to the sequence predicted from the translated DNA sequence. This difference proved to be an error involving the substitution of the tyrosine/cysteine residue at position #6 of peptide #11-4 with a proline residue (see Table 4 and Figure 19). The proline residue was apparently masked by a contaminating DTT peak that appears during the microsequencing procedure (D. Watson, personal communication). As a result of this error, only 15/20 of the nucleotides in oligo #11-4, with 64 possible combinations, were available for hybridization.

DNA sequencing and hybridization of the 770 bp HindIII/Sall to TIV DNA revealed an open reading frame that extended into the adjacent HindIII fragment (#26b, 2471 bp). The complete ORF was subsequently found to be located on HindIII #26b. This fragment was cloned in both orientations into the XbaI site of M13 mp19 by a partial fill of the sticky ends of both vector and insert. The resulting clones, called MH26 and MH26op, were deleted by the Dale procedure and sequenced. Two subclones were also made from this

Table 4. List of capsid protein tryptic peptides selected for sequencing.

Tryptic Peptide	Amino acid sequence
5	(MET/ALA) (GLY/TYR) (LEU/PHE) (ALA/VAL) ILE THR GLN LYS
11-1	ASN THR GLY ASN ALA ALA PHE (TYR/GLY) GLN GLU TRP (TYR) VAL SER ILE
11-2	GLN ASN TYR VAL PRO LEU THR ASN ALA SER PRO (ARG)
11-4	TYR MET TYR GLY GLY (TRP/CYS) THR ALA THR ALA TYR PHE
12	GLU ALA THR ILE THR PHE ASN ASP LEU VAL ALA ALA ARG
16	(ALA/GLY) GLY ASP TYR LEU LEU GLN THR TRP LEU ^v
19	VAL ASN ILE PRO (HIS/PRO) VAL THR LEU SER GLY LEU GLY ASN THR TYR (SER) LEU (LYS)

fragment to complete a gap in the Dale deletions. These fragments, a 1.05 Kbp Sall/BamHI and a 400 bp BamHI/Sall were cloned into M13 mp19 and referred to as ST1 and ST2, respectively. The sequencing strategy used is illustrated in Figure 18A, B and the gene sequence is shown in Figure 19B.

The DNA sequence in Figure 19A revealed an open reading frame of 464 amino acids beginning with the first methionine at base #610 and terminating at base #2004 (TAA). This would yield a polypeptide with a calculated molecular weight of 50830.49 Da and an estimated pI of 6.34. A hydropathy plot of this protein, shown in Figure 19B, revealed a predominantly hydrophobic protein with some hydrophilic residues. Alanine, serine, asparagine, leucine, and threonine constitute the predominant amino acids while cysteine (2 residues), lysine, histidine, tryptophan and methionine make only a small contribution to the protein. An inspection of the nucleotide sequence preceding the start of the capsid coding protein region revealed it to be extremely A-T rich (75%). This compares with 68% A-T for the coding region. In addition, a number of direct and inverted repeat sequences were located upstream from the first methionine codon of the ORF (Figure 19A). No such sequences were found at the 3' end, however a eukaryotic polyadenylation signal (AATAAA) was located ~200 bp from the termination codon.

Amino acid sequence comparisons were made with published sequences available in two data banks (GenBank, M. Zuker, Division of Biological Sciences, NRC). No proteins of obvious identity were found.

e. Characterization of S24b: sequence related to peptide #5

Several other tryptic peptides were subsequently sequenced and only one of significant length (#5) could not be matched with the putative capsid DNA sequence (Table 4). This peptide gave an ambiguous amino acid sequence for the first four residues and may have several possible origins which include: a) the capsid protein itself; b) a co-purified protein or protein functionally associated with the capsid protein; c) a contaminant appearing during micro-sequencing procedure (D. Watson, personal

Figure 18. Sequencing strategy for the TIV capsid gene.

(A) Sizing of M13 mp19 Dale deletion clones. Single stranded DNA from each clone was hybridized in solution with the complementary sequence of the full length parent (MS26op) then S1 nuclease digested and electrophoresed in 0.7% agarose. DH26 clone numbers and the full size insert (MS26) are also indicated. Markers include lambda DNA digested with HindIII/EcoRI, pUC19 linearized with EcoRI, M13 mp19 RF DNA digested with EcoRI, and plasmid pBT11 digested with HindIII (11 Kbp). (B) Sequencing strategy of MH26 and MH26op using Dale clones and subcloned fragments.

A

B

Figure 19. Nucleotide sequence and deduced amino acid sequence of HindIII fragment #26b containing the TIV capsid gene.

(A) Capsid gene and amino acid sequences. Peptides sequenced are aligned with the amino acid sequence (see Table 4). Other open reading frames located on HindIII #26b are listed below. (B) Hydropathy plot of capsid protein using the parameters specified by Kyte and Doolittle (1982) with a window size of 9.

TS = transcription start region (see Figure 25)

A-T = putative promoter regions

IR = inverted repeats

DR = direct repeat

AATAAA = eukaryotic transcription termination signal

ORF 2 = 74 amino acids (aa); #686 - #910

ORF 3 = 60 aa; #1472 - #1654

ORF 4 = 70 aa; #1724 - #1936

ORF 5 = > 147 aa; #416 - #1 (reverse complement strand)

AAGCTTTT 9

GAAGGTGTTGAAAGTCTACTGAAATAGGCTTCATTAGCATTTTATTTTGTCCACAAATTCATTATTTTAAAGGCTGTTCTTCACCTTTATTCGCAT 109
 ATTCAAAGTAATCGATTAATTTTTTTGAATATGGACGATATCATCCATGAACATAAACCAAACTTCATAATATATAGTATGGAGTAACGGGTTAATTAA 209
 ACCATTGATTCCTTTAATTTGTTTGGATTAATGAGGTTAAATCATATAAATTTTCTATTJTITTTAAATTTTTCGAGCAATTTTAAATTTGAT 309
 TTAACCAAAACAACTCTCTACTTTAATTTGTTAOGGTTGGTACTTTTAAACCATTAAATTTTATTTTGGAGGAAGAACAACGCTTTATTAAGCGTTGG 409
 AATCCATTAATCGCTTGTTTTATCATAGGTTATTTTTTAACTATAAAAAATACTAAATTACTACAGTTAECATATGTCGGCATTAGTTCTCCTTCAT 509
 ATTTTCGTATTTTATACCCITAAATTTAACCTAATCAATTTCTACATTTATTTTTGGGTTCAAAATTTTACCGCAATATTGCTACTAATAAATTAAC 609

A-T A-T A-T TS

ATG TCT ATG TCC TCA TCG AAT ATA ACC TCA GGG TTT ATC GAT ATC GCC ACT TTT GAC GAA ATC GAA AAA TAT ATG 684
 met SER MET SER SER SER ASN ILE THR SER GLY PHE ILE ASP ILE ALA THR PHE ASP GLU ILE GLU LYS TYR MET

30 35 40 45 50
 TAT GGC GGC CCA ACA GCA ACA GCA TAC TTT GTT AGA GAA ATT AGA AAG TCG ACT TGG TTC ACT CAA GTA CCA GTT 759
 TYR GLY GLY PRO THR ALA THR ALA TYR PHE VAL ARG GLU ILE ARG LYS SER THR TRP PHE THR GLN VAL PRO VAL

11-4 55 60 65 70 75
 CCA CTA TCT AGA AAT ACT GGT AAT GCG GCT TTT GGA CAA GAA TGG TCG GTA TCT ATA TCA CGT GCT GGA GAT TAT 834
 PRO LEU SER ARG ASN THR GLY ASN ALA ALA PHE GLY GLN GLU TRP SER VAL SER ILE SER ARG ALA GLY ASP TYR

80 85 11-1 90 95 100
 TTG TTG CAG ACC TGG TTA CGA GTC AAT ATC CCA CCA GTT ACT CTT AGT GGT CTA CTT GGT AAC ACT TAC TCT TTA 909
 LEU LEU GLN THR TRP LEU ARG VAL ASN ILE PRO PRO VAL THR LEU SER GLY LEU LEU GLY ASN THR TYR SER LEU

16 105 110 19 115 120 125
 AGA TGG ACC AAA AAT TTA ATG CAT AAC TTG ATT CGT GAA GCC ACC ATT ACC TTT AAT GAT TTG GTT GCA GCT CGA 984
 ARG TRP THR LYS ASN LEU MET HIS ASN LEU ILE ARG GLU ALA THR ILE THR PHE ASN ASP LEU VAL ALA ALA ARG

130 135 140 12 145 150
 TTT GAT AAC TAT CAT TTG GAT TTC TGG TCT GCT TTC ACC GTA CCT GCC AGC AAA CGC AAT GGG TAT GAT AAC ATG 1059
 PHE ASP ASN TYR HIS LEU ASP PHE TRP SER ALA PHE THR VAL PRO ALA SER LYS ARG ASN GLY TYR ASP ASN MET

155 160 165 170 175
 ATT GGT AAT GTC TCT TCT TTA ATT AAT CCA GTT GCT CCG GGT GGT ACT TTG GGT AGC GTA GGT GGT ATT AAC CTT 1134
 ILE GLY ASN VAL SER SER LEU ILE ASN PRO VAL ALA PRO GLY GLY THR LEU GLY SER VAL GLY GLY ILE ASN LEU

180 185 190 195 200
 AAT CTT CCA CTT CCA TTT TTC TTC TCT CGA GAT ACT GGT GTA GCA CTA CCA ACA GCT GCT CTA CCT TAC AAT GAG 1209
 ASN LEU PRO LEU PRO PHE PHE PHE SER ARG ASP THR GLY VAL ALA LEU PRO THR ALA ALA LEU PRO TYR ASN GLU

205 210 215 220 225
 ATG CAA ATC AAC TTT AAT TTC AGA GAT TGG CAT GAG CTT TTG ATT TTG ACT AAC AGT GCT CTA GTA CCA CCA GCA 1284
 MET GLN ILE ASN PHE ASN PHE ARG ASP TRP HIS GLU LEU LEU ILE LEU THR ASN SER ALA LEU VAL PRO PRO ALA

230 235 240 245 250
 AGT CCA TAT GTT CCA ATT GTT GTA GGT ACT CAT ATT TCA GCT GCT CCA GTT TTA GGA CCA GTT CAA GTA TGG GCT 1359
 SER PRO TYR VAL PRO ILE VAL VAL GLY THR HIS ILE SER ALA ALA PRO VAL LEU GLY PRO VAL GLN VAL TRP ALA

255 260 265 270 275
 AAC TAT GCC ATC GTC TCC AAC GAA GAA CGT CGT AGA ATG GGT TGT GCC ATT CGA GAC ATT TTG ATT GAA CAG GTT 1434
 ASN TYR ALA ILE VAL SER ASN GLU GLU ARG ARG ARG MET GLY CYS ALA ILE ARG ASP ILE LEU ILE GLU GLN VAL

280 285 290 295 300
 CAA ACG GCA CCA CGT CAA AAT TAT GTA CTT TTG ACC AAT GCT AGT CCA ACA TTT GAT ATT CGT TTC TCT CAT GCA 1509
 GLN THR ALA PRO ARG GLN ASN TYR VAL PRO LEU THR ASN ALA SER PRO THR PHE ASP ILE ARG PHE SER HIS ALA

305 310 11-2 315 320 325
 ATC AAA GCA TTA TTC TTT GCT GTA CGA AAT AAA ACA TCT GCA GCA GAA TGG TCA AAT TAT GCT ACT TCT TCT CCA 1584
 ILE LYS ALA LEU PHE PHE ALA VAL ARG ASN LYS THR SER ALA ALA GLU TRP SER ASN TYR ALA THR SER SER PRO

330 335 340 345 350
 GTT GTT ACT GGT GCA ACG GTT AAC TAC GAA CCA ACA GGT TCT TTT GAC CCT ATT GCC AAT ACA ACA TTG ATT TAT 1659
 VAL VAL THR GLY ALA THR VAL ASN TYR GLU PRO THR GLY SER PHE ASP PRO ILE ALA ASN THR THR LEU ILE TYR

355 360 365 370 375
 GAG AAC ACT AAT CGT TTG GGT GCC ATG GGA TCA GAT TAC TTC TCT TTG ATT AAT CCA TTC TAT CAT GCT CCA ACT 1734
 GLU ASN THR ASN ARG LEU GLY ALA MET GLY SER ASP TYR PHE SER LEU ILE ASN PRO PHE TYR HIS ALA PRO THR

380 385 390 395 400
 ATT CCA TCA TTC ATT GGA TAT CAT TTG TAC TCA TAT TCT CTT CAC TTT TAT GAC TTG GAT CCG ATG GGT TCT ACC 1809
 ILE PRO SER PHE ILE GLY TYR HIS LEU TYR SER TYR SER LEU HIS PHE TYR ASP LEU ASP PRO MET GLY SER THR

405 410 415 420 425
 AAT TAC GGT AAA CTC ACT AAT GTG TCT GTT GTA CCC CAA GCT AGT CCG GCA GCA ATT GCG GCA GCA GGA GGT ACT 1884
 ASN TYR GLY LYS LEU THR ASN VAL SER VAL VAL PRO GLN ALA SER PRO ALA ALA ILE ALA ALA ALA GLY GLY THR

430 435 440 445 450
 GGT GGT CAA GCA GGT TCA GAT TAC CCT CAA AAT TAT GAA TTT GTC ATA TTA GCT GTC AAT AAT AAT ATT GTC AGA 1959
 GLY GLY GLN ALA GLY SER ASP TYR PRO GLN ASN TYR GLU PHE VAL ILE LEU ALA VAL ASN ASN ASN ILE VAL ARG
 455 460 465
 ATA TCA GGT GGA GAA ACA CCA CAA AAT TAC ATA GCA GTT TGT TAA GGTAATTTGTAACGCTCCACAAGAGGCGGAAGTGGTCTC 2043
 ILE SER GLY GLY GLU THR PRO GLN ASN TYR ILE ALA VAL CYS ***
 GTGAGAGACCGATATTGAGGTTTTATCAACCTTAATTTGAATCATGAATTAACATGATACTTTGGTACCGTCTAGTCGGCTTAATATGTCGGGCTAATGGT 2143
 CTTTTTTGATCATCAAGTGGCTATAAGTGGTACGTCGACGACAGTCGACACCTAGTGGTTT AATAAAGGTTTTTTACCCAAATTAAGTGGAAACAGGCAA 2243
 GGTGATGAAAACGGTCAAAATTCAGATAGTCTCGGGGGCTATTTTGGACAAGACCGTCGGTGCAGCTAATGCGTAAGCATCAGTGATATCGCTATCGAC 2343
 TGGGTCATCAATCGGTTGCTCTATCTGACTTTTTAAAGTCTCCAGGATGGCTCAATGTACAGTCAGCCCGCAGTAAGGTGTATTCGAGCTGTCTTCAG 2443
 GATAAAAGTAACTTGAAAAAGAAGCTT 2471

communication). To insure that this peptide was indeed not derived from the capsid protein, a mixed oligonucleotide (192 combinations) was made from a portion of this peptide by back-translation of the amino acid sequence (Figure 20A). The mixed oligonucleotide was end labelled and used as a hybridization probe against digests of TIV DNA. This data, shown in Figure 20B, C revealed, unexpectedly, a single strong hybridizing fragment, considering the mixed nature of the oligonucleotide. The second Sall fragment 4.4 Kbp in size (#24b; S24a corresponds to the AB1 gene) was identified and cloned in both orientations in M13 mp19 (MS24b, MS24bop). Hybridization of this fragment to restriction digests of total TIV DNA (Figure 21A, B) identified a number of fragments that cannot be accounted for by the total size of the cloned fragment. These results suggest that Sall #24b may contain sequences that are repeated in the genome. In order to identify the transcript(s) that is encoded within Sall 24b, this fragment was radiolabelled and used to hybridize against a Northern blot. This hybridization shown in Figure 21C, identified a major transcript about 1.35 Kb in size and a weaker transcript about 1.65 Kb in size.

In parallel experiments, it was of interest to isolate the thymidine kinase (tk) gene from TIV by heterologous hybridization using both the vaccinia virus and herpes virus tk genes. Initial hybridizations with both of these full length genes did not give reproducible hybridization to the TIV genome. A core consensus sequence located within an EcoRI/HinfI (65 bp) fragment of the vaccinia virus gene was then subcloned into M13 mp19 (Figure 22A) and used as a hybridization probe against digested TIV DNA. These results shown in Figure 22B, C identified Sall fragment #24b as a likely candidate for containing this gene. Further experiments involving the isolation of this gene will be carried out in the near future.

E. Capsid gene and protein expression

a. Gene copy number in TIV

The HindIII fragment #26b containing the capsid gene with a total of about 1.1 Kbp

Figure 20. Characterization of tryptic peptide sequence #5 identified during amino acid sequencing of the capsid protein.;

(A) Back translation of peptide #5 to DNA sequence. Peptide #5 yields an oligonucleotide sequence with 192 combinations. (B) Restriction endonuclease digests of TIV DNA fractionated on an 0.7% agarose gel. (C) Hybridization of end-labelled oligo #5 to an alkaline transfer (Hybond) of the gel shown in (B). See legend to Figure 18 for a description of the markers (M).

B = BamHI

E = EcoRI

H = HindIII

S = Sall

A

PEPTIDE #5

MET GLY LEU ALA ILE PRO THR GLN LYS
 ALA TYR PHE VAL

PROBE SEQUENCE (REVERSE COMPLEMENT)

B

B E H S M

C

B E H S M

Figure 21. Characterization of Sall #24b fragment identified by oligo #5 by nucleic acid hybridization.

TIV DNA was digested with various restriction endonucleases, electrophoresed in an 0.7% agarose gel (A) and alkaline transferred to nylon (Hybond) membrane. Random primer labelled insert DNA from MS24b (Sall #24b cloned into M13 mp19) was hybridized to this blot (B) and a Northern blot (C) of uninfected (U) and infected (I, 12 h p.i.) *E. acraea* total RNA (6 μ g). The RNA was alkaline transferred to nylon (Hybond) membrane as described in Materials and Methods, section J.

M = markers; see legend to Figure 18

U = Undigested TIV DNA

B = BamHI

E = EcoRI

H = HindIII

P = PstI

S = Sall

Xb = XbaI

Xh = XhoI

A **B** **C** U I

← 1.65 Kb

← 1.35 Kb

Figure 22. Hybridization of vaccinia virus thymidine kinase gene to TIV DNA.

A highly conserved region of the vaccinia virus tk gene was cloned into M13 mp19 (see Materials and Methods section I) and verified by sequencing (A). Digests of TIV DNA were electrophoresed in 0.7% agarose (B) and alkaline blotted to nylon (Biotrans) membrane. A radiolabelled single stranded DNA probe was prepared by primer extension of the M13 sequencing primer on clone TKC, and hybridized to this blot (C) under low stringency conditions (42°C for 16 h) and washed under relaxed conditions (2X SSC/0.1% SDS, 30 min. at RT). Markers (M) are lambda DNA digested with EcoRI/HindIII. The ~2 Kbp HindIII/HincII fragment of pJ5 (VV-tk; arrow) is included as a positive hybridization control.

A

S

E

CCCAA TCA ATT ACA GAT TTC TCC GTG ATA GGT ATC GAT GAA GGA CAG TTC TTT CCA GAC ATT GTT GAATTC
 SER ILE THR ASP PHE SER VAL ILE GLY ILE ASP GLU GLY GLN PHE PHE PRO ASP ILE VAL

LEGEND

EcoRI : BamHI
 EcoRI : EcoRI
 HindIII : HindIII
 SalI : SalI
 Multiple Cloning Site

TKC

B

C

of 5' and 3' flanking sequence, was radiolabelled and hybridized to restriction digests of TIV DNA. This result shown in Figure 23A, B revealed that the capsid gene is located only on the L DNA component of TIV and that it is present in L as a single copy. A restriction endonuclease map of EcoRI #1 (~25 Kbp) containing the capsid gene is shown in Figure 23C.

b. Transcript analysis

Hybridization of the HindIII #26b fragment to a Slot blot of TIV-infected *E. acraea* total RNA extracted at various times after infection revealed that transcription of this gene increased as the infection progressed (Figure 24D). Northern blot analysis also revealed that this gene codes for a transcript of about 1.8 Kb in size (Figure 24C).

The transcription initiation site for the capsid gene was determined by primer extension analysis of mRNA using reverse transcriptase. This data shown in Figure 25 indicated that the RNA start site was within an A-T rich sequence (5'-TGCTACTAAT-3'), 19 nucleotides upstream from the first methionine of the capsid ORF. A second primer that was designed to initiate reverse transcription at C, one nucleotide upstream from the first methionine codon of the ORF (see Materials and Methods, section Q), gave a start site within the same region (data not shown). A-T rich sequences that may represent putative promoter regions are also shown in Figure 25B.

c. In vivo labelling of capsid protein in insect cells

To detect newly synthesized proteins in TIV-infected *E. acraea* cells, both uninfected and infected cells were pulse labelled with ³⁵S-methionine for a period of 1 hour, at various times after infection, and harvested at the end of the labelling period. The cells were starved for methionine for 1 hour prior to labelling by using a methionine minus medium. These experiments were done in parallel with the time course RNA experiments mentioned above (section C, a). These time course samples were analyzed by SDS-PAGE and fluorography and the results are shown in Figure 26A. A number of bands appear during the replication cycle that do not appear in the control samples. The most prominent

Figure 23. Hybridization of capsid gene and flanking sequences to the TIV genome.

TIV DNA was digested with various restriction endonucleases, electrophoresed in 0.7% agarose (A), and alkaline transferred to nylon (Hybond) membrane. Insert DNA from pH26b was radiolabelled by using random primers and hybridized to this blot (B). (C) Partial restriction map of EcoRI fragment #1 containing the capsid gene. For sites outside HindIII #26b, only those restriction fragments containing a corresponding site within the boundaries of HindIII #26b are indicated.

M = markers; see legend to Figure 18

U = Undigested TIV DNA

B = BamHI

E = EcoRI

H = HindIII

P = PstI

S = Sall

Xb = XbaI

Xh = XhoI

Figure 24. Capsid gene transcript size and expression in *E. acrea* cells.

Uninfected (U) and infected (I) *E. acrea* total RNA were electrophoresed in a 1% agarose gel with formaldehyde. (A) Molecular weight markers are yeast (Y; *Schwanniomyces alluvius*) ribosomal RNA and an RNA ladder (M) from BRL. (B) EtBr stained gel containing uninfected (12 h p.i.) and infected (16 h p.i.) RNA (7 μ g). (C) Duplicate samples of the RNA (10 μ g) shown in (B) were transferred to nylon (Hybond) membrane by electroblotting, and hybridized with random primed labelled pH26b insert DNA (C). The capsid transcript size is indicated. (D) A slot blot containing uninfected (U) and infected *E. acrea* total RNA was hybridized with the probe used in (B) as described in the legend to Figure 12.

Figure 25. Mapping of the transcription start point of the TIV capsid gene by primer extension analysis.

End-labelled PE2 primer was annealed to 20 μ g of total TIV-infected *E. acrea* RNA (16 h p.i.) or total yeast RNA as a control and a cDNA copy of the transcript was made using AMV reverse transcriptase (A). The RNA of the DNA:RNA hybrid was then removed by alkali, and the product was analyzed on a sequencing gel along side a sequencing ladder using PE2 and the capsid gene in MH26 (B). The transcription start region (arrows) predicted from the size of the primer extension product are indicated. A-T rich regions upstream from the first ATG of the capsid ORF are shown.

A

mRNA

AUG

5

3'

5'

B

A C G T

TTV Con.

Content

• Ent

T

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Figure 26. A fluorogram of *in vivo* ^{35}S -labelled proteins from uninfected and TIV-infected *E. acraea* cells.

(A) Cells were starved for methionine for one hour, then labelled with ^{35}S -methionine for one hour at the designated times (hours). Samples were then washed with medium containing methionine, lysed in RIPA buffer, analyzed by SDS-PAGE (4% stack/10% base) and fluorography. (B) SDS-PAGE and fluorography of ^{35}S -labelled TIV obtained from *E. acraea* cells.

A **B**

of these have molecular weights of 54, 46 (capsid), 39, and 33 KDa as determined by SDS-PAGE.

To identify the capsid protein from amongst the other labelled proteins, western blotting analysis was carried out. This was necessary because the anticapsid antibody would not precipitate the ^{35}S -labelled capsid protein under a variety of the experimental conditions tested. To do this experiment, *in vivo* proteins were labelled from 20-24 h p.i. and were separated by SDS-PAGE. Following electrophoresis, gels were silver stained, or western blotted and probed with the anticapsid antibody using a non-radioactive secondary antibody. These results shown in Figure 27 revealed that the capsid protein is amongst the top three intensely labelled proteins late in infection. A comparison of the *in vivo* labelled proteins with ^{35}S -labelled virus particles (Figure 26A, B) indicated that the capsid protein represents the most abundantly labelled protein that is packaged within virions.

d. *In vitro* translation of RNA

Several attempts were made at identifying the capsid mRNA translation product made by cell free translation of total RNA isolated from TIV-infected *E. acrea* and *A. albopictus* cells harvested at 12 and 16 h p.i. A typical *in vitro* translation result which includes an immunoprecipitation experiment using anticapsid antiserum is shown in Figure 28A. Although equivalent amounts of RNA were used for samples from both uninfected (lanes 3 and 4) and infected (lanes 5 and 6) cells, the uninfected samples produced more reaction products (lane 3). A band that migrated at the expected position of the capsid protein appeared in lane 5, and ran with the same mobility as the globin band (lane 1). Some other minor differences are also evident between the two samples, however the major difference is a reduced synthesis of proteins in the sample from infected cells. Immunoprecipitation of the samples using the anticapsid antiserum resulted in no specific bands appearing at the expected position of the capsid protein (see lanes 4 and 6). A longer exposure (7 days) of the immunoprecipitated samples resulted in non-specific bands appearing in the uninfected sample (lane 4; data not shown). Western analysis of the IVT,

Figure 27. Identification of *in vivo* labelled capsid protein by western analysis.

(A) A fluorograph of proteins labelled *in vivo* at 30 h (lane 1), 36 h (lane 2) and 20-24 h p.i. (lane 3). Markers are ^{14}C -labelled low molecular weight markers from BRL. (B) Silver stained gel of *in vivo* labelled *E. acraea* proteins at 20 h (uninfected; lane 1) and 20-24 h p.i. (infected; lane 2). Lanes 3 and 4 contain TIV virions and purified capsid protein, respectively. (C) Western analysis of a duplicate gel in (B) probed with the anticapsid antiserum as described in the legend to Figure 9. The capsid protein is indicated (dot). X-ray film was exposed for 24 h with one intensifying screen.

A

M 1 2 3

Fluorograph

B

1 2 3 4 M

Silver Stain

C

1 2 3 4

Western Blot

Figure 28. Analysis of *in vitro* translation products made from uninfected and infected *E. acraea* total RNA.

E. acraea total RNA (8 μ g) was *in vitro* translated using a rabbit reticulocyte lysate system (BRL and Amersham), and the resulting peptides analyzed by SDS-PAGE and fluorography (A). Lanes 1, 3 and 5 contain a water control, uninfected cell RNA, and infected cell RNA, respectively (15% of translated products loaded). Lanes 2, 4 and 6 show the corresponding immunoprecipitations with the anticapsid antibody (85% of translated products loaded). Immunoprecipitations of IVT products were done as described in Materials and Methods, section N, b. Markers (M) are 14 C-labelled proteins (BRL). (B) Western analysis of IVT products as described in the legend to Figure 9. Lanes 1, 2 and 3 contain IVT products of a water control, uninfected and infected cell RNA (10 μ g), respectively. Lanes 4 and 5 contain purified capsid protein and TIV virions, respectively. The position of the capsid protein is indicated (arrow).

products using the anticapsid antiserum (Figure 28B), indicated that the capsid protein was inefficiently synthesized or not at all, in this *in vitro* system.

e. Immunofluorescence microscopy

Over the course of this study, ~10 attempts were made to determine the intracellular location of the capsid protein (see Materials and Methods, section C, b). Antisera obtained from 5 rabbits were tested at various dilutions with and without blocking agents, against uninfected and TIV-infected *E. acrea* and *A. albopictus* cells. The cells were fixed with two types of aldehyde fixatives using combinations of the two, or with methanol. A general diffuse fluorescent staining pattern was often observed in the cell cytoplasm using these methods. However this pattern was also observed when preimmune antiserum was used. The secondary antibody showed little to no staining when a PBS control was substituted for the primary antiserum.

f. Relative abundance of the capsid protein

The techniques most commonly used for the detection of proteins, namely protein stains such as silver or Coomassie Blue, or radiolabelling with ^{35}S -methionine, rely on a specific property of the protein being examined (see Discussion, C). The preceding experiments in this thesis suggested that there are definite discrepancies between these techniques. A comparison of capsid abundance using all three techniques mentioned above indicated that this 50 KDa protein appeared as the most abundant in the virion (Figure 29). However, major differences were noticeable when some of the other minor proteins were compared, the most prominent of these having molecular weights of 29 KDa and 33 KDa (indicated by dots in Figure 29). Some of the extra bands appearing in ^{35}S -labelled TIV (Figure 29C) may represent contaminating host proteins co-purifying with the virus.

g. Capsid protein expression in *E. coli*

The experiments presented thus far have provided very strong evidence that the capsid gene which was isolated and sequenced, codes for the protein against which the

Figure 29. A comparison of TIV proteins using various protein detection methods.

Purified TIV was analyzed by SDS-PAGE (see legend to Figure 9) and either silver (A) or Coomassie blue (B) stained. (C) Fluorograph of ^{35}S -methionine labelled virions from *E. acraea* cells. The capsid protein (arrow) and minor proteins are indicated (dots; see text).

antibody was made. More direct evidence can be provided by expressing the full capsid gene in a heterologous system and detecting the protein product with the anticapsid antibody. These studies should also provide the ground work necessary for isolation of sufficient quantities of purified capsid protein in order to study the assembly properties of this protein. First, it was of interest to determine if the native TIV capsid promoter could function in *E. coli* cells and produce capsid protein. To test such a possibility, HindIII fragment #26b containing the full capsid coding sequence, 609 bp of 5' sequence and 449 bp of 3' sequence was cloned into plasmid pUC19. The resulting recombinant plasmid called pH26b was tested for its ability to express capsid protein in *E. coli* JM101 cells. Figure 30 shows a western analysis of *E. coli* cells transformed with pH26b and grown for 5.5 and 18.5 h. The anticapsid antiserum showed no additional bands appearing in these samples as compared to the control. Therefore, it appears that TIV capsid protein was either inefficiently synthesized or not at all in *E. coli* cells, when under the control of TIV promoter sequences.

A second approach to capsid protein expression in *E. coli* cells involved creating a fusion between the α -peptide of the pUC19 plasmid and the capsid protein. Under these conditions, the promoter sequences employed would belong to the *lacZ* gene. A recombinant clone called pLC26 containing capsid sequences fused with the *lacZ* gene was constructed. After the initial isolation of this gene and subsequent DNA sequencing it was thought that pLC26 contained an in frame fusion with the *lacZ* gene. This clone was then used for expression studies in *E. coli* JM101 cells. However, a re-analysis of the DNA sequence revealed that this clone was in fact out of frame with the *lacZ* ORF (see Figure 31A, B). The hybrid gene created by this fusion shown in Figure 31A, contains 16 amino acids of the α -peptide at the N-terminus fused with the capsid gene that lacks 13 N-terminal amino acid residues from the first methionine. The remaining sequences downstream from this region represent those derived from pH26b.

Untransformed and pUC19 transformed JM101 cells along with the pLC26

Figure 30. Expression study of the TIV capsid gene in *E. coli* cells using the native capsid promoter elements.

Plasmid pH26b containing the capsid gene and 609 bp of 5' sequence was transformed into *E. coli* cells. Cells were grown and lysates were analyzed by SDS-PAGE (4% stack/10% base) and Coomassie Blue staining (A). Proteins were western transferred to nitrocellulose and immunoreacted with anticapsid antiserum as described in Materials and Methods, section M.

M = protein markers

a = purified capsid protein (50 KDa)

b = *E. coli* cells transformed with pH26b and grown 18.5 h

c = *E. coli* cells transformed with pH26b and grown 5.5 h

d = *E. coli* cells transformed with pUC19 and grown 18.5 h

A

KDa M a b c d

200-

97.4-

68-

43-

25.7-

18.4-

12.3-

B

a b c d

50KDa

Figure 31. Fusion of the *lacZ* α -peptide of pUC19 with the TIV capsid gene for capsid protein expression in *E. coli*.

(A) The 1852 bp *Cl*I/*Eco*RI fragment of pH26b encoding amino acids 13-464 and the 3' end from pH26b was fused with the α -peptide at the *B*amHI site of pUC19. Arrow indicates direction of transcription. (B) Verification of the fusion by DNA sequencing of the *lacZ*/capsid junction.

A

ClaI/EcoRI

BamHI/EcoRI

Cohesive
Ligation
(EcoRI)

Partial Fill in
with dGMP and
Klenow enzyme

Blunt-ending
with *E. coli*
DNA polymerase I

Blunt-end
Ligation

LEGEND

B = BamHI

Bg = BglI

C = ClaI

E = EcoRI

H = HindIII

X = XbaI

mcs = Multiple Cloning Site

LacZ' (mcs)

Capid Sequence

... AGG TCG ACT CTA GAG GAT GGC GAG TTT TGA CCAATCGAAAAATAT ATG ...
... ARG SER THR LEU GLU ASP ...

B

GATC

recombinant were subsequently analyzed by SDS-PAGE, followed by Coomassie Blue staining and western analysis. Cells were grown with or without IPTG as inducer of the *lacZ* promoter. Induction with the gratuitous inducer IPTG results in repressor protein inactivation and the induction of transcription and translation of the *lacZ* gene. The data shown in Figure 32B shows that the capsid gene product was detectable by the anticapsid antibody. This product was not produced in sufficient quantities to be visualized by Coomassie Blue staining (Figure 32A). Figure 32B revealed several bands appearing in the recombinant construct lanes that do not appear in the control lanes. These bands correspond to sizes of 15, 17, 28, 30, 41, 43, and 45 KDa. Most of these bands also appear at a lower intensity in the uninduced pLC26 sample.

F. Presence of a small icosahedral particle

On one occasion over the course of this study, during conventional purification of TIV from *G. mellonella*, it was found that the sucrose gradient fractionation yielded a much wider top component band. Upon further purification of this band by a second linear sucrose gradient, EM observations revealed that this band contained numerous small icosahedral particles ~ 26 nm in diameter that were not as intensely stained as "full" or "empty" TIV virions which measured about 150 nm (see Figure 33A, B). These small particles were non-occluded but were sometimes associated loosely with membranous material or engulfed within a membrane. However, most often these particles appeared free, or arranged in arrays. Small particles were reported by Yule (1971), in *G. mellonella* larvae, and were estimated to be about 50 nm in size. In this study, it was initially thought that they may be a contaminating latent virus induced from the larvae. However, SDS-PAGE analysis, Coomassie Blue (not shown) and silver staining (Figure 33C) revealed the presence of a major band co-migrating with the major TIV capsid protein, and some other minor bands. Further, no nucleic acid other than the TIV DNA regular components (L and S1) were isolated from this sucrose fraction. Whether the 26 nm particles are comprised of aggregated capsid protein and/or breakdown products of TIV,

Figure 32. Expression of the capsid protein sequence in *E. coli* cells.

The pLC26 plasmid shown in Figure 31 was used for expression in *E. coli* JM101 cells with (+) or without (-) IPTG as an inducer. (A) Coomassie Blue staining of control (JM101, pUC19) and recombinant (pLC26) samples. (B) Western analysis of a duplicate gel in (A) as described in the legend to Figure 9. Capsid specific protein products are indicated (dots).

A

M JM101 pUC19 PLC26 TIV M

-	+	-	+	-	+
---	---	---	---	---	---

Coomassie

B

JM101 pUC19 PLC26 TIV

-	+	-	+	-	+
---	---	---	---	---	---

KDa
-200

-97.4

-68

-43

-25.7

-18.4

-12.3

Western Blot

Figure 33. Comparison of TIV and small icosahedral (sv) virus particles from *G. mellonella*.

(A) and (B) Negatively stained electron micrographs of TIV and small icosahedral particles, respectively. Silver stain (C) and western analysis (D) of TIV (lane 1) and sv (lane 2) particles. (E) Western analysis using ^{125}I protein A (see Materials and Methods section M, d) of TIV frozen and thawed ~10 times (lane 2). Lane 1 contains purified capsid protein. Western analysis was done as described in the legend to Figure 9. Bar represents 100 nm.

A

B

C

KDa
200-

TIV_{SV}

97.4-

66-

43-

25.7-

18.4-
12.3-

D

TIV_{SV}

E

50KDa
TIV

or the product of a latent viral infection has not yet been determined. Repeated attempts were made in this study to obtain a homogeneous sample from this fraction that was devoid of the larger TIV particles, but such a homogeneous sample was difficult to obtain because of the tendency of these particles to aggregate. The possibility exists that the 26 nm particles were generated by degradation of the TIV capsid.

The capsid protein was found to be quite stable since long term storage at 4°C (over 3 years) resulted in at least 30% of the protein remaining intact and of the expected size (46 KDa) as seen by SDS-PAGE followed by silver staining and western analysis (Figure 33C, D). The degraded capsid protein formed discrete bands as a consequence of long term storage at 4°C (Figure 32D). This phenomenon was also observed by western analysis of virus that had been extensively frozen and thawed (Figure 32E).

IV. Discussion

A. TIV Genome

In an earlier study, we reported (Tajbakhsh *et al.*, 1986) that DNA purified from *G. mellonella* TIV virions contains four DNA components: L (> 176 Kbp), S1 (10.8 Kbp), S2 (~6 Kbp) and S3 (~3.5 Kbp). L and S1 DNA components are seen consistently in ethidium bromide stained agarose gels of total TIV DNA and are in roughly equimolar amounts, whereas S2 and S3 DNAs were made visible only by hybridization of S1 DNA to Southern blots of total TIV DNA. We believe that these latter DNA components may be deletions of L and/or S1. The L and S1 DNA components appear to have some related sequences, however the location of S1 DNA sequences on L remains unclear.

The possibility that S1 and L are both essential for TIV replication has not been tested in this thesis. However, there are several examples of viruses with more than one component. The genome of herpesvirus consists of two covalently linked DNA components in a linear arrangement (L: long-82%; S: short-18%), each containing unique sequence (Roizman and Batterson, 1985). In more relevant work, with a mosquito iridescent virus (RMIV), Wagner and Paschke (1977) suggested that this virus may contain two identical DNA molecules. Other studies by Krell *et al.* (1982) have revealed that the *Campoletis sonorensis* virus, which belongs to the recently discovered polydnavirus family, contains a multipartite genome. This viral genome is believed to consist of at least 28 molecules of covalently closed, superhelical, double-stranded DNA. The size of these molecules ranges from 6 to 21 Kbp and they are non-equimolar in distribution (Krell *et al.*, 1982; Blissard *et al.*, 1986).

The presence of a small DNA component (S1) existing separately from the main genomic DNA is a novel finding among the large double-stranded DNA containing viruses. To fulfill any long term goal of solving TIV genomic organization, it was necessary to provide evidence that S1 DNA is packaged within virions and is TIV specific. By treating

TIV particles with nucleases and subsequently purifying total DNA, it was demonstrated here that S1 DNA is indeed of viral origin and is packaged within virions. Furthermore, CsCl fractionation of TIV particles and extraction of total viral DNA from the "empty", "partially filled" and "full" particles confirmed our earlier finding (Tajbakhsh *et al.*, 1986) that partially filled virions contain a higher S1 to L ratio than does the main viral fraction. To ensure that this and other TIV preparations used in this thesis were free of other contaminating particles, TIV suspensions were regularly monitored by electron microscope examination. These studies were then extended to determine if the S1 DNA component is made by infected cells. The results reported here revealed that S1 DNA is indeed synthesized in both *E. acraea* and *A. albopictus* cells. Additionally, the intracellular ratio of S1 to L DNA was at least 10 fold greater than in mature virions where the ratio for these DNA components is approximately 1:1 (Figure 2; Tajbakhsh *et al.*, 1986). In some of the experiments involving cDNA hybridizations to total TIV DNA, it was observed that both L and S1 DNA hybridized to cDNA. Although these results suggest that transcribed sequences are present on S1 DNA, we do not know in fact, whether S1 DNA serves as the transcriptional template. None of the genes identified in this thesis (capsid, AB1, AB2, LW1, and putative tk) hybridize to S1 DNA. From the data presented thus far the presence of more S1 than L DNA in partially filled capsids implies that the S1 DNA component, by virtue of its smaller size, is preferentially packaged into empty viral capsids first followed by the L component.

The high levels of S1 DNA in infected cells argues that S1 DNA may replicate autonomously in insect cells. Alternatively, S1 DNA may be generated from L DNA during infection in a way that is analogous to the "onionskin" model proposed by Sambrook *et al.* (1974). This model assumes that repeated activation of an origin of replication would ultimately result in production of extrachromosomal copies and of intrachromosomal duplications and deletions. These events could account for the release of free cyclic molecules (Borgaux *et al.*, 1982). Although S1 DNA has sequences common to L

(Tajbakhsh *et al.*, 1986), it is not certain if all of the S1 DNA sequences are exclusive to L DNA. The results reported in this thesis clearly demonstrated that S1 DNA contains TIV specific sequences. It would be of interest to determine if purified S1 DNA can replicate autonomously in insect cells, or if this DNA component requires coinfection with TIV. If the former is true, then S1 will be a good candidate for the development of an expression vector for insect cells.

A preliminary comparative analysis, not presented in this thesis, was carried out between our TIV isolate and a TIV isolate that was propagated in *G. mellonella* (material was provided by M. Kelly, Dept. of Agriculture Zoology, Queen's University of Belfast, Belfast, Ireland). Total DNA from this Irish TIV isolate revealed the presence of only the L DNA component in ethidium bromide stained agarose gels. Further, hybridization of TIV genes (capsid and AB2) to restriction endonuclease digests of both genomes revealed differences in the hybridization pattern between the two isolates. However, the estimated genome sizes are about the same as that which we reported earlier (Tajbakhsh *et al.*, 1986). Clearly, more extensive analyses must be carried out between these two isolates and other iridescent viruses in order to determine their evolutionary relationships.

The estimated genome size of TIV from my thesis work was obtained by averaging totals from restriction digests and eliminating the submolar fragments. My estimate of 176-247 Kbp is within the range of earlier studies (Bellet and Inman, 1967) which indicated that the molecular mass of this genome is 1.26×10^8 to 1.55×10^8 Da ($\sim$ 190-235 Kbp). This value is also in close agreement with the size of IV22 DNA, isolated from a *Simulium* species, aquatic Blackfly larvae (Diptera), which was found to be 173-220 Kbp by restriction endonuclease analysis (Hibbin and Kelly, 1981). Further, genomic size heterogeneity in the uncloned viral preparation of IV22 was suggested by these authors as one of the reasons for the presence of submolar fragments. More recently, Ward and Kalkmakoff (1987) reported a genome size of 192.5 Kbp for IV9 isolated from *Wiseana cervinata*, and the presence of submolar fragments in restriction digests of total IV9 DNA.

Serial passage of this virus in *G. mellonella* larvae resulted in a complete loss of one of these submolar fragments. These authors also attributed the presence of submolar fragments to a mixed population of virus in their preparations. The genomic size variation observed with TIV was only partly resolved by the finding of the smaller "S" DNA components associated with this virus. It is likely that measurement error of large molecular weight fragments accounts for some differences. As mentioned previously (see Introduction, section I, a), it has been demonstrated that several members of the *Iridoviridae* family have genomes that are circularly permuted and terminally redundant. The observed heterogeneity in the termini of these viral genomes could also account for some of the variability in genome size seen with TIV. However, preliminary electron microscope studies carried out in collaboration with Dr. K.G. Murti (St. Jude Children's Research Hospital, Memphis, Tennessee) have failed to demonstrate that the TIV genome is circularly permuted. Further EM studies and analysis of the genomic termini with 5' lambda exonuclease and T4 DNA polymerase are planned for the immediate future to determine if this is in fact the case.

Another potential source of heterogeneity in genomic size measurement involves the methylation of cytosine to 5-methylcytosine in DNA which would affect cutting by various restriction endonucleases. Methylation has been suggested to be involved in the regulation of eukaryotic and, in some cases, viral gene expression (Razin and Riggs, 1980; Youssoufian *et al.*, 1982). In this thesis, agarose gel analysis was done with TIV DNA digested with the isoschizomeric nucleases, HpaII and MspI. This preliminary analysis revealed that unlike FV3 (Willis and Granoff, 1980) and FLDV (Wagner *et al.*, 1985) DNAs, TIV DNA does not contain methylated CpG sequences. These results agree favorably with those of Thomas (1961) which indicated that no methycytosine or 5'-hydroxymethylcytosine residues were present in TIV DNA. Therefore, methylation may not contribute much to TIV genomic size variation. It would be of immediate interest to obtain genomic size estimates of a plaque pure TIV from a homogeneous tissue culture

system when it is available.

B. Semipermissive Replication of TIV

Host cells that are infected by viruses can be classified as permissive, semipermissive, or nonpermissive for viral replication. Viruses that infect their host cells and produce viable progeny virions are said to replicate permissively. Those that cannot replicate in a host cell, most commonly because the cells lack the necessary receptor, are said to be nonpermissive with that host. Probably the most interesting scenario is the semipermissive replication of a virus where limited viral replication takes place. Often, investigation of the semipermissive replication of a virus provides us with the opportunity to study a "window" in the viral replication cycle in order to identify some of the factors required for viral pathogenesis. These studies often involve the study of viral mutants that are defective in a particular function.

In this thesis, it was shown that *G. mellonella*-TIV replicated semipermissively in the Dipteran cell line *A. albopictus* and that the infection was aborted at a late stage in TIV replication, possibly during the assembly of progeny virions. By light microscope autoradiography it was shown that, as with TIV-infected *E. acraea* cells, TIV drastically reduced *A. albopictus* DNA synthesis. Light and electron microscope analysis showed that VC morphology appeared similar in both of these cell lines although VCs in *E. acraea* cells were often much larger presumably due to the larger size of these cells. Analysis of total DNA from the infected cells revealed that both the L and S1 DNA components were present in the permissive and semipermissive host cells. Transcript analysis also showed that, at least the major viral transcripts were made in both *E. acraea* and *A. albopictus* cells. The experiments done here were not sensitive enough to detect the production of all viral transcripts, therefore, the possibility that some viral transcripts were not synthesized in *A. albopictus* cells cannot be formally excluded. The kinetics of infection between the two cell lines with respect to DNA and mRNA synthesis and formation of viroplasmic centers, was also found to be similar. An extensive electron microscope analysis of *A. albopictus*

cells was then carried out in order to visualize the presumptive block in these semipermissive cells. This analysis showed that greater than 90% of VCs in *A. albopictus* cells were devoid of progeny virions or partially assembled capsids even as late as 96 h p.i., while progeny virions were clearly detectable in VCs as early as 16h p.i. in *E. acraea* cells. These EM studies also revealed that as late as 96 h p.i., as a rough estimate, up to 15% of the infected cells contained virus particles that were retained within vesicles heterogeneous in size. These cells appeared more like skeletal structures as the infection progressed, with little cytoplasm remaining. The source of these virus particles is believed to be from the inoculum.

Our finding that a very minor portion of the infected *A. albopictus* cells contained progeny virions is not surprising due to the fact that the inoculum source was not plaque purified virus. A heterogeneous virus population derived from a systemic infection of the larvae would likely contain some virus particles that could replicate in *A. albopictus* cells. Our experiments have indicated that the titres of such a virus population are very low and experiments will have to be done to isolate sufficient quantities of *A. albopictus*-TIV in order to make a comparison with *E. acraea* and *G. mellonella* purified virus.

The host range of TIV is broad, spanning three separate insect orders, however, this study showed that among the Diptera, at least one host is semipermissive in tissue culture. It is of interest to note that TIV was originally isolated from the Dipteran *Tipula paludosa* (Xeros, 1954). It has been propagated in *G. mellonella* since 1966 and its host range may have been modified due to continual passage in this host. It would also be of interest to determine the extent of TIV replication in *A. albopictus* larvae.

Since the data presented in this thesis indicated that the block in viral replication appears to be at a late stage, possibly during assembly of progeny virions, it was of interest to determine if at least the major TIV structural protein was made in these cells. These experiments revealed that the TIV capsid protein was indeed present in the infected *A. albopictus* cells, although the levels found in infected *E. acraea* cells were about 2 fold

greater when equivalent amounts of total infected cell protein were analyzed. This discrepancy can be accounted for by the higher infectivity of the *E. acraea* cells by TIV (~ 2-3 fold). Although the possibility exists that the anticapsid antiserum was detecting capsid protein originating from virus particles in the inoculum for the *A. albopictus* cells, the major contribution most likely represents *de novo* capsid protein synthesis. The number of virus particles internalized by the *A. albopictus* cells as qualitatively determined by electron microscopy, cannot fully account for the levels of capsid protein detected in these cells.

A number of factors could possibly account for the observed block in progeny virion formation. First, a critical host function such as a gene product may be lacking in the *A. albopictus* cell line, or alternatively, an essential viral factor may not be efficiently expressed in this cell line. Such restrictions can conceivably occur at numerous levels such as a cellular or viral gene product essential for replication, or a failure of the virus to modify a host function necessary for virus production. Second, a media component may be below a critical concentration or not present at all during growth of *A. albopictus* cells. For example, Shahrabadi and Lee (1986) showed that calcium is important in maintaining the structure of rotavirus (*Reoviridae*) virions. The minimum concentration of CaCl_2 required in the growth medium to maintain maximal virus yield was determined to be approximately 0.17 mM. Viruses that were produced in the absence of calcium were found to be exclusively incomplete single-shelled particles. Additionally, a reduction in the levels of the major capsid protein was observed in the absence of calcium. These authors speculated that either a threshold level of the capsid protein must be made before viral particle assembly takes place, or the absence of calcium resulted in its failure to be incorporated into progeny virions rendering it more susceptible to degradation by cellular proteases. The *E. acraea* (Grace's) and *A. albopictus* (MEM) media used in this study contained 6.8 mM and 1.8 mM concentrations of CaCl_2 (see Table 5, Appendix). These concentrations are at least 10 fold greater than the minimal requirement for rotavirus.

However, this does not exclude the possibility that calcium or some other media component is present at sub-threshold levels for TIV replication. A brief survey of the Grace's and MEM media components (see Appendix) revealed lower levels of certain components in MEM such as magnesium and potassium. Kurtti and Munderloh (1985) list some of the amino acid requirements for proper growth of mosquito cells, however the threshold levels required for TIV replication in these cells are not known. Experiments can be done in the future to determine if there are any components missing in the *A. albopictus* medium that are essential for TIV replication.

The use of viral mutants to study the effects of specific lesions in the viral replication cycle has been well documented in the literature (Moss, 1985; Horwitz, 1985; Roizman and Batterson, 1985). Probably the best studied semipermissive cell system is the infection of African green monkey kidney cells with human adenovirus type 2 (Ad2; Eggerding and Pierce, 1986). Replication of human adenoviruses is often incomplete in most cultured cells of nonhuman origin. For example, the infection of rodent or monkey cells by Ad2 is semipermissive, resulting in limited viral replication and up to a 5 log reduction in virus production (Gallimore, 1974; Baum, 1977; Eggerding and Pierce, 1986). The lesion in viral replication in these cells involves aberrant late gene expression at the level of transcript processing and translation (Hashimoto, 1973; Nakajima and Oda, 1975; Klessig and Chow, 1980). The semipermissive replication of adenovirus in other established cell lines is still not well understood (Eggerding and Pierce, 1986).

The present study described the semipermissive replication of TIV, a broad host range insect virus, in *A. albopictus* cells. This viral/cell system provides an opportunity to study the factors that define host range and permissiveness among the insect iridescent viruses. These cells have been used to a limited extent to study the replication of other insect iridescent viruses. For example, *A. albopictus* cells were found to be permissive for IV22 replication (Kelly, 1976), however, CIV replication was found not to occur in this cell line (Cerutti *et al.*, 1981), although this virus was still capable of inducing cell fusion of the

A. albopictus cells (Cerutti and Devauchelle, 1979).

The establishment of cell lines from primary cells of a host organism has been a routine practise mainly for the study of viral replication *in vitro*. Cells that have undergone continual passage often have chromosome numbers, growth rates, cell morphologies, and nutrient requirements that are very different from their counterparts *in vivo* (Mitsuhashi, 1981). Although *in vitro* studies provide a convenient framework for the study of viral pathogenesis, it is necessary to exercise extreme caution when extrapolations are made back to the original host organism. Furthermore, two cell lines obtained from the same organism can vary greatly in their properties or their susceptibility to viral infection. For example, Mitsuhashi (1981) showed that *A. albopictus* cells (NIAS-AeAl-2) were susceptible to infection by CIV, whereas an earlier study (Cerutti *et al.*, 1981) using another strain of the same cell line (ATC-15), reported CIV to be non-permissive in these cells. It would be of interest to study the replication of TIV in other *A. albopictus* cell lines, to test for permissivity and to make parallel comparisons with TIV replication in the semipermissive *A. albopictus* C6/36 cells.

C. Isolation of the TIV Capsid Gene

Initially, the strategy chosen for the isolation of the capsid gene relied on the presumed abundance of the capsid transcript in infected cells. To do this, fragments were chosen that hybridized most intensely against radiolabelled cDNA made from total RNA by using oligo dT as primer. Two genes called AB1 and AB2 were subsequently isolated after such an analysis. The transcript sizes for these genes were determined by Northern analysis to be 1.1 and 0.75 Kb, respectively. The capsid transcript was expected to be at least 1.4 Kb (coding information) in size not including 5' or 3' untranslated sequences. Although some transcripts may run aberrantly on a denaturing agarose gel (e.g. LW1 gene, present study) possibly due to extensive secondary structure (Lee-Chen and Niles, 1988) the observed discrepancy in predicted size of the capsid mRNA and the observed sizes of the AB1 and AB2 transcripts necessitated devising a second strategy for isolation of the

capsid gene. The alternate strategy employed is discussed further on in this discussion. To examine the appearance of the AB1 and AB2 transcripts after infection of *E. acrea* cells with TIV, cloned fragments carrying these genes were radiolabelled and hybridized against a slot blot of time course RNA. Slight differences were observed in the AB2 transcript level as compared to the AB1 transcript expression during infection. The level of the AB2 transcript was greater than the AB1 transcript at 4, 8, 10 and 12 h p.i. After this period, the transcript levels for these two genes appear similar. Additionally, both of these genes showed a steady increase in the level of expression until 14 h p.i. when the transcript levels stabilized. Although some of this increase may be a reflection of an increased number of cells being infected, such a possibility was minimized by the routine "superinfection" conditions used for the infection of these cells with TIV. In other words, an increase in the concentration of TIV used during infection resulted in no corresponding increase in the number of infected cells. Under these conditions, a high multiplicity of infection would be simulated such that all susceptible cells should be infected by the virus.

One of the long term goals of this study is to isolate and characterize TIV genes in order to evaluate the regulatory mechanisms involved in gene expression in insect cells. To fulfill some of these requirements, the AB1 and AB2 genes reported here are currently being analyzed in detail by DNA sequencing. Furthermore, the progressive increase in the levels of the AB1 and AB2 transcripts during TIV infection, and their relatively small size make these genes amenable to molecular analysis. Attempts to isolate cDNA clones for the AB1, LW1 and capsid genes using the bank from which AB1 cDNA clones were isolated (8 h p.i.) proved unsuccessful. As a result, the question of polyadenylation of TIV transcripts remains unresolved. Sequencing of the AB1 cDNA clones and comparisons with the corresponding genomic DNA sequence should determine if these clones were generated by internal priming with oligo dT due to A rich sequences in the TIV genome. Since FV3 genes were found to lack poly(A) tails, a survey of TIV transcript sequences should help answer questions of polyadenylation among the insect iridescent viruses and

assist in the classification of the *Iridoviridae* members (D.B. Willis, personal communication).

Since the cDNA hybridization strategy was not sufficient to locate the capsid transcript on the TIV genome, an alternate strategy was employed which made use of tryptic peptides of the capsid protein. These tryptic peptides were sequenced and mixed DNA oligonucleotides were subsequently synthesized using information obtained by reverse-translation of the amino acid sequences. A 20-mer oligonucleotide (#11-4) which was made from one of these peptides, identified two *Sall* DNA fragments #23 and #33 when hybridized against the TIV genome. The former, which did not hybridize after a higher stringency wash, was shown to be a result of non-specific hybridization. Northern blot analysis with a portion of fragment #23 identified a transcript of 3.6 Kb. This transcript was occasionally found to run as multiple bands by Northern blot analysis if the sample was not fully denatured. DNA sequencing of an open reading frame in this region revealed that the predicted amino acid sequence did not match that of peptide #11-4. This gene has been called LW1 and sequencing by W.A. Home (MSc student) revealed an open reading frame of 863 amino acids with a predicted molecular weight of 95.6 KDa and estimated pI of 10.9. This gene showed no significant DNA sequence identity with the capsid gene.

a. Sequence characterization of the TIV capsid gene

DNA sequencing of *Sall* #33 revealed the location of peptide #11-4 on this DNA fragment. However, a discrepancy which resulted from an error during peptide sequencing, allowed only 15/20 of the nucleotides in oligo #11-4 to be available for hybridization to the TIV DNA. This mismatch can account for the relatively weak hybridization signals that were obtained with the radiolabelled oligo #11-4 probe. Finer mapping experiments using oligo #11-4 and the 770 bp *HindIII*/*Sall* fragment located on the right end of *Sall* #33 revealed that the putative capsid gene was located on *HindIII* fragment #26b. DNA sequencing of this fragment revealed an open reading frame of

464 amino acids with a predicted molecular weight of 50.83 KDa and an estimated pI of 6.34. This gene was found to be present in the TIV genome as a single copy, and had a transcript size of 1.8 Kb as determined by Northern analysis. The DNA sequence was not found to be interrupted by introns. This was not unexpected as introns have not yet been shown to be present in DNA viruses that replicate in the cytoplasm (Moss, 1985). As mentioned in the Introduction, vaccinia virus and FV3 genes lack typical prokaryotic or eukaryotic consensus regulatory sequences. Primer extension analysis of the TIV capsid gene indicated a putative transcription start point 19 nucleotides (nt) upstream from the first ATG of the capsid ORF. A CCAAT sequence was located 129 nt upstream from the ATG, and three A-T rich sequences that can act as putative promoter sites, are present 39, 55 and 81 nt upstream from the ATG. Sequence information is available for only two FV3 genes (Willis *et al.*, 1984; Beckman *et al.*, 1988). Only one of these (ICR489) contained TATA (AATAAAA at -41; 72 nt upstream from ATG), CCAAT (at -104) and GC rich sequences (at -180) similar to eukaryotic motifs (Beckman *et al.*, 1988). These FV3 motifs are similar in appearance and location when compared with the A-T rich and CCAAT sequences of the TIV capsid gene. A eukaryotic polyadenylation signal was located at the 3' end of the capsid gene, but attempts to define this 3' end by mung bean nuclease treatment of the protected capsid transcript were unsuccessful (data not presented). DNA sequence comparisons were made between the TIV capsid gene and sequences present in two sequence banks (Dr. M. Zuker, NRC). No obvious similarities were observed with previously published sequences.

The identity of the putative capsid gene was further verified by sequencing of several other tryptic peptides of the capsid protein. Only one of significant length (#5) with an ambiguous N-terminal portion could not be matched with the putative capsid DNA sequence. This peptide sequence (#5) was not found to be present in any of the reading frames located on HindIII #26b. Nevertheless, to determine if peptide #5 was derived from the capsid protein, an oligonucleotide was created and hybridized against TIV DNA.

These experiments resulted in the identification and isolation of Sall fragment #24b which hybridized to a major (1.35 Kb) and a minor (1.65 Kb) transcript after Northern analysis. Preliminary experiments using the core consensus sequence from the vaccinia virus thymidine kinase (tk) gene suggested that Sall #24b may contain the TIV thymidine kinase gene. The justifications for determining the presence of such a gene in TIV are many-fold. First, because TIV is a cytoplasmically replicating virus, it would be expected that like vaccinia virus, this virus encodes its own tk gene. Further, this may be a non-essential gene for TIV replication, as is the case with vaccinia virus (Moss, 1985). The isolation of such a gene from TIV could provide a site of integration of foreign genes into this locus if the cellularly encoded enzyme is sufficient to allow TIV replication. This system has been successfully exploited with vaccinia virus (Weir *et al.*, 1982; Panicali and Paoletti, 1982). Additionally, since the tk gene is one of the most extensively studied genes (Kit, 1985), isolation of the TIV tk gene should allow comparisons to be made with those that have been isolated, in order to better understand the regulation of these enzymes. Furthermore, no insect virus tk genes have been isolated to date (Boyle *et al.*, 1987), therefore, the isolation of a TIV tk should facilitate evolutionary comparisons. In other experiments described in this thesis, it was found that the herpes virus tk gene did not hybridize specifically to the TIV genome. This finding was not surprising since it was expected that the vaccinia virus and TIV tk genes would be more closely related because both viruses replicate in the cytoplasm, whereas herpes virus replicates in the nuclei of infected cells.

b. Expression of capsid in insect cells

The expression of the TIV capsid protein was studied in infected *E. acraea* cells by *in vivo* labelling of total proteins and by western analysis. These experiments revealed an increase in the production of the capsid protein from 14 h to 36 h p.i. Cell associated capsid protein was found to be the third most abundant *de novo* synthesized protein in infected cells as determined by ³⁵S-methionine labelling, however, analysis of ³⁵S-labelled TIV indicated that the capsid protein was the most abundant protein in virions.

During the course of this study, several commonly used techniques were employed to identify the most abundant protein in virions, in other words, the capsid protein. These techniques involved the use of protein binding reagents such as silver and Coomassie Blue, and radioactive labelling with ^{35}S -methionine. A preliminary comparison of these techniques was conducted in order to evaluate the relative abundance of the capsid protein compared to the other virion proteins. Such a comparison revealed, as expected, that the 50 KDa capsid protein is the most abundant protein in the virion. However, some striking differences were evident when some of the other minor proteins were examined. For example, silver staining of virion proteins revealed the presence of an intensely staining 33 KDa protein, and a 29 KDa that was barely detectable. In contrast, Coomassie Blue staining of an identical viral preparation revealed these bands to be of equivalent staining intensity. These dramatic differences can be attributed to the specific properties of the protein that is being detected. Tal *et al.* (1985) reported a strong correlation between intensity of response to Coomassie dyes and the basicity of a protein, which depends on the number of lysine, histidine, and arginine residues, and the nature of the N-terminal amino acid. These authors also reported that hydrophobic interactions of the dye molecule with the polypeptide backbone adjacent to positively charged amino acids, enhances the binding. The Coomassie R molecule contains two sulfonic groups and three nitrogens, two of which are likely positively charged under the staining conditions (Tal *et al.*, 1985). According to these studies, the binding of Coomassie R to proteins is a consequence of electrostatic forces between the sulfonic groups and the basic amino acids as well as hydrophobic interactions with the peptide backbone. The number of dye molecules bound was found to be approximately proportional to the number of positively charged amino acids in the protein, therefore, estimation of the relative molar ratios of proteins in a sample is possible if the sequence is known. In this thesis, it was found that the capsid protein contains 35 basic amino acid residues, the predominant one being arginine (20 residues).

Although silver staining of proteins in gels is reported to be up to 100 times more

sensitive than Coomassie Blue staining, the basis of the silver stain is not as well defined and extreme caution has been suggested for quantitation of proteins following silver staining (Nielsen and Brown, 1984; Chuba and Palchaudhuri, 1986). Additionally, lipopolysaccharides have been shown to stain with silver (Tsai and Frasch, 1982). Nielsen and Brown (1984) suggested that the different shades of blue, yellow, red, and gray that characterize different proteins stained with silver, can be predicted if the amino acid sequence is known. Further, they suggested that the color of protein/silver complexes is related to the binding of silver to the side chains of charged amino acids. In contrast, Chuba and Palchaudhuri (1986) showed a strong correlation existed between increased staining with silver and the cysteine content of the protein. Regarding the mechanism of action, Dion and Pomenti (1983) reported that protein-aldehyde adducts that result from glutaraldehyde pretreatment, directly participate in metallic silver deposition in a manner that resembles latent image formation in the photographic process. Although the precise mechanisms for these protein stains remains obscure, it is evident that quantitation of proteins using Coomassie Blue staining gives a more reliable indication of the relative quantities of proteins than does staining with silver.

Lastly, quantitation using ^{35}S -methionine labelling is dependent on the methionine content of the protein. In the present study, translation of the capsid DNA sequence revealed that the capsid protein contains 9 methionine residues. For a more accurate evaluation of the relative abundance of proteins, it would be suitable to label proteins with a mixture of labelled amino acids such that none of these precursor molecules are limiting during the synthesis of the protein(s). This approach should help minimize some of the obvious biases inherent in the techniques discussed above.

In the course of this study, several attempts were made to synthesize capsid protein *in vitro* by cell-free translation of total RNA using a rabbit reticulocyte system. Western analysis of the *in vitro* translation products using anticapsid antiserum did not detect capsid protein synthesis under these conditions. This result is consistent with the findings of

Raghow and Granoff (1983) that late FV3 mRNAs were translated poorly in cell-free protein-synthesizing extracts prepared from rabbit reticulocytes, wheat germ, or cultured baby hamster kidney cells. In contrast, these authors reported that early FV3 mRNAs were efficiently translated under similar conditions. Further, Raghow and Granoff (1983) reported that polypeptides made in reticulocyte extracts supplemented with a 0.5 M KCl wash from ribosomes of FV3-infected BHK cells, restored the translation of late FV3 mRNAs to the levels resembling those obtained *in vivo*. This effect of the KCl wash, which released initiation factors, was not reproduced when ribosomes were used from uninfected cells. These experiments implicated a virus-specified or modified initiation factor(s) present in FV3-infected cells. It would be interesting to determine if similar mechanisms are involved in the translation of TIV messages.

Several attempts were made to detect the distribution of the TIV capsid protein in infected *E. acrea* and *A. albopictus* cells by indirect immunofluorescence. This series of experiments was unsuccessful because of the inability of the anticapsid antibody to detect the native antigen. These findings were corroborated by the inability of the anticapsid antibody to immunoprecipitate ³⁵S-labelled capsid protein that was synthesized in TIV-infected *E. acrea* cells. However, the anticapsid antiserum was routinely used to detect the capsid protein in its denatured state on western blots. The ability of the antibody to detect antigen on western blots and not in the native state is not unexpected since injection of a foreign antigen into an organism often leads to its breakdown in the host. Some of the factors that effect the antibody response include the primary amino acid sequence of the polypeptide and its conformational determinants (Maurer and Callahan, 1980). In the present study, the capsid antigen was presented to the rabbits in a denatured state as a consequence of SDS-PAGE. Therefore, it was not surprising that the antibody did not recognize this protein in its native state.

c. Expression of capsid in *E. coli* cells

The experiments discussed thus far have provided very strong circumstantial

evidence that the gene identified as the capsid gene codes for the protein from which amino acid sequence was obtained and against which antibody was raised. To obtain direct evidence that this was indeed the sought after gene, it was necessary to show that the product of this isolated gene could be identified by the anticapsid antibody. Initial expression studies in *E. coli* using the native TIV capsid promoter showed that the TIV promoter is not recognized in *E. coli* cells. A construct (pLC26) was subsequently created by fusing a portion of the α -peptide sequence from pUC19 plasmid, with the capsid gene sequence. This pLC26 construct was expressed in *E. coli* cells by induction of the *lacZ* promoter with the gratuitous inducer IPTG. Western analysis of the expressed proteins showed that the translated products of the capsid gene were indeed detectable by the antibody. However, DNA sequencing of pLC26 revealed a stop codon (TGA) in frame with *lacZ* immediately downstream from the *lacZ*/capsid junction. The finding that this clone expressed capsid protein in *E. coli* cells was therefore unexpected. This observed expression likely resulted from internal initiation of translation of the hybrid transcript. An examination of the capsid sequence revealed no consensus *E. coli* ribosome binding sequences (GGAGG; Curry and Tomich, 1988) immediately upstream of the methionine codons. Since the levels of expressed capsid protein increased after induction of the *lacZ* promoter with IPTG, this increase in capsid protein expression was most likely resulted from elevated levels of the hybrid transcript. The capsid specific products that were expressed in *E. coli* cells migrated with calculated molecular weights ranging from 15 to 45 KDa. These products were not detected when the native TIV promoter was used for expression in *E. coli*. The presence of two inverted repeats located at the 5' end of the capsid gene likely rendered this region unfavorable for continuous transcription from the *lacZ* promoter by acting as premature termination sites (Platt and Bear, 1983).

It was found in this study that cycles of freezing and thawing or long term storage of TIV particles resulted in the degradation of the capsid protein into discrete bands. This observation that there are preferred sites for cleavage suggest a highly ordered structure

for this protein. These findings are in agreement with Manyakov (1977) that TIV remained infectious after 2 months of storage at 4°C. It would be of interest to express the native capsid protein in *E. coli* cells and study the biophysical properties of the purified protein to elucidate the mechanisms involved in this observed cleavage. Further, the self-assembly properties of the native capsid protein could be studied after purification of the recombinant protein from *E. coli* cells. The preliminary investigation of small icosahedral particles in this study suggested that such a phenomenon may occur *in vivo*. Expression in *E. coli* cells can be achieved by placing the entire capsid coding region under the regulatory sequences of a prokaryotic gene such as *lacZ*. Similar experiments were carried out with the polyoma virus VP₁ capsid protein where Salunke *et al.* (1986) demonstrated that *E. coli* expressing the recombinant gene formed oligomers resembling dissociated capsomeres of viral capsids. These assemblies were found to be stable at low ionic strength in the presence of calcium (Salunke *et al.*, 1986). In these studies, it was necessary to obtain VP₁ that lacked posttranslational modification and was free of other minor viral capsid proteins, to allow the study of the intrinsic structural properties of the major capsid protein (Salunke *et al.*, 1986). X-ray diffraction studies had earlier revealed (Rayment *et al.*, 1982) that the design of the polyoma virus capsid does not conform to the quasi-equivalence theory proposed by Caspar and Klug (1962) which implied that the capsid was constructed from 12 pentameric and 60 hexameric capsomeres. By velocity sedimentation, image analysis, and electron microscopy of the bacterially expressed VP₁, Salunke *et al.* (1986) indicated that nonequivalent bonding occurs among molecules of the VP₁ protein in an all-pentamer capsid. It was suggested that three types of bonds could account for the assembly of 72 pentamers in the capsid where switching of bond specificity occurs among nonequivalent bonding states (Rayment *et al.*, 1982; Salunke *et al.*, 1986). The use of the heterologous *E. coli* expression system allowed these investigators to study the assembly properties of the native polyoma virus capsid protein in an environment that was free of other viral constituents. Such a heterologous system might also be useful in studying the

self-assembly properties of the TIV capsid protein. Evidence was presented in this thesis for the existence of small (~26 nm) particles that were most likely composed of TIV capsid protein. If this was in fact the case, this protein may possess self-assembly properties *in vivo*, either alone, or in association with other components. A well documented example of such a phenomenon is provided by hepatitis B virus where incomplete viral coat protein, carbohydrate, and lipid constitute particles lacking viral DNA, that are characteristic of hepatitis B virus infection (Robinson, 1985). Preliminary attempts to isolate nucleic acid from the impure small particles found in the present study only showed the presence of substantive amounts of TIV DNA and no other types.

In conclusion, my thesis work has provided evidence that TIV has a multipartite genome, a finding that is unique among the large cytoplasmically replicating dsDNA viruses. In addition, an *A. albopictus* cell line was reported to be semipermissive for TIV replication where the replication cycle was aborted late in infection, likely before the assembly of progeny virions. Since a plaque assay is not yet available for TIV, this should be a valuable system for the study of TIV pathogenesis. Further, this virus-cell system should provide some answers on TIV host range. In other studies, the TIV capsid gene was isolated, sequenced, and the expression of the protein was carried out in insect and bacterial cells. The availability of this cloned capsid gene should also allow further expression studies to be carried out in insect cells or heterologous systems such as *E. coli* or yeast in order to study the biophysical and functional properties of this protein. The availability of this gene and the four other genes described in this thesis should allow sequence comparisons to be made with consensus regulatory sequences from other systems. Finally, the studies carried out here have made TIV amenable to further in depth molecular analysis.

Appendix

Table 5. Comparison of media used for *E. acraea* (Grace's) and *A. albopictus* (Minimal Essential Medium [MEM]) cells¹.

COMPONENT	GTC-100 (mg/L)	A.a. Medium (mg/L)
Inorganic Salts:		
CaCl ₂ (anhyd.)	750.00	200.00
KCl	4100.00	400.00
MgCl ₂ ·6H ₂ O	2280.00	—
MgSO ₄ ·7H ₂ O	2780.00	200.00
NaCl	—	6800.00
NaHCO ₃	350.00	2200.00
NaH ₂ PO ₄ ·H ₂ O	1013.00	140.00
Other Components:		
Alpha-Ketoglutaric acid	370.00	—
Fructose	400.00	—
Fumaric acid	55.00	—
D-Glucose ¹	700.00	1000.00
Malic acid	670.00	—
Succinic acid	60.00	—
Sucrose	26680.00	—
Phenol red	—	10.00
Amino Acids:		
β-Alanine	200.00	—
L-Alanine	225.00	—
L-Arginine-HCl	700.00	126.00
L-Asparagine	350.00	1500.00
L-Aspartic acid	350.00	1330.00
L-Cysteine	22.00	24.00
L-Glutamic acid	600.00	1470.00
L-Glutamine	600.00	292.00
Glycine	650.00	750.00
L-Histidine	2500.00	—
L-Histidine-HCl·H ₂ O	—	42.00
L-Isoleucine	50.00	52.00
L-Leucine	75.00	52.00
L-Lysine-HCl	625.00	72.50
L-Methionine	50.00	15.00
L-Phenylalanine	150.00	32.00
L-Proline	350.00	11.50
DL-Serine	1100.00	1050.00
L-Threonine	175.00	48.00
L-Tryptophan	100.00	10.00
L-Tyrosine	50.00	36.00
L-Valine	100.00	46.00
Vitamins:		
Biotin	0.01	—
D-Ca pantothenate	0.02	1.00
Choline chloride	0.20	1.00
Folic acid	0.02	1.00
D-Inositol	0.02	2.00
Niacin	0.02	—
Nicotinamide	0.02	1.00
Para-aminobenzoic acid	0.02	—
Pyridoxine-HCl	0.02	1.00
Riboflavin	0.02	0.10
Thiamine-HCl	0.02	1.00
Supplements:		
Fetal bovine serum	10%	10%
Bactotryptose broth	0.26%	—
Garamycin	50 lg/ml	—
Penicillin/Streptomycin	—	5000 units/5000 lg

¹ According to the manufacturer (GIBCO, BRL).

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