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UNIVERSITY OF ALBERTA

REGULATION OF GENE EXPRESSION IN SHOPE FIBROMA VIRUS

BY

COLIN G. MACAULAY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING 1990

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED REGULATION OF GENE EXPRESSION IN SHOPE FIBROMA VIRUS

SUBMITTED BY COLIN G. MACAULAY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY.

Dr. G. McFadden (Supervisor)

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Date: Dec 8, 1989

ABSTRACT

A comprehensive transcriptional map for the entire 12.4 kb terminal inverted repeat (TIR) region of Shope fibroma virus (SFV) genome has been determined. Northern blotting and S1-nuclease mapping were used to show that the entire TIR is transcribed into poly A^+ RNA directed towards the termini of the genome. Fifteen transcriptional initiation sites were mapped, 12 within the TIRs and 3 within the unique sequences close to the junction between the left TIR and the unique internal sequences. Eleven of these initiation sites correspond to the 5' ends of major open reading frames (ORFs) and four map within ORFs or to the 5' ends of minor ORFs. All the expressed ORFs are tandemly arranged and oriented towards the hairpin terminus. These ORFs are transcribed at early times, and many have the concensus transcriptional termination sequence from vaccinia at their 3'-ends, while only one minor ORF has the consensus late transcriptional initiation site found in vaccinia. The region upstream of the terminal ORF (T1) was isolated and shown to behave as an early promoter using transient expression assays in infected cells. The promoter domain, localized by 3' and 5' deletion analysis, was found to comprise about 28bp including the transcriptional initiation site and a stretch of eight 'A' residues from position -18 to -11 which were critical for promoter activity. The transcriptional machinery of *leporipoxviruses* and *orthopoxviruses* was shown to be functionally conserved using a transient expression system and by constructing a recombinant vaccinia virus in which the bacterial chloramphenicol acetyl transferase gene (CAT) is driven by the SFV T1 promoter, and a recombinant SFV in which the CAT gene is driven by the vaccinia 7.5-kDa early/late promoter. Both the T1 and the 7.5-kDa early promoters were shown to function in their heterologous genomic environment the same way as they do in their homologous genomic environment in terms of their temporal regulation, and the site of initiation of transcription.

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I would first like to express my deep appreciation for the support of my supervisor Dr. G McFadden. Not only did he (along with Dr. A. R. Morgan), start my career with financial support as a summer student, but he has, and still does, set an impressive example of academic professionalism, and intellectual thoroughness. His uncommon patiences and copious enthusiasm are standards by which others are measured.

All members of the lab have contributed to the stimulating and enjoyable atmosphere in which I have lived for the past six years. The unfailingly consistent technical help of R. Maranchuck and A. Wills have greatly simplified work in the lab. The sequencing of the entire TR of SFV and analysis of these sequences by C. Upton, with contributions by R. Maranchuck, L. DeLange, and J. Macen, was especially important in the analysis of the transcriptional information I obtained in chapter III of this thesis.

I would like to thank D. Hruby for the gift of the vaccinia TK insertion vectors pVV5.1 and pVV3.0 which were used in the analysis of the T1 promoter in chapter IV.

I gratefully acknowledge the help of A. Wills and R. Bradley in preparing the photographs of this work.

The Department of Biochemistry and the Alberta Heritage Medical Foundation have provided the financial support which has allowed me to pursue this career.

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ABBREVIATIONS AND DEFINITIONS

Ara C:	arabinosylcytosine, an inhibitor of DNA replication
AUG:	the initiating codon for the translation of mRNA
bp:	base pair
CAT:	chloramphenicol acetyl transferase
DMSO:	dimethyl sulfoxide
dNTPs:	the four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and TTP)
dsDNA:	double stranded DNA
dsRNA:	double stranded RNA
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetate acid
ffu:	focus forming units, the amount of Shope fibroma virus present in a sample as measured by its ability to produce foci on susceptible cells
foci:	an aggregation of cells infected by SFV so they look like small piles on an uninfected monolayer
IBT:	isatin-β-thiosemicarbasone
kbp:	kilobasepair, 1000 basepairs
-kDa:	kilodalton, 1000 daltons
-MDa:	megadalton, 1,000,000 daltons
m.o.i.:	multiplicity of infection, expressed in pfu/cell or ffu/cell
mRNA:	messenger RNA
MRV:	malignant rabbit fibroma virus, a recombinant between SFV and myxoma

NP40:	a nonionic detergent
ORF:	open reading frame
pfu:	plaque forming units, the amount of vaccinia virus present in a sample as measured by its ability to produce plaques on susceptible cells
RNA:	ribonucleic acid
SFV:	Shope fibroma virus
1XSSC:	0.15M NaCl and 0.015M Na citrate
ssDNA:	single stranded DNA
TIR:	terminal inverted repeat
TK:	thymidine kinase
Tris-HCl/Tris:	tris (hydroxymethyl) aminomethane hydrochloride
Triton X-100:	a nonionic detergent
ts:	temperature sensitive
Vac:	vaccinia virus
VETF:	vaccinia early transcription factor
VLTF-1:	vaccinia late transcription factor 1

RESTRICTION ENZYMES NOTATIONS

Bam HI:	B, Bam
Bgl I:	BI
Bgl II:	Bg
Cla I:	Cl.
Dde I:	Dd
Eco RI:	Ec, R1
Hha I:	H
Hind III:	H III

Kpn I:	Kp
Pst I:	Ps, P1
Pvu I:	Pv
Sal I:	Sa
Sma I:	Sm
Sph I:	Sp
Sst I:	S I
Sst II:	S II
Xba I:	Xb
Xho I:	Xo

CHAPTER I
REVIEW OF POXVIRUS GENE EXPRESSION

A. INTRODUCTION

The orderly expression of important genes is a fundamental mechanism by which the biological processes of all organisms are controlled. The study of how gene expression is regulated in eukaryotic viruses has been very rewarding and many of the models for regulation, and many of the techniques developed for these systems, have been more generally applicable to other systems. Poxviruses are one of the largest and most complex of the eukaryotic viruses. They are dispersed throughout the wild and have been isolated from most vertebrates and some insects. Classification of a virus as a poxvirus (table I) is based on certain common properties such as their large size, complex morphology, a dsDNA genome with hairpin termini, and a cytoplasmic location for their replicative cycle. Although all poxviruses share these properties each member tends to be very species specific and in fact the host species forms one of the criteria for the classification of the various poxvirus genera. Members within a genera are serologically cross reactive using a neutralization test, while members between genera show limited nonneutralizing cross reactivity (Fenner and Ratcliffe, 1965; Joklik, 1968). The pathology that each poxvirus has for its host is unique to that member. For example both myxoma virus and Shope fibroma virus are *leporipoxviruses*, they show extensive serological cross reactivity and their genomes cross hybridize at low stringencies (Block *et al.*, 1985; Fenner and Ratcliffe, 1965). Although they belong to the same genus, myxoma produces a highly invasive fatal disease in cottontail rabbits called myxomatosis, whereas SFV only produces a benign tumor-like growth at the site of innoculation from which the rabbit recovers and is left immune to both the SFV and Myxoma viruses (Fenner and Ratcliffe 1965; McFadden, 1988). So it is very important to remember that the biology of each poxvirus is unique to

Table I

***Poxviridae* Family**	
Subfamily ***Chordopoxvirinae*** (vertebrate poxviruses)	
Genera	**Members**
Orthopoxvirus orthos: Grk.'straight, correct'	Vaccinia, Variola, Monkeypox, Cowpox, Rabbitpox, Buffalopox, Camelpox, Ectromelia (mousepox).
Possible *orthopoxviruses*	Camivorepox, Elephantpox, Racoonpox.
Leporipoxvirus lepori: Ltn., *lepus, leporis* 'hare'	Myxoma virus, Rabbit (Shope) fibroma virus, Squirrel fibroma virus, Malignant rabbit fibroma virus, Hare firbroma virus.
Avipoxvirus avi: Ltn., *avis* 'bird'	Fowlpox, Canarypox, Juncopox.
Capripoxvirus capri: Ltn., *caper, capri* 'goat'	Sheeppox, Goatpox.
Parapoxvirus. para: Grk., *para* 'by the side of'	Orf, Bovine pustular stomatitis virus, Chamois contagious Ecthyma virus, Milker's node virus.
Suipoxvirus sui: Ltn., *sus* 'swine'	Swinepox.
Yatapoxvirus	Tanapox, Yaba monkey tumor virus.
Unclassified.	Molluscum contagiosum.
Subfamily ***Entomopoxvirinae*** (insect poxviruses).	
Genera	**Members**
Group A.	*Melolontha melolontha*, and related members from *Coleptera.*
Group B.	*Amsacta mori* virus, and related members from *Lepidoptera.*
Group C.	*Chironomus luridus* virus, and related members from *Diptera.*

refs. Dales and Pogo, 1981; Fenner *et al.*, 1989; Knight *et al.*, 1989; Mathews, 1982; McFadden, 1988; Moss, 1985.

that member, since most of what is known about the molecular biology of poxviruses has been determined with the *orthopoxvirus* vaccinia. It is not known how well conserved the molecular features are between the various poxvirus members.

The prophylactic use of vaccinia virus as a live vaccine led to the world-wide eradication of smallpox (Fenner, 1985; Henderson, 1988), and with the current use of recombinant DNA technology to clone and express foreign genes within poxvirus genomes, many novel vaccines for both human and veterinary use are now possible (Fathi *et al.*, 1986; Mackett *et al.*, 1982, 1984; Mackett and Smith, 1986; Moss and Flexner, 1987; Piccini *et al.*, 1987; Piccini and Paoletti, 1986; Taylor *et al.*, 1988a, b). This has intensified the study of the molecular biology of poxviruses, and in order to help understand how poxvirus gene expression is regulated it is useful to review some of the physical and biological properties of poxviruses using vaccinia virus as the prototypic member.

B. PHYSICAL AND BIOLOGICAL PROPERTIES OF THE ORTHOPOXVIRUS VACCINIA

1. *Physical Properties*

The physical properties of poxvirus virions have been reviewed in detail by Dales and Pogo (1981). The morphology of the *ortho-*, *lepori-*, *avi-*, and *capri*-poxviruses are indistinguishable though they may vary somewhat in size. Electron microscopy of whole negatively stained vaccinia virus shows that it is a very large brick shaped particle surrounded entirely by a lipid envelope, with randomly arrayed small tube-like structures (surface tubule elements), covering the surface of the particle. Fig. 1 schematically illustrates an electron micrograph of a thin section of an intracellular vaccinia virion. It is composed of a biconcave nucleoprotein core particle with two lateral bodies and is surrounded by an envelope. The *parapoxviruses* are more ovoid rather than brick shaped

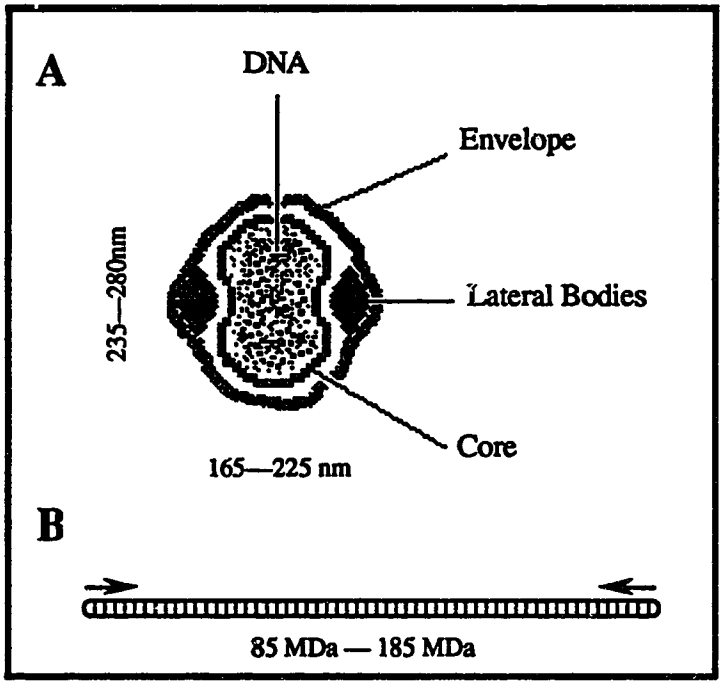


Fig. 1 Pysical properties of poxvin ses.
(A) A schematic illustration of an electron micrograph of a thin section of vaccinia virus. Based on Fig. 3 in Dales and Pogo (1981). (B) The linear dsDNA viral genome featuring hairpin termini and terminal inverted repeats.

and their surface tubule elements appear in a helical array instead or randomly. The insect poxviruses can have a very different morphology and shall not be considered at all. There is no RNA, and a very small amount of carbohydrate associated with the virus. The envelope is acquired intracellularly during viral assembly and not by budding out of the cytoplasm, though extracellular virus may contain a second envelope derived from the golgi membranes.

Using two dimensional isoelectric focusing gels, at least 110 polypeptides have been associated with the virion and at least 279 new polypeptides are made in vaccinia infected cells (Dales and Pogo, 1981; Carrasco and Bravo, 1986). When a nonionic detergent is used to remove the viral envelope during the isolation of viral core particles, approximately 30-40% of the viral protein is solublized, although no enzymes are released (Paoletti *et al.*, 1974b). The function of the lateral bodies remains unknown.

The poxvirus genome consists of a single large linear dsDNA molecule, which can vary in size from 85-MDa for *parapoxviruses* to 185-MDa for fowlpoxvirus, with hairpin termini and terminal inverted repeats (TIRs) (Fig. 1B). Poxvirus genomes tend to be very AT rich, ranging from 78-84% AT for the *entomopoxviruses*, to 60-68% AT for the vertibrate poxviruses. The exception is the *parapoxviruses*, which only shows 37% AT richness. Restriction enzyme maps for the genomes of a number of different poxviruses have been determined, the vaccinia virus genome is 186 kbp (Moss, 1985), myxoma virus is 163 kbp (Russel and Robbins, 1989), SFV is 160-163 kbp (Cabriac *et al.*, 1985; DeLange *et al.*, 1984b; Wills *et al.*, 1983), Yaba monkey tumor virus is 145 kbp (Kilpatrick and Rouhandeh, 1987), *Capripoxviruses* are 143-146 kbp (Gershon and Black, 1988), raccoonpox virus is 215 kbp (Parsons and Pickup, 1987), *parapoxvirus* (orf) is 138-140 kbp (Robinson *et al.*, 1987), and the restriction enzyme profiles of 38 different *orthopoxviruses* have been compared (Esposito and Knight, 1985). Adjacent to the hairpin termini within the terminal inverted repeats, the *orthopoxviruses* have a series of tandemly repeated sequences while the *leporipoxviruses* have a series of imperfect palindromic

sequences, the function of either of which is unknown (Upton *et al.*, 1987a).

2. *The Replicative Cycle*

A complete review of all the different aspects of the virus replicative cycle will not be given, instead its basic features are provided as a frame work on which to discuss the regulation of poxviral gene expression. The vaccinia replicative cycle is depicted in Fig. 2, and it is separated into six convenient steps: adsorption and penetration, uncoating, early gene expression, DNA replication, late gene expression, and the assembly and morphogenesis of mature progeny viruses.

a) Adsorption and Penetration

No specific cellular receptors have been identified though virus surface antigens which elicit neutralizing antibodies have been described (Dales and Pogo, 1981). The virus binds to the cell surface and enters the cell by membrane fusion at either the cell surface or from within phagocytic vesicles.

b) Uncoating

Uncoating of the viral DNA has been separated into two steps that have been defined biochemically and by electron microscopy.

Uncoating I. This first step occurs by the fusion of the viral envelope with the cytoplasmic envelope of the cell and results in the release of naked cores into the cytoplasm. When viral phospholipid is labeled, acid soluble radioactivity is first detected intracellularly and then in the medium, while labeled proteins associated with the envelope no longer sediment with virus particles. The lateral bodies have been seen to remain associated with the viral envelope or to remain loosely attached to the core particles from which they eventually fall off (Dales and Pogo, 1981; Joklik,1968). This first step is

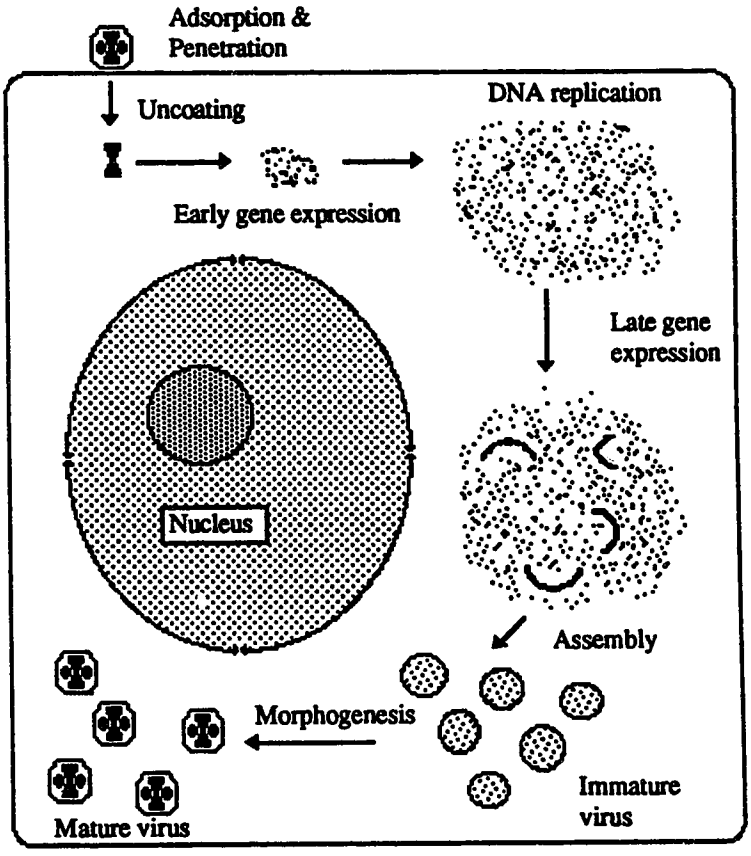


Fig. 2. The replicative cycle of vaccinia virus.

entirely dependent on cellular processes, no virus functions are involved, and it is not prevented by protein synthesis inhibitors. UV or heat inactivated virus particles can still enter the cell and be uncoated to virus cores.

Uncoating II. This second step is seen to occur by the disappearance of the viral core particle. The core structure breaks down and the viral DNA becomes DNase I sensitive. This step requires virus gene expression and is inhibited by protein synthesis inhibitors. Thus, there is a lag time of 30-60 minutes in order that a trypsin-like protease called the uncoating protein can be synthesized (Joklik and Beaker, 1964; Pedley and Cooper, 1989). The uncoating protein in cells infected by one genera of vertibrate poxvirus can uncoat other genera as demonstrated by the reactivation of heat or UV inactivated virus of one genera by a wild type virus of a different genera during a mixed infection (Joklik, 1966). The efficiency of uncoating probably depends on the cell type and its metabolic state, but more virus undergoes uncoating I than uncoating II, and not all of the virus entering the cell is uncoated. The highest levels of uncoating seen with vaccinia virus is 70%. The virus particle to infective unit ratio is at best 2.4-10 for vaccinia, but more typically it is 20-40 and can be as high as 50-1000 due to clumping of the virus particles (Dales and Pogo, 1981; Joklik, 1966).

c) Early Gene Expression

Gene expression in poxviruses has been divided into two phases designated early and late (see section D for a more detailed discussion). Early genes are transcribed prior to DNA replication and late genes are transcribed after the initiation of DNA replication. Early gene expression occurs as soon as uncoating I has occured, and these genes include those involved in the expression and replication of viral DNA, such as RNA and DNA polymerases, thymidine kinase, capping enzymes etc., as well as some viral structural genes.

d) DNA Replication

DNA replication requires early viral gene expression and occurs after the viral cores have been broken down (uncoating II), and the DNA is present in viral factories within the cytoplasm. At an multiplicity of infection of 400 virus particles/cell the incorporation of ^{3}H-thymidine into progeny viral DNA starts at about 2 hrs post-infection, has maximum rate at 2.5-3 hrs post-infection, and decreases to low levels at about 5 hrs post-infection (Joklik and Becker, 1964; McFadden and Dales, 1982). When the amount of viral DNA within the infected cell is measured by Southern blotting analysis, it is seen to increase steadily between 3 hours and 10-12 hours post-infection. The reason ^{3}H-thymidine is poorly incorporated at late times is not understood but it may reflect drastic changes in the intracellular nucleotide pools at later times in the infection (reviewed by Traktman, 1989).

e) Late Gene Expression

Late gene expression starts after the initiation of DNA replication. In the presence of inhibitors of DNA replication late gene expression does not occur, and early gene expression continues. What signals this switch from early gene to late gene expression is unknown. The products of late genes comprise virus structural proteins and those enzymes required for the assembly and morphogenesis of progeny virus. Transcription of late genes starts at about 2hrs. post-infection, has a maximum rate at about 8 hrs., and then tapers off to a rate that is half that found at its peak for the next 20hrs. (Joklik, 1968; Moss, 1985).

f) Assembly and Morphogenesis

Crescent shaped envelopes are the first virion structures seen within the cytoplasmic virus factories at about 5hrs post-infection. The appearance of envelopes requires early gene expression and they can be detected in the presence of inhibitors of DNA replication (Dales and Pogo, 1981). Fully spherical envelopes enclosing a viroplasmic matrix of viral

DNA and protein comprise the immature virus particle, which then matures as seen by the differentiation of the viroplasmic matrix into a core and lateral bodies. This late stage maturation process is associated with the proteolytic processing of major virion proteins and drugs such as isatin-β-thiosemicarbazone (IBT) and rifampicin can inhibit this process. By measuring the packaging of viral DNA by its resistance to DNase I, Joklik and Becker (1964), show that only 30-50% of the nascent viral DNA is packaged. Packaging requires continuous protein synthesis, starts at about 5hrs post-infection and then increases steadily between 7.5hrs and 24hrs.

Vaccinia does not have a specific lysis function, though some of the mature virus particles are seen at the ends of long cytoplasmic extrusions called microvilli which can breakdown and release the virus. Less than 10% of the progeny virus is released into the medium, so that plaque titration assays can be done without an agar overlay. The virus appears to be transmitted from cell to cell quite well and this horizontal spread is not inhibited by neutralizing antiserum. Some strains of vaccinia promote cell fusion and cause syncytia formation. The yield of vaccinia virus from a single step growth curve is approximately several hundred pfus/cell (Joklik, 1966).

C. NUCLEAR REQUIREMENTS

Poxviruses are unique among the DNA viruses in that biochemical and electron microscopic studies have shown that the entire replicative cycle is localized to the cytoplasm of the infected cell, and requirements for host cell nuclear functions, if any, are not well defined (reviewed by Moyer, 1987). Early experiments showed that vaccinia can undergo a normal replicative cycle in cells which have been enucleated with cytochalasin B, until late times where the morphogenesis of immature virus into mature virus is blocked, suggesting that some nuclear function is required for this stage in the replicative cycle (Hruby *et al.*, 1979a; Pennington and Follet, 1974; Prescott *et al.*, 1971).

In the presence of the drug α-amanitin, which is known to inhibit the host RNA

polymerase II but not the vaccinia RNA polymerase (Baroudy and Moss, 1980; Costanzo *et al.*, 1970; Nevins and Joklik, 1977a), virus maturation is blocked and the proteolytic processing of some late proteins is inhibited (Hruby *et al.*, 1979b; Silver *et al.*, 1979; Silver and Dales, 1982). A cell line whose RNA polymerase II is resistant to α-amanitin allows for the production of mature progeny virus (Silver *et al.*, 1979). Morrison and Moyer (1986), as well as Bloom *et al.*, (1989), have found that shortly after infection, specific proteins which are normally associated with the cell nucleus and include a nuclear lamin-like protein, and the large subunit of RNA polymerase II (170-kDa), are released into the cytoplasm. This cellular RNA polymerase II subunit has also been found to be packaged within virions. These results have given rise to the speculation that a host cell RNA polymerase II subunit is important for the transcription of some late vaccinia virus genes required for morphogenesis. However, it should be noted that vaccinia is known to package other heterologous nonviral proteins as well (Franke and Hruby, 1987). An α-amanitin resistant mutant of vaccinia virus has been isolated and localized to the *HindIII* N fragment (ORF N2 or M1) (Tamin *et al.*, 1988; Villareal *et al.*, 1984, 1986). This mutant not only allows for the production of mature virus in the presence of α-amanitin, but it also allows for the production of mature virus in enucleated cells at 37% of the levels found in whole cells. It appears that this mutation overcomes the block imposed by cytochalasin enucleation of cells and by α-amanitin. There is still no direct evidence that any nuclear functions are required for the expression and replication of the viral genome. The fact that vaccinia encodes many of the enzymes required for the expression and replication of its genome suggests a significant autonomy from the host cell nucleus.

D. THE TEMPORAL REGULATION OF VACCINIA GENE EXPRESSION

1. *Different Sets of Proteins are Synthesized at Early and Late Times*

The first clues that poxvirus gene expression is temporally regulated were made by those studying the effects of inhibitors of protein synthesis, transcription, or DNA

replication, on the production of virally induced enzymes in infected cells. A number of new enzymatic activities such as thymidine kinase (TK) (Kit *et al.*, 1962; McAuslan and Joklik, 1962), DNA polymerase (Green *et al.*, 1964; Green and Pina, 1962; Magee, 1962), DNA ligase (Sambrook and Shatkin, 1969), and deoxyribonuclease (Hanafusa, 1961; McAuslan 1965), can be detected upon infection of a cell by vaccinia. The TK activity is produced continuously after infection until viral DNA synthesis begins, whereupon TK synthesis stops. This "switch off" phenomenon requires viral DNA replication and transcription since when either of these two are blocked by UV irradiation or actinomycin D, then the synthesis of TK activity is not switched off (Jungwirth and Joklik, 1965; McAuslan, 1963a, b; McAuslan and Joklik, 1962). Infecting cells in the presence of the antipoxvirus drug IBT, which causes the breakdown of polyribosomes at late times, or the addition of protein synthesis inhibitors to infected cells at different times during an infection, has been used to demonstrate that protein synthesis is required after DNA replication in order for mature virus to be produced (Joklik and Becker, 1964; Salzman *et al.*, 1963; Woodson and Joklik, 1965). So it is clear that in order for a complete replicative cycle to occur, protein synthesis is required before and after DNA replication, and that some of the proteins are sythesized prior to, and not after DNA replication.

By pulse-labeling infected cells with radio-labeled amino acids at different times during the infection and then resolving the labeled proteins by polyacrylamide gel electrophoresis, it has been demonstrated that distinct sets of proteins are made before replication (early times), and after DNA replication (late times). If viral DNA replication is inhibited, then the synthesis of the early protein set is prolonged (Esteban and Metz, 1973; Moss and Salzman, 1968; Salzman and Sebring, 1967; Pennington, 1974). A few of the early proteins and many of the late proteins correspond to virion structural proteins (Cohen and Wilcox, 1968; Holowczak and Joklik, 1967; Salzman and Sebring, 1967). Though some of the differences between early and late protein synthesis may be due to regulation at the translational level (Golini and Kates, 1984; Hruby and Ball, 1981; Vassef *et al.*, 1982),

much of this temporal regulation occurs at the transcriptional level.

2. *Different Sets of RNAs are Synthesized at Early and Late Times*

Using hybridization and sedimentation analysis Oda and Joklik (1967), demonstrated that early transcription differs from late transcription. Early viral RNA is smaller (10-12S), than late viral RNA (16-20S). Late mRNA contains sequences not found in early mRNA but all the sequences transcribed at early times are also transcribed at late times. In other words, the sequences transcribed at early times are a subset of the sequences transcribed at late times. Like the protein synthesis pattern, the early transcription pattern is maintained if DNA replication is inhibited.

Paoletti and Grady (1977) used solution hybridization to measure the amount of viral DNA that is transcribed at early and late times. By hybridizing ^{3}H-viral DNA with excess early or late RNA they found that 25% of the viral DNA forms hybrids with early RNA and 52% of the DNA forms hybrids with late RNA. Similar findings where late viral RNA has twice the sequence complexity of early viral RNA have been made by others (Boone and Moss, 1978; Kaverin *et al.*, 1975; Oda and Joklik, 1967; Varich *et al.*, 1979). It has also been noted that there is a significant amount (up to 15%) of dsRNA present at late times, which has a sequence complexity the same as the late viral RNA (Boone *et al.*, 1979; Colby *et al.*, 1971; Varich *et al.*, 1979).

When northern blots of early RNA are probed with specific viral DNA fragments the transcripts appear to be of a distinct size, and generally, both the 5' and 3' ends can be defined by S1 analysis (Venkatesan *et al.*, 1982; Wittek *et al.*, 1980b). Yeun and Moss (1986) isolated and sequenced a number of cDNA clones for the vaccinia early gene that encodes an epidermal growth factor-like gene, and found the 3' ends to be clustered 50-100 nucleotides downstream from the ORF. Northern blots of late viral RNA generally do not show any distinct transcripts at late times, though some specific late genes, such as the cowpox 160-kDa gene, do have transcripts of a defined size (Patel and Pickup, 1987).

Transcription of most late genes continues into the sequences downstream of the coding ORF and no defined termination sites can be detected by S1 analysis (Cooper *et al.*, 1981b; Golini and Kates, 1984; Mahr and Roberts, 1984b; Rosel and Moss, 1985; Weinrich and Hruby, 1987; Weir and Moss, 1984). This explains the marked difference in size distributions between the early and late transcripts. The fact that the early transcripts are a subset of the late transcripts, and the fact that transcription can occur from both DNA strands, provides an explanation of how a significant amount of complementary RNA can be made at late times. Another distinguishing feature of some late transcripts is that they can contain a stretch of up to 50 'A' residues attached to the 5' end. This poly (A) head has been detected by, (1) cDNA cloning, (2) noting the difference between the position of the mapped transcriptional initiation site when using S1 nuclease analysis or primer extension, and (3) sequencing the RNA directly during the primer extension reaction (Bertholet *et al.*, 1987; Patel and Pickup, 1987; Schwer *et al.*, 1987). This correlates with information from Boone and Moss (1977), who studied type I and type II CAP structures on the 5'-ends of vaccinia RNA (see appendix II). They found that for early RNA the N1 position was 55% G and 45% A residues, while for late RNA it was 11% G and 89% A residues. In summary, there are clear differences between early and late viral RNA in terms of structure, sequences transcribed, and genes encoded, which corresponds to the differences seen between the early and late viral proteins.

Early transcription occurs immediately after the first uncoating and is a prerequisite for the second stage uncoating. This means that the virus must package the enzymes required to transcribe the genome at early times. When purified vaccinia virions are permeablized with the nonionic detergent NP40 and incubated in the appropriate buffer (ex. 50mM Tris. pH8.4, 0.05% NP40, 6mM $MgCl_2$, 5mMDTT, 3mMATP, and 1mM each of CTP, GTP, UTP), viral RNA is synthesized and extruded (Ensinger *et al.*, 1975; Kates and McAuslan, 1967; Kates and Beeson 1970a; Munyon *et al.*, 1967; Nevins and Joklik, 1975; Wei and Moss, 1974). The sequence complexity of this *in vitro* RNA has been

variously shown to be less than early RNA (Kates and Beeson, 1970a), the same as early RNA (Nevins and Joklik, 1975), and the same as late RNA (Boone and Moss, 1978; Paoletti and Grady, 1977). Boone and Moss (1978) suggest that when disaggregation procedures such as sonication are used in virus purification, some of the transcriptional regulation is lost and early RNA made either *in vivo* or *in vitro* in fact shows the same complexity as late viral RNA.

If early or late RNA is isolated from infected cells and translated *in vitro* the products are the same sizes as those seen *in vivo* when infected cells are pulsed with radiolabeled amino acids at both early and late times. Viral mRNA made by permeablized cores *in vitro* is capped, polyadenylated, and functional when purified and added to an *in vitro* translation system. When the transcription and translation systems are coupled *in vitro*, synthesis of *bona fide* viral proteins is observed (Jaureguiberry *et al.*, 1975; Beaud *et al.*, 1972; Cooper and Moss, 1978, 1979a, b; Pelham *et al.*, 1978). These *in vitro* synthesized products have the same size profile as proteins detected at early times *in vivo*, except that proteins larger than about 40-50-kDa are not seen. These larger species are observed when early RNA extracted from infected cells is translated *in vitro*, so it appears that, regardless of the sequence complexity, RNA made *in vitro* is functional mRNA and represents a subset of the mRNA produced at early times *in vivo*.

Some investigators have found that high molecular weight (20-30S) virion associated RNA is synthesized *in vitro* under conditions where ATP is depleted. This large RNA is capped, not polyadenylated, can be pulse labeled and chased into the 8-12S mRNAs that are normally released from cores (Nuss and Paoletti, 1977; Paoletti 1977a, b). There may be a precursor/product relationship between the high molecular weight core-associated RNA and the released mature mRNA but the nature of this relationship is not clear. There is no evidence for splicing of poxvirus mRNA: furthermore using UV inactivation studies with *in vitro* translation of RNA from purified virus to determine the target size of the coding sequences, Bossart *et al.*, (1978 a, b) demonstrated that

multicistronic transcripts are not produced, and there is only one coding sequence per RNA molecule. Products of *in vitro* translation of the high molecular weight RNA are identical to the 8-12S mRNA.

Thus it has been demonstrated that transcription of the vaccinia genome is temporally regulated and that distinct sets of genes are expressed before and after DNA replication. How this regulation occurs is currently being examined by many laboratories by trying to identify and characterize both the trans-acting factors essential for transcription and the cis-acting sequences which control the elements of the genome to be expressed.

3. *Enzymes and Factors Required for Transcription*

As mentioned previously, vaccinia virions contain all the enzymes required for the synthesis of early mRNA, and the individual components which have been isolated so far are listed in table II. Other enzyme activities isolated from virions or from infected cells are discussed in appendix II. Encapsidation of an enzymatic activity does not prove it is a viral-encoded enzyme, but where it is known, all enzyme activities that are induced in infected cells or contained within virions, are specified by the viral genome. Identification of these components has been done in three ways. One way has been to purify and characterize enzymatic activities that are clearly important for transcription, such as the RNA polymerase (discussed below). A second way has been to develop *in vitro* transcription systems that allow the use of standard biochemical techniques to identify components essential for transcription, such as the vaccinia early transcription factor, the vaccinia late transcription factor, and the early termination factor (discussed in part 4). A third way that has been used to identify viral genes involved in transcription, and their possible functions, is through the use of poxvirus genetics, (discussed in section F).

A ssDNA-dependent RNA polymerase activity has been purified from both vaccinia virions and vaccinia-infected HeLa cells (Baroudy and Moss, 1980; Ishama and Nagata, 1988; Nevins and Joklik, 1977a; Spencer *et al.*, 1980). When examined by

Table II

Virion Associated Transcriptional Enzymes	
RNA polymerase	Capping enzyme complex:
Poly (A) polymerase	-polynucleotide triphosphatase
Nucleotide phosphohydrolase I + II	-guanylyltransferase
Vaccinia early transcription factor ATPase	-(guanine-7)-methyl transferase
	-(nucleoside-2')-methyl transferase

See appendix II for a complete description of other virion associated activities.

polyacrylamide gel electrophoresis the virion associated enzyme was composed of 140, 137, 37, 35, 31, 22, and 17-kDa subunits while the activity purified from infected HeLa cells was composed of 135, 130, 77, 35, 19.5, 16.5, and 13.5-kDa subunits. Like cellular eukaryotic RNA polymerase II, the viral enzyme is a complex multisubunit enzyme containing two large subunits plus a number of smaller subunits. Extensive purification of polymerase activity has allowed the development of antisera against some of the individual subunits. These subunits have then been mapped onto the viral genome by screening a λgt11 expression library (Broyles and Moss, 1986), and by immunoprecipitating the *in vitro* translation products from a reaction programmed with hybrid-selected RNA (Jones *et al.*, 1987; Morrison *et al.*, 1985). These antibodies appear to be virus specific and do not inhibit host RNA polymerase II or III activities (Jones *et al.*, 1987).

The vaccinia genes for the two largest RNA polymerase subunits (147 and 132-kDa), and for the 22-kDa subunit have so far been identified and mapped (see appendix I). The 147-kDa subunit from vaccinia and the 137-kDa subunit from cowpox, which is highly related to vaccinia, are homologous to the largest and second largest subunits, respectively, from the RNA polymerase of *Esherichia coli*, and the RNA polymerase II of *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Broyles and Moss, 1986; Patel and Pickup, 1989). Six conserved domains are found between the 147-kDa vaccinia subunit and the largest subunits of *S. cerevisiae* RNA polymerase II and III, and *D. melanogaster* RNA polymerase II. The first N-terminal conserved domain in the vaccinia and eukaryotic subunits is not shared with the *E. coli* RNA polymerase large subunit, although the other five domains are shared. Considering the wide differences between these enzymes in terms of the complexity of the sequences which are recognized, these domains probably represent functions which are conserved among all RNA polymerases, such as RNA chain elongation. Some monoclonal antibodies to the purified vaccinia RNA polymerase large subunit cross react with the large subunit of a eukaryotic RNA polymerase indicating the presence of at least one conserved antigenic epitope (Morrison

and Moyer, 1986). Although sequence homology and antigenic conservation between these RNA polymerase subunits exists, rifampicin and α-amanitin, which are known to specifically inhibit the prokaryotic RNA polymerase and the eukaryotic RNA polymerase II, respectively, do not affect the vaccinia RNA polymerase. The α-amanitin resistance mutation in a BALB/c 3T3 mouse cell line has been mapped to within conserved domain number four of the RNA polymerase II large subunit (Bartolomei and Carden, 1987; Broyles and Moss, 1986), while the α-amanatin resistance locus of vaccinia doesn't map to any of the known viral polymerase subunits (Tamin *et al.*, 1988).

A common feature among the eukaryotic RNA polymerase II large subunits, that is not shared with the vaccinia 147-kDa subunit, is a C-terminal domain consisting of a tandemly repeated heptapeptide whose consensus sequences is Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This consensus sequence is well conserved among the yeast and mammalian enzymes, but is more diverged in drosophila. Mutational analysis and the use of monoclonal antibodies in an *in vitro* transcription system, indicate that this C-terminal tail is required specifically for accurate promoter-directed initiation of transcription, and may do so by directly interacting with RNA polymerase II transcriptional factors (Allison *et al.*, 1988; Bartolomei *et al.*, 1988; Nonet *et al.*, 1987; Smith *et al.*, 1989; Thompson *et al.*, 1989b; Zehring *et al.*, 1988). If the viral RNA polymerase has to only recognize two sets of genes (early and late), then it may not need such a mechanism for promoter recognition.

Although no antigenic relationship has been described between the 147-kDa vaccina subunit and the *E. coli* β' subunit, the E. coli RNA polymerase can transcribe viral DNA in a limited fashion *in vitro* (Dahl and Kates, 1970; Esteban *et al.*, 1985). Similarly, *E. coli* plasmid DNA can be shown to be transcribed to a limited degree when transfected into poxvirus-infected cells (C. Macaulay, unpublished observations, and Miner *et al.*, 1988). The vaccinia virus early transcription factor (VETF), binds specifically to vaccinia early promoters (see below), and this binding stimulates an endogenous ATPase activity. This ATPase activity is stimulated to a greater extent when VETF binds to pUC19 or M13

replicative form DNA, than when it binds to whole calf thymus DNA (Broyles and Moss, 1988). It is, however, not known if viral promoters are accurately recognized by the *E. coli* RNA polymerase or if prokaryotic promoters are specifically recognized by the poxvirus RNA polymerase. The purification and characterization of other enzymes involved in viral transcription is further discussed in appendix II.

4. *In Vitro Transcription Systems*

To help identify the factors which are involved with early gene transcription, *in vitro* systems have been developed using either whole cell extracts of infected cells (Fogleson 1985; Puckett and Moss, 1983), or crude extracts of purified vaccinia virions (Golini and Kates, 1985; Rohrmann *et al.*, 1986; Rohrmann and Moss, 1985). Virion extracts are much less complex and have little contaminating cellular material so they have been used more extensively for the purification and characterization of the components required for early viral gene transcription. Crude virion extract will transcribe single stranded (ss) or double stranded (ds) viral DNA templates. A ssDNA template measures the elongation ability of the RNA polymerase, while a dsDNA template measures the ability to initiate transcription in a promoter specific fashion. When a double stranded viral DNA template is used, crude virion extracts will only transcribe early genes, late genes are not recognized and the major transcriptional initiation sites seen *in vitro* correspond to those seen *in vivo*, although there are many additional spurious nonspecific initiation sites. Transcription is not only sensitive to actinomycin D, but it is also sensitive to high concentration of novobiocin, an *E. coli* topoisomerase II inhibitor, and transcription in both permeablized virions and crude virion extracts requires ATP with a hydrolyzable β-γ bond (Broyles and Moss, 1987b; Gershowitz *et al.*, 1978; Shuman *et al.*, 1980a).

When a crude virion extract is fractionated on a glycerol gradient various enzymatic activities can be detected (Broyles and Moss, 1987b). An RNA polymerase activity which uses both single and double stranded DNA as template is detected. Co-sedimenting with

the RNA polymerase are guanylyltransferase and nucleotide phosphohydrolase-I (NPH-I or ATPase-I) activities. The poly (A) polymerase and topoisomerase I activities are well separated from the RNA polymerase, nevertheless both the RNA polymerase activity and a DNA dependent ATPase activity are still sensitive to high concentrations of novobiocin (0.5-1.0 mM). This ATPase activity, which requires a hydrolyzable β-γ bond, is needed for transcription on dsDNA templates but not on ssDNA templates and thus may be required specifically for the initiation of transcription, and not for the elongation process.

Further purification of the RNA polymerase activity leads to the loss of the ability to use dsDNA, but not ssDNA, as a template. By adding different fractions of a virus extract to the semipurified RNA polymerase and then testing for the restoration of transcription on a dsDNA template, a vaccinia early transcription factor (VETF) was identified (Broyles and Moss, 1988; Yuen *et al.*, 1987). VETF is a heterodimer composed of an 82-kDa and a 77-kDa subunit. The complex binds to early promoters in a gel retardation assay, and point mutations which reduce VETF binding also reduce promoter strength. VETF contains a DNA dependent ATPase activity similar to NPH-I except that it is stimulated more by dsDNA than by ssDNA and antisera to NPH-I does not affect VETF ATPase activity (Broyles and Moss, 1988). The VETF ATPase is therefore a strong candidate for the ATPase required for transcription on a dsDNA template.

In a similar fashion, the protein factors and sequence requirements for the termination of early transcription have been identified. Transcription in crude virion extracts was shown to terminate about 50 nucleotides downstream of the sequence TTTTTNT (T_5NT), even if the downstream sequences were not viral (Rohrmann *et al.*, 1986; Yuen and Moss, 1986, 1987). Deletion analysis showed that this sequence was required in order to get termination of transcription (Yuen and Moss, 1987), and a time course for the *in vitro* synthesis of RNA demonstrates that the termination of transcription occurs where the 3'-end of the transcripts occur. Both the terminated and the runoff transcripts are polyadenylated, indicating transcript cleavage is not part of 3'-end

processing. Transcription by RNA polymerase II terminates somewhere downstream of the sequence AAUAAA in the RNA which is the cleavage and polyadenylation signal required for 3' end formation (Birnstiel *et al.*, 1985). By adding nucleotide analogues to an *in vitro* transcription system Shuman and Moss (1988a) demonstrated that the actual signal for termination of early transcription was U5NU in the RNA. Termination in vaccinia is similar to rho independent termination in *E. coli*, except that the poxvirus sequence is not palindromic. Termination is not 100% efficient *in vitro* or *in vivo*, and if read through occurs then transcription continues until the next termination signal (Bajszar *et al.*, 1983; Golini and Kates, 1984). In two cases consensus termination sequences have been found within early genes and these do not appear to be recognized *in vivo* (Patel and Pickup, 1989). A more purified RNA polymerase activity does not terminate trancription. By adding fractions of the virus extract back to this RNA polymerase activity to restore termination Shuman *et al.*, (1987), demonstrated that the capping enzyme complex is the termination factor. Guanylyl- and methyl- transferase activities are not required so the RNA does not have to be capped to be terminated. Some of the capping enzyme activity was shown to cosediment with the RNA polymerase activity on gylcerol gradients (Broyles and Moss, 1987b). Fuerst and Moss (1989), showed that in cells coinfected with one recombinant virus containing the T7 RNA polymerase gene under the vaccinia 7.5 early/late promoter, and a second recombinant virus containing the bacterial *lac* Z gene flanked by T7 promoter and terminator sequences, large quantities of *lac* Z transcripts are made but only 5-10% are capped. This would indicate that the capping enzyme acts in a complex with the viral RNA polymerase. Both early and late transcripts are capped, so the capping enzyme must be present at both times. Why the termination signals are not recognized at late times is unknown.

When whole cell extracts are made from infected cells at late times they will specifically transcribe both early and late genes using a dsDNA template (Puckett and Moss, 1983; Schwer and Stunnenberg 1988; Wright and Moss, 1987, 1989). These

extracts add 5' poly (A) heads to the late transcripts as is seen *in vivo*, and pulse chase experiments indicate that this poly (A) tract is probably not added posttranscriptionally. Late transcription, unlike early transcription, does not seem to require ATP with a hydrolyzable β-γ bond. Fractionation of the late extracts is not as well developed as the virion extracts. However, the late extracts have been fractionated by different ion exchange chromatography systems into a number of components, which by themselves can not specify late template transcription, but when mixed togeather they permit transcription (Wright and Moss, 1989). The active component of one such complementing fraction, which lacked any RNA polymerase activity, sedimented on a glycerol gradient at 3.6S. This is equivalent to a 45-kDa globular protein and it is designated the vaccinia late transcription factor-1 (VLTF-1). VLTF-1 has not yet been further characterized and it is not known if it binds to late promoters, but it is not required for early template transcription *in vitro*. Miner and Hruby (1989b), have found a late promoter binding activity specific to infected cell extracts using gel retardation assays but details of how it relates to VLTF-1 are not available.

The evidence gathered so far indicates that vaccinia virus transcribes its genome in a temporally regulated manner within the cytoplasm of infected cells. Since the poxviral transcriptional machinery recognizes only viral genes and not host cell genes (Cochran *et al.*, 1985a), poxviruses must have their own unique cis-acting promoter sequences as well.

E. ORTHOPOXVIRUS PROMOTERS

The analysis of poxvirus promoters is closely tied to the development of recombinant vaccinia viruses since an active poxvirus promoter is required for the efficient expression of foreign genes. Virtually any intronless gene can be expressed in a recombinant vaccinia virus, and this has led to the extensive use of vaccinia as a eukaryotic expression vector, and to the investigation into what constitutes a vaccinia promoter.

Vaccinia virus promoters have been functionally defined as those sequences which allow for the expression of heterologous genes in either recombinant viruses or in transient expression systems. Though randomly cloned viral DNA sequences can be tested for promoter activity, candidate promoters are typically chosen as sequences upstream from, and including, the transcriptional initiation site of a mapped viral gene. In order to test a putative promoter sequence for activity, it is fused to the coding segment of a gene whose product can be either easily detected, or positively selected for. If the putative promoter sequence is fused to a gene whose product is easily assayed, then this construct can be tested directly in a transient gene expression assay, or a recombinant virus can be constructed and the expression of the marker gene assayed in infected cells.

A transient expression system is the quickest and most convenient method to test for promoter activity. It involves transfecting the promoter/marker-gene construct into infected cells, and then assaying cell lysates for the marker gene product or transcript. This technique has been shown to be somewhat biased towards late promoters (Cochran *et al.*, 1985a, b). This is probably because early promoters on the plasmid DNA are exposed to the viral early-transcriptional machinery for only a short time between uncoating II, when the viral core breaks down, and DNA replication, when early gene expression is normally shut off. Thus if a very weak early promoter is tested, there may not be enough marker gene product made in that short period of time to be detected. To overcome this, the promoter/marker-gene construct can be cloned into a vaccinia insertion vector and inserted into the viral genome to produce a recombinant virus. Once the recombinant virus is made and plaque purified then the amount the marker gene product and transcript within infected cells can easily be tested. Both of these methods are particularly u[illegible]l for testing the activity of promoters which have been altered by *in vitro* mutagen[illegible] Marker genes which have been used in such assays include the bacterial chlor[illegible]ol acetyl transferase (CAT) gene (Mackett *et al.*, 1984; Cochran *et al.*, 19[illegible] as the *Esherichia coli lac* Z and *gal* K genes which express β-galactosidase [illegible]kinase

respectively (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986; Shepard *et al.*, 1987).

The third method that has been used to test for promoter activity takes advantage of the methodology that is used to construct recombinant poxviruses. A putative promoter sequence is fused to a marker gene whose protein product can be positively selected for. This promoter-marker gene construct is ligated into a viral insertion vector which is then transfected into infected cells. A viral insertion vector is a plasmid which contains the viral gene into which you want the promoter-marker gene fusion to be inserted in the recombinant virus. In the simplest fashion this is accomplished by engineering a convenient restriction enzyme site into the middle of the target gene such that a segment of DNA containing the promoter-marker gene fusion can be inserted into this site. When the resulting plasmid is transfected into infected cells, a small proportion of the viral genomic DNA can recombine with the flanking viral target sequences in the transfected plasmid in such a way that the promoter marker gene is inserted into the viral genome. The virus from this infection is collected and then plated out under selective conditions so that only recombinant viruses which express the marker gene will grow. In other words among randomly cloned viral DNA fragments only those which act as promoters will allow for the growth of recombinant virus (Vassef *et al.*, 1985). This technique has also been used for the deletion analysis of a known promoter (Coupar *et al.*, 1987). It should be considered, however, that the marker gene product or transcript is not being assayed directly when using this method. A variety of promoters of different strengths have not been tested so it is not known how sensitive this method is or how strong the promoter must be in order to give enough of the marker gene product to overcome the selective pressure. The thymidine kinase gene is the only marker gene for which this technique has been used; nevertheless other genes which have been used to positively select for recombinant vaccinia virus, such as the bacterial neomycin resistance (Neo) gene, or the *E. coli* xanthine guanine phosphoribosyl transferase (Ecogpt) gene, should also work (Boyle and Coupar, 1988a; Falkner and Moss, 1988; Franke *et al.*, 1985).

Using these techniques a number of *Orthopoxvirus* promoters for both early and late genes have been identified and are listed in Table III. Early and late promoters behave as expected in that early promoters only transcribed the marker gene at early times and this is not reduced by inhibitors of DNA replication (expression is in fact augmented by such inhibitors), while late promoters only transcribe the marker gene at late times and this is prevented by inhibitors of DNA replication. Extensive point mutational analysis to identify the important promoter sequences has not yet been published, but 5'-deletion analysis of the 7.5-kDa, TK, and CPV 38-kDa early promoters, and the 7.5-kDa, A1, 28-kDa, p11, and D13 late promoters have been published and are listed in Table IV. These show that the 5' boundary of the sequences which are absolutely critical for early and late promoter activity are all contained within about 36 bp or less of the transcriptional initiation site. These are minimal *orthopoxvirus* promoters and sequences farther upstream can significantly augment promoter activity. Assuming that this is a general finding, the sequences upstream of the transcriptional initiation sites for the mapped poxvirus genes are listed in appendix III. Attempts to predict significant sequence conservation upstream of early transcriptional start sites have been made (Mars and Beaud, 1987; Pluciennïczak *et al.*, 1985; Vassef, 1987). However, because early promoters can vary so much in terms of overall expression levels, it is not clear how much sequence conservation one should expect, and no strictly conserved elements have been identified (Lee-Chen *et al.*, 1988). Analysis of these promoter sequences indicates that they are very AT rich and stretches of A residues have been shown to be important in the 7.5-kDa early promoter. When C residues were substituted for A residues at positions -13 and -26, both promoter activity and VETF binding were reduced (Yuen *et al.*, 1987).

Analysis of sequences at the transcriptional initiation sites for late genes indicates that the sequence A/T A/T TAAAT Pu Pu is somewhat conserved (Hänggi *et al.*, 1986; Rosel *et al.*, 1986). The TAAAT motif is often part of the sequence TAAATG where the ATG is the initiating methionine for the ORF. Transcription initiates at a TAAAT sequence

Table III

ORTHOPOXVIRUS PROMOTERS	
Early Genes	**References**
7.5-kDa	Mackett *et al.*, 1982; Cochran et al., 1985a,b.
TK	Mackett *et al.*, 1984; Weir and Moss, 1983, 1987b.
CPV 38-kDa	Ink and Pickup 1989.
147-kDa RNA pol.	Barberyron *et al.*, 1987.
36-kDa (J3)	Barberyron *et al.*, 1987.
pT series	Mars and Beaud, 1987.
pF	Coupar *et al.*, 1987; Panicali *et al.*, 1983; Panicali and Paoletti, 1982; Shepard *et al.*, 1987.
Late Genes	
28-kDa	Weir and Moss, 1984, 1987a.
p11	Bertholet *et al.*, 1985, 1986; Hänggi *et al.*, 1986; Magistris and Stunnenberg, 1988.
7.5-kDa	Cochran *et al.*, 1985b.
A1	Miner *et al.*, 1988.
D13 (L65)	Miner *et al.*, 1988.
CPV 160-kDa (ATI)	Patel *et al.*, 1988.

Table IV

5'-DELETION ANALYSIS	
Late Promoters	
p11	AAAAATATAGT†AGAATTTCATTTTGTTTTTTTCTATGCTATAAATGAATT
28-kDa	TCGGTACGGGTATTCATTTAT†CACAAAAAAAA†CTTCTCTA AATGAGTCTA
7.5-kDa	CAAACCCACC†CGCTTTTTATAGTAA†GTTTTTCACCCATAAATAATAAATAC
D13 proximal	AT†TTCTCTACGGAGTTTATTGTAAGCTT†TTTCCATTTTAAATAGAAAATGAA
D13 distal	AG†GAAAAGGTCATAAATA†CAATAAACTTACTAAGAGATAAAGACGGCGTT
Early Promoters	
CPV 38-kDa	GTTTATAC†AAGATT†GAAAATATATTTCTTTTTATTGAGTG N65 GCCATGGA
7.5-kDa	TTCT†CGTAAAA†GTAGAAAATATATTCTAATTTATTGCACG N43 TCATGAAA
TK	AAGT†CGAATAAAGT†GAACAATAATTAATTCTTTATTGTCACATGAACGGC

A comparison of the sequences of the orthopoxvirus promoters which have been analysed by 5'-deletion analysis. The major transcriptional start sites (*) are indicated, and the sequences between the marks (†), indicates the 5' boundary of the promoter. When these sequences are removed the promoter is no longer active. The mark in the p11 promoter indicates the 5' position of the sequences which still gives promoter activity.

in 21 out of the 31 late promoter listed in appendix III. Deletion analysis (3'), of some TAAAT containing late genes indicates that the transcriptional initiation site is absolutely required for promoter activity (Bertholet *et al.*, 1986; Hänggi *et al.*, 1986; Miner *et al.*, 1989b), while changing TAAAT to TGAAT has been shown to inactivate the p11 and 28-kDa late gene promoters (Wright and Moss, 1987). It has already been mentioned that some late transcripts have 5' poly (A) heads, and it is thought that this non template directed adenylation might occur by RNA polymerase slippage on the multiple A residues during initiation of late gene transcription (Magistris and Stunnenberg, 1988), as has been shown to occur for the T7 RNA polymerase (Martin *et al.*, 1988).

It is not known if the transcriptional initiation sites for the early *orthopoxvirus* promoters are absolutely required. Coupar *et al.*, (1987), have performed 3'-deletion analysis of the pF promoter and show that the native initiation site is not required for promoter activity. However, they tested the deletions by the ability to express the herpes virus TK gene, and to rescue TK$^-$ vaccinia virus. S1-nuclease mapping indicated that the transcriptional initiation site of the deleted promoter had not changed from that of the wild type promoter, relative to the upstream promoter sequences, despite the apparent change in sequence. However, the sequences which were substituted for the wild type initiation site were not presented. Ink and Pickup (1989), have done linker scanning mutations of the cowpox virus 38-kDa early gene, and show that the sequences between -27 and -8 are required for promoter activity while the sequences form -8 to -4 are not required. The transcriptional initiation site itself was apparently not required for promoter activity though this data was not shown. Thus *orthopoxvirus* early and late promoters are contained within about 36 bp of the transcriptional initiation site and at least for the late genes tested, they include the transcriptional initiation site.

Inspection of the putative promoter sequences in appendix III will show that not all late genes use the TAAAT motif, in fact one early gene (#15), uses this sequence and some

late genes do not have this sequence (#10, 11, 12, 13, 15, 18, 20, 22, 29), or do not use it even if they have it (#8). There is no evidence that the two classes of late genes, those with and those without the TAAAT transcriptional initiation site, are of a different temporal class. Vos and Stunnenberg (1988), claim that there is an intermediate class of promoters (ORFs I8 and I3), where transcription starts sometime between when DNA replication begins and late transcription begins. However, they only see this pattern of transcription on transfected plasmids in the presence of hydroxyurea, or on the viral genome when the infection is done in the presence of hydroxyurea which is then replaced with cycloheximide. No evidence is presented to suggest that this temporal pattern of expression occurs in the absence of drug. It is known that some late genes are artifactually transcribed in the presence of cycloheximide, during which only early genes are normally transcribed (Lee-Chen and Niles, 1988b). It must be noted that though we broadly classify *orthopoxvirus* genes as either early or late, this does not mean that all the early genes simultaneously start and stop transcription at precisely the same moment or that all the late genes start transcription simultaneously. Using S1 nuclease or primer extension analysis to measure the steady state levels of a particular transcript Weinrich and Hruby (1987), found that the temporal regulation of a late gene cluster at the HindIII A/D junction is complex. Although all the genes are of the late class, transcription can start at different times for different genes. Similarly Tamin *et al.*, (1988), found that though both M1 and N2 are early genes, N2 transcripts are seen from 0.5-1hr post-infection while M1 transcripts are seen from 0.5-12hrs post-infection.

There is a class of genes which are transcribed at both early and late times called the expressed genes. While some have separate and distinct early and late transcriptional start sites (7.5-kDa, D2, I3, and H6 genes), others have a late start site and a separate early/late start site (CPV 132 and H6). These early/late promoters have not been well characterized but high steady state levels of RNA using the same transcriptional start site are seen at both early and late times so it has been assumed that they are being transcribed. It is not clear if

these genes are in fact transcribed at late times or if the transcripts made at early times are very stable and persist into late times. Using actinomycin D, Sebring and Salzman (1967), showed that bulk early RNA has a half life of about 120 minutes, which is approximately when DNA replication would normally start, while the late RNA half life is about 13 minutes. Oda and Joklik (1967), have also found that in the presence of actinomycin D early RNA is more stable than late RNA, but only in HeLa cells and not in L cells where their stabilities are similar. It is not known if there is a specific signal which allows early transcripts to be degraded when late gene expression starts or why the steady state levels of some transcripts remain elevated well into late times (ex. M1; Tamin *et al.*, 1988). If a 3' to 5' exonuclease is responsible for the short half-life of late viral RNA then the extra length due to a lack of termination of transcription could extend the time for which late mRNA remains functional.

F. GENETICS

A large number of mutant *orthopoxviruses* have been isolated, and with the use of marker rescue to physically map the mutations onto the viral genome, these isolates are proving to be valuable in helping to determine the functions of some of the viral genes. While some of these mutant poxviruses have been isolated as variants of natural populations which display a host-range or drug resistant phenotype, most have been made by mutagenizing virus *in vitro* and then selecting for individual plaques with a drug resistant or temperature sensitive phenotype (reviewed by Condit and Niles, 1989). Some of these mutants have provided some insight into how *orthopoxvirus* gene expression is regulated.

One group of mutants are those which have temperature sensitive mutations in the subunits of the RNA polymerase complex. Temperature sensitive mutations in each of the two largest subunits (147-kDa and 135-kDa), and the 22-kDa subunit have been isolated and described as having a defective late phenotype (Ensinger, 1987; Hooda-Dhingra *et al.*,

1989; Thompson *et al.*, 1989a). In these mutants late gene expression is reduced and/or delayed at the nonpermissive temperature. The degree to which late gene expression is reduced is variable and depends on the individual mutant. All the mutations in the RNA polymerase subunits appear to be assembly mutants such that if the virus is grown at the nonpermissive temperature, new RNA polymerase is not assembled correctly. If the virus is grown at the permissive temperature then new functional RNA polymerase is assembled and it remains functional even if the temperature is shifted up to the nonpermissive temperature. When grown at the nonpermissive temperature late viral RNA is not made so late protein synthesis does not occur, progeny virus is not assembled, and early gene transcription is not suppressed. DNA replication occurs but the resolution of long concatomeric progeny genomes into monomer sizes does not occur (DeLange, 1989; Hooda-Dhingra *et al.*, 1989; Merchlinsky and Moss, 1989b). This indicates that late genes are transcribed by newly synthesized RNA polymerase, early genes are transcribed by the packaged RNA polymerase, early transcription is not turned off by DNA replication *per se*, and resolution of concatomeric progeny viral genomes requires at least one late gene function.

Another complementation group with a defective late phenotype, maps to the NPH-I or ATPase-I gene in the HindIII D fragment (Kunzi and Traktman, 1989). There are two mutants in this group (ts36 and ts50), both of which show normal early gene transcription and DNA replication. Host cell protein synthesis is not inhibited, early viral gene transcription is not shutoff at late times, and late viral gene expression is severely reduced. The role of this ATPase in late gene expression is unknown, but it does cosediment with the virion RNA polymerase on glycerol gradients (Broyles and Moss, 1987b).

One temperature sensitive mutant (ts22), has an abortive late phenotype where at about 8-10 hours after infection at the nonpermissive temperature, the viral RNA and rRNA is degraded into discrete fragments (Pacha and Condit, 1985). This same phenotype is seen when wild type virus infects cells in the presence of the drug IBT. It is not yet known

if IBT affects the same gene as ts22, but both seem to prevent the virus from inhibiting the induction of an increase in the levels of 2-5A [(2'-5')pppA(pA)n], within the cell (Cohrs *et al.*, 1989). 2-5A activates a latent cellular RNase which leads to the breakdown of RNA and is the same pathway used by interferon to create an antiviral state within the cell (Joklik, 1985). It is not known if interferon is involved in this response.

With the ability to mutagenize viral genes *in vitro* and then introduce single site mutations back into the genome it is clear that poxvirus genetics will be very valuable in helping to elucidate the functions of some of the genes involved in the regulation of expression of the poxvirus genome.

G. THE EARLY/LATE TRANSCRIPTION SWITCH

This review raises important questions about how the early to late gene transcription switch is regulated. Any model for this regulation must accommodate the fact that somehow the late transcriptional machinery is activated by DNA replication while the early transcriptional machinery is inactivated by late gene expression. One model would be that late genes are transcribed only from progeny viral genomes and not from the infecting parental DNA. However, there is no evidence that these two genomic DNAs are physically different. It may be possible that the replicative machinery itself activates the expression of one or more genes which would in turn activate the newly assembled late transcriptional machinery. Such an intermediate class of genes has not yet been been detected but candidate genes would be those that could make regulatory modifications of the late and/or early transcriptional machinery such as phosphatases or kinases. A ts-mutant in this gene would also be predicted to have a defective-late phenotype. All of the components of the early transcriptional machinery including the RNA polymerase and VETF are within the cell at late times since they are packaged into the progeny virions. Regardless of how early and late transcription is controlled, this regulation is lost when whole cell extracts are made from infected cells at late times. In these extracts both early and late viral templates are

specifically transcribed *in vitro* (Wright and Moss, 1989). It has not been reported whether early genes are transcribed *in vitro* if these late extracts are made in the presence of phosphatase inhibitors. If they are not transcribed then phosphorylation may regulate the activity of the early transcriptional machinery such that at late times it would be inactive but could be reactivated during maturation of the progeny virus particles.

The protein components of the early and late transcriptional machinery may be different. The subunit composition of the virion associated RNA polymerase may be different from the late RNA polymerase. When these two ssDNA dependent RNA polymerase activities are purified and compared by polyacrylamide gel electrophoresis, the RNA polymerase from the infected cell contains a 77-kDa subunit that is not seen in the virion associated activity. It has also been found by S1-nuclease analysis that the second largest RNA polymerase subunit in cowpoxvirus (RPO-132), has two transcriptional initiation sites (Patel and Pickup, 1989). The predominant start site is seen at both early and late times and occurs within the ORF very near the N-terminus. A minor late start site is detected just 5' to the ORF and these late transcripts can potentially encode the entire gene. Assuming that translation of the transcripts originating at the early/late start site occurs at the next available AUG codon then this gene product would be 8 amino acids shorter than the full length product. This N-terminal sequence could be important in regulating early/late transcriptional activity and/or packaging of the RNA polymerase complex. No N-terminal protein sequence of the RPO-132 is available. Other differences between the early and late transcriptional systems are that early transcription requires ATP with a hydrolyzable β-γ bond, while late transcription does not have this requirement *in vitro*; however, a ts-mutation in a viral ATPase (NPH-I) severely reduces late gene expression. Further differences between the early and late transcriptional systems will undoubtedly be found as both of the *in vitro* transcription systems are compared, and as ts-mutants affecting virus gene expression are found and characterized.

H. SHOPE FIBROMA VIRUS

Vaccinia is the prototypic poxvirus; however, other poxviruses are very different from this *orthopoxvirus* and it is not clear how well conserved the molecular features are between the different members of the poxvirus family. One group of poxviruses whose biology is distinct from the *orthopoxviruses* are the so called tumorigenic poxviruses. This is not a genera of poxviruses but comprises those members which induce in their natural hosts, a benign proliferation of tissue at the site of infection. Members of this group include the the *leporipoxvirus* SFV, the *yatapoxvirus* yaba monkey tumor virus, and an unclassified human poxvirus which causes *molluscum contagiosum*. SFV is the best characterized of these viruses and is the subject of the work of this thesis.

SFV was first isolated from a subcutaneous tumor-like growth on a wild cottontail rabbit by Richard E. Shope, who demonstrated that this lesion could be transmitted to a healthy rabbit by an extract of the tumor (Shope, 1932a, b). Although SFV is often referred to as a tumorigenic poxvirus, this is a bit of a misnomer. Unlike other tumor viruses, SFV has a very limited interaction with the nucleus of the infected cell, and most evidence indicates that SFV infected cells in the "tumor" or in tissue culture do not proliferate (Scherrer, 1968; Kato, 1966). Histologically the proliferating cells in the tumor-like growth appear to be uninfected fibroblasts which make up the surrounding epidermal connective tissue so "...the tumor can better be considered a local hyperplastic connective tissue reaction to the virus than as a true neoplastic process....." (Shope, 1932b). A detailed description of the biology of SFV will not be given here, instead an outline of the basic growth pattern *in vivo* and in tissue culture shall be given to facilitate the comparison with what we know about vaccinia virus. For a review of the *lepori-* and *orthopoxvirus* infections in rabbits see McFadden (1988), and references contained therein.

When SFV is innoculated subcutaneously into an adult rabbit the following pattern is seen (Kato *et al.*, 1963a; Shope, 1932a): By day 3-5, the tissue at the site of innoculation thickens and over the next 5-7 days it continues to thicken and the swollen

region becomes well circumscribed so that it takes on a tumor-like appearance. On days 8-11, the tumor is at its maximum size, roughly 30-50mm in diameter, and it contains the maximum amount of virus. By day 10-11, the tumor gets a reddish-purple spot in the center which spreads as the tumor becomes necrotic and by day 18-25 it scabs over and will eventually fall off. No secondary lesions are seen except in newborn or immunocompromized adult rabbits (Allison, 1966; Allison *et al.*, 1966; Yuill *et al.*, 1964). Tumor regression is associated with both humoral and cell mediated immune responses to the virally infected cells (Sell and Scott, 1981).

In tissue culture systems SFV grows well on primate kidney cells and on rabbit cells. At low multiplicities the infected cells aggregate together and pile up into foci so the virus can be titered and this is expressed in focus forming units (ffu). Like vaccinia, SFV is very cell associated and titrations can be done without an agar overlay. The overall steps in the replicative cycle of SFV, are probably the same as for vaccinia, but the virus grows more slowly and is much less cytopathic (Bohn, 1980). Ikuta *et al.*, (1978), pulsed RK-13 cells with radiolabeled amino acids at various times after infection with SFV, vaccinia, and cowpox, and then resolved the labeled proteins by polyacrylamide gel electrophoresis. It is clear that for these viruses distinct sets of proteins are made at early and late times during the infection but the kinetics for the early to late switch is different for each virus. For the *orthopoxviruses* vaccinia and cowpox, late gene products are first seen at 4-5 hours and 5-6 hours after infection respectively. The background of host cell protein synthesis is quite high at early times, decreases at late times and is gone by 11-12 hours post-infection. For SFV, late proteins are not synthesized until 8-9 hours after infection and though host cell protein synthesis is diminished at late times, it is still present 23-24 hours post-infection. So the switch from early to late gene expression occurs much later in SFV than in vaccinia and host cell protein synthesis is not as inhibited.

By measuring the incorporation of ^{3}H-thymidine into SFV infected cells Ewton and Hodes (1967), and Scherrer (1968), demonstrated that viral DNA synthesis begins at 4-5

hours post-infection, peaks at 8-9 hours post-infection and then declines to background levels by 14-15 hours post-infection. DeLange and McFadden (1986), have found that transfected plasmid DNAs are replicated in SFV infected cells and that nascent plasmid DNA is first seen at 8 hours post-infection and it continues to accumulate up to 24 hours post-infection, with a small amount of accumulation between 24 and 48 hours. Thus this situation may be similar to vaccinia where viral DNA is seen to accumulate even after ^{3}H-thymidine incorporation is reduced (Traktman, 1989). By measuring the incorporation of ^{3}H-thymidine Scherrer (1968), also noted that by four hours post-infection, host cell DNA replication is reduced by 50% while Ewton and Hodes (1967), show that incorporation of ^{14}C-uridine into rRNA is reduced more than 40% by 8.5-11 hours post-infection. Thus, like vaccinia, SFV reduces host cell gene expression and DNA replication, but more slowly and less severely. Vaccinia forms plaques on susceptible cells in tissue culture while SFV forms foci and these piled up cells stain as live cells. SFV is not only less cytopathic and grows more slowly, but the yield of virus is about ten times less than vaccinia virus. The burst size for vaccinia virus is roughly 100 pfu/cell (Joklik, 1966), while Israeli and Sachs (1964), found that in ten experiments the yield of SFV ranged from 2-36 ffu/cell, while Israeli (1966) found in three experiments that the average yield was ten ffu/cell. Like vaccinia, a number of induced enzymatic activities are seen in SFV-infected cells, including DNA polymerase (Chang and Hodes, 1967, 1968b), RNA polymerase (Chang and Hodes 1968a), TK (Barbanti-Brodana *et al.*, 1968), and topoisomerase I (Upton *et al.*, submitted). These have not been extensively characterized but it looks as though SFV may contain the same complement of enzymes as does vaccinia.

One of the most interesting reports on SFV in the early literature are those that describe isolates of the virus which have lost their tumor inducing capacity (Andrews, 1936; Andrews and Shope, 1936; Shope, 1936). These isolates still grow in rabbits but instead of inducing a tumor-like growth they induce a necrotic inflamation at the site of innoculation. Unfortunately samples of these isolates are not available. However, many

orthopoxvirus isolates which show reduced virulence or altered growth patterns, such as the host range mutants, have been reported. When these isolates are examined closely it has been found that most of them are associated with deletions or rearrangements within the terminal portion of the viral genomes. This has been found for vaccinia (Dallo and Esteban, 1987; Drillien *et al.*, 1981; Gillard *et al.*, 1985, 1986; Kotwol *et al.*, 1989; Kotwol and Moss, 1988; Moss *et al.*, 1981; Paez *et al.*, 1985; Paez and Esteban, 1988; Panicali *et al.*, 1981; Perkus *et al.*, 1986), rabbitpox virus (Lake and Cooper, 1980; Moyer *et al.*, 1980b), and monkeypox virus (Esposito *et al.*, 1981). An examination of the restriction enzyme profiles of the genomes of many *orthopoxviruses* shows that the central region is quite conserved, while the terminal portion of the genomes are variable (Esposito and Knight, 1985). Thus the center of the poxvirus genomes probably contains genes which are essential for virus growth, while the terminal regions contain nonessential genes, some of which could influence host-range or virus virulence. So it is possible that naturally occuring isolates of SFV which show differences in tumor production could be due to alterations in the terminal portions of the genome. The Boerlage strain of SFV, which is no more virulent than other strains, but is reported to be more invasive and to induce larger, though histologically identical, tumors at the site of innoculation (Febvre, 1962; Strayer *et al.*, 1984), does have a small deletion within the terminal inverted repeat which results in the truncation of the C-terminal portion of the T6 ORF (Upton *et al.*, 1988). It is not known if this lesion is responsible for the altered growth phenotype. Recently a new *leporipoxvirus* called malignant rabbit fibroma virus (MRV) was isolated from a stock of SFV that was passaged in rabbits (Strayer *et al.*, 1983b). This virus has been shown to be a recombinant between SFV and myxoma (Block *et al.*, 1985, Upton *et al.*, 1988). Sequences from the TIR of SFV (ORFs T5 fusion, T6-T8), have replaced sequences from the left end of the myxoma genome, and a segment spanning the junction between the TIR and the unique internal sequences at the left end of the SFV genome (ORFs T5 fusion, T6-SFGF, T11 fusion), have replaced sequences at the right end of the

myxoma genome. The exact nature of the myxoma sequences which were replaced is not yet fully deduced, but they do include the myxoma homologue of an epidermal growth factor-like gene (Upton *et al.*, 1987b). MRV and myxoma are reported to have slightly different tumorigenic properties *in vivo*, though the nature of these differences have not been thoroughly investigated (Strayer *et al.*, 1983a; Strayer and Sell, 1983). This work indicates that the ends of the *leporipoxvirus* genomes are somewhat plastic, and like those of the *orthopoxviruses*, they may contain nonessential genes that affect virus growth.

I. THESIS OBJECTIVE

Much of what is known about the molecular biology of vaccinia virus gene expression has been determined in the last decade, and at the time that this work was started virtually nothing was known about the molecular biology of the other genera of poxviruses. The purpose of this work was to compare the transcriptional systems of SFV and vaccinia virus. The vaccinia TIRs were one of the few transcriptionally well characterized regions in poxviruses, so the TIRs of SFV were transcriptionally mapped in order to compare the two systems and to characterize a region which may be important to the unique biology of SFV. In order to test how related the transcriptional systems of these two genera were, an early promoter from the TIR of SFV was chosen for detailed analysis.

CHAPTER II
MATERIALS AND METHODS

A. CELLS and VIRUSES

1. *Cell Culture Medium*

For all the tissue culture in this thesis Dulbecco's modified Eagle's medium (DME) (Flow laboratories or Gibco) was used. This was supplemented with sodium bicarbonate (3.7g/ml), penicillin G potassium (Ayerst; 250 I. U./ml), Streptomycin sulfate (Boehringer Mannheim; 200mg/l), and either 5% calf or 5% fetal calf serum. Only adherent cell lines were used in this work and they were grown in either disposable plastic dishes and flasks (Corning, Costar, or Nunc), or for large cultures, 1330 cm^2 glass roller bottles (Bellco) were used. Open dishes or flasks were incubated at 37°C, in 95% air, 5% CO_2 and 95% humidity.

2. *Cell Lines*

The monkey kidney cell line BGMK was obtained from S. Dales (Department of Microbiology and Immunology, University of Western Ontario), and was used for the growth and titration of poxviruses. The human thymidine kinase negative (TK^-) osteosarcoma cell line H143 (Rhim *et al.*, 1975; Campione-Piccardo *et al.*, 1979), was obtained from Dennis Panicali (Applied Biotechnology Inc. Cambridge MA), and used to select for, and plaque purify recombinant TK^- vaccinia virus. The monkey TK^- cell line T22 was obtained from J. Smiley (McMaster University, Hamilton, Ont.), and was used to select for SFV TK^- virus. SFV does not form foci on these cells but they were used to passage virus in the presence of bromodeoxyuridine (BUdR), before screening for recombinant (blue) foci on BGMK cells.

3. *Viruses*

The two poxviruses used in this thesis are vaccinia virus (strain WR), and Shope fibroma virus (strain Kasza), both of which were obtained from the American Type Culture

Collection (ATCC). The handling and production of stocks of SFV is essentially the same as for vaccinia only the virus grows more slowly, to a much lower titer, and it is more restricted in its host range. The basic steps involved in the production of vaccinia virus have been outlined by Mackett *et al.*, (1985).

a) The routine production of virus stocks.

Semiconfluent BGMK cells were infected at about a multiplicity of one by replacing the medium with a small volume of virus innoculum (6-8mls/roller bottle, or 2-3mls/150cm^2 flask), and then placing the roller bottles back on their roller or the flasks on a rocking platform in a 37°C room. After adsorption (1 hour for vaccinia, 2-3 hours for SFV), medium was added to the containers and incubation continued for about 48 hours. If many of the infected cells had detached and were floating in the medium they were collected by centrifugation. The infected cells were removed from the container using sterile 1X SSC (0.1M sodium chloride, 0.015M sodium citrate) and collected by centrifugation (1000gs, 5minutes). The pellet of infected cells was resuspended (10ml/10^8cells) in cold RSB (reticulocyte swelling buffer; 10mM Tris-HCl pH 8.0, 2mM $MgCl_2$), and incubated on ice for 15 minutes. The cells were then broken open by dounce homogenization until most of the cells were lysed (checked by microscopy), and this solution was stored at -70°C to be used as a crude innoculum. When higher titered virus stocks were required the virus was partially purified by pelleting the cellular debris (1000gs, 5 minutes, 4°C). The supernatant was collected into a sterile container, the pellet was resuspended with a second volume of cold RSB and dounce homogenized. The debris was pelleted and the cycle repeated with a third volume of cold RSB. The supernatants were pooled and the virus was pelleted (25000gs, 60 minutes, 4°C). The pellet was resuspended in a small volume of RSB (about 2mls/10^8cells), and sonicated twice for 30 seconds each time (Bransonic 1510 sonicator using a 50ml cup-tip probe filled with ice water, at full power), in order to break up the clumps of virus and cellular debris. The virus was then partially purified by pelleting through a cushion of sucrose. For small

volumes, 4.5mls of virus in RSB was placed into a sterile 10ml Oak Ridge type polycarbonate tube (Nalgene), and then underlayed with an equal volume of sucrose (36% sucrose, 50 mM Tris-HCl pH 8). This was centrifuged in an SW41 rotor at 25000gs, 60 minutes, 4°C). The supernatant was discarded and the virus pellet resuspended in a small volume of RSB or medium.

b) Titration of virus stocks.

Vaccinia and SFV were both titered on BGMK cells in the same fashion except that vaccinia forms plaques after 24-48 hours while SFV forms foci after 4-6 days. For both viruses 1:10 serial dilutions in DME were prepared (0.05mls into 0.5mls) and 0.2mls was used to innoculate semiconfluent BGMK cells in Costar six-well dishes. After adsorption (1hour for vaccinia, 2-3 hours for SFV), 2.5 mls of medium was added to each well and the dishes incubated until plaques or foci developed. The cells were then fixed by removing the medium, washing the monolayer in PBS (137mM NaCl, 2.7mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2), adding 2mls of neutral buffered formalin (NBF) to each well (4% formaldehyde, 29.2 mM Na_2HPO_4, 30.5 mM NaH_2PO_4, 145mM NaCl, pH adjusted to 7 or higher with solid sodium carbonate) and incubating for 10 minutes at room temperature. The NBF was removed and the cells stained with 0.1% crystal violet (1ml/well) for 5 minutes at room temperature. The monolayers were washed with running tap water, air dried, and then the plaques or foci were counted.

c) Plaque purification of recombinant viruses.

The virus stock was diluted sufficiently in order to give individual plaques on a monolayer of cells in 100mm tissue culture dishes. For vaccinia virus, plaques form very quickly so the cells were overlayed with agar (1.3% low melting point agarose [BRL], 1X DME, 5% calf serum) right after adsorption; however, for SFV the foci take many days to develop so the agar overlay was not placed on the cells until foci were just begining to form. The desired plaques or foci were picked with a sterile Pasteur pipet, and the agar plug with the attached infected cells was transfered to 0.5mls DME. This was freeze-

thawed three times, sonicated, and used as innoculum to grow up small stocks of the picked virus. Selective agents or indicators were included in the agar overlay as required.

B. RNA PURIFICATION

RNA was purified from uninfected and infected BGMK cells for a number of experiments in this thesis. I found the most reproducible method involved homogenizing the cells in a guanidine thiocyanate solution and pelleting RNA through a cushion of $CsCl_2$ (Chirgwin *et al.*, 1979; Glisin *et al.*, 1974). This was used for both large and small scale preparations. With the exception of the guanidine thiocyanate solution, glyoxal solution, organic solvents, ethanol, and solutions containing Tris, all solutions used for RNA work were treated with diethylpyrocarbonate (DEP). The solutions were made 0.1% in DEP, mixed thoroughly, left to stand overnight, and then autoclaved.

1. *Large Scale RNA Preparations Using One or More Roller Bottles of Cells.*

The cells were removed with 1X SSC and pelleted as described in the preparation of viruses. The cell pellet was dissolved in 6M guanidine thiocyanate, 5mM sodium citrate pH7, 0.5% sodium *N*-lauroylsarcosine and 0.1M 2-mercaptoethanol (10-20mls/10^9 cells), and the mixture homogenized using a Servall tissue homogenizer for three 30 second bursts. $CsCl_2$ was added to 1g/2.5mls, and the solution was layered onto 2.5mls of 5.7M CsCl in a tube for a Beckman SW 50.1 swinging bucket rotor. This was centrifuged for 12-16 hours, at 20°C and 36000 rpm. The supernatant was decanted, the walls of the tubes dried with a Kimwipe, and the RNA pellet dissolved in 0.1% sodium dodecyl sulfate (SDS). This solution was extracted with phenol:choloroform:isoamyl alcohol (49:49:2), followed by a chloroform and ethanol precipitated by adding one tenth volume of 3M NaOAc pH 5.5 plus two volumes of ethanol. The RNA was stored at -20°C as an ethanol precipitate (stable for years), and when needed it was pelleted and resuspended in either water or 0.1% SDS.

2. *Small Scale Preparations of RNA from Cells in 100mm Plastic Tissue Culture Dishes*

The medium was removed from a dish, the monolayer washed with PBS, and 1-2 mls of guanidine thiocyanate solution was added to the dish. The cells were scraped into the solution using a rubber policeman and the mixture would become viscous as the cells dissolved and the DNA was released into solution. When a number of dishes were being used the guanidine thiocyanate solution from one dish was scraped directly into the next dish using the rubber policeman. In this way 5 dishes of cells could be readily processed with just 2 mls of solution. This mixture was homogenized by passage through a 26 guage needle 3-5X, and the RNA purified by pelleting through $CsCl_2$ as described above. Late RNA, early RNA, and immediate-early RNA were isolated from cells infected in the absence of inhibitors, or in the presence of arabinosyl cytosine (Ara C, 40 μg/ml), or in the presence of cycloheximide (100 μg/ml), respectively.

3. *Isolation of Poly A^+ RNA*

Poly A^+ RNA was isolated by chromatography on oligo-dT cellulose (Collaborative Research) as outlined in Maniatis *et al.*, (1982). One volume of 2X loading buffer was added to total cellular RNA (1X loading buffer= 20mM Tris-HCl pH7.6, 0.5M LiCl, 1mM EDTA, and 0.1% SDS). This was heated to 65°C, cooled to room temperature and passed through a 1 ml column. The eluate was collected and reapplied five times heating and cooling between each application. The column was subsequently washed with five column volumes of wash buffer (20mM Tris-HCl pH7.6, 0.1M LiCl, 1mM EDTA, and 0.1% SDS), and the bound RNA was eluted with 60°C elution buffer (20mM Tris-HCl pH7.6, 1mM EDTA, 0.05% SDS). Fractions containing the eluted RNA were pooled and ethanol precipitated.

4. *Quantitation of RNA*

During this work, the measurement of the amount of RNA in a sample was often required. The need for ribonuclease free cuvettes made adsorbance measurements inconvenient so a fluorimetric assay described by Morgan *et al.*, (1979), was used. This

assay measures the increase in the fluorescence of ethidium bromide when it intercalates into the double stranded regions of DNA or RNA. A fluorimeter was set up under standard conditions such that 500ng of calf thymus DNA in 1.2mls of pH8 buffer (0.5µg/ml ethidium bromide, 5mM Tris pH8.2, 0.5mM EDTA), gives a reading of 70. A standard fluorescence curve for different RNA concentrations was prepared by making serial dilutions of a stock of yeast RNA that was 10.5 mg/ml as determined by its adsorbance at 260nm (assuming 1 A260=40 µg/ml). The fluorescence of each of these dilutions was measured as seen in table V, and plotted onto a graph (Fig. 2). This graph was plotted using Cricket Graph (Cricket Software) on a Macintosh Plus (Apple Computer Inc.) and the slope of a straight line interpolated through the points was calculated to be 49. I had originally plotted this data by hand and my value for the slope was 52.45, so all the RNA concentrations in this thesis were done using this later value. One caveate of using this method that must be kept in mind is that the standard curve was constructed using total yeast RNA which is primarily ribosomal RNA and contains a lot of secondary structure. Measuring total cellular RNA will, therefore, be quite accurate; however, measuring poly A^+ RNA in which most of the rRNA has been removed will not be as accurate.

C. NORTHERN HYBRIDIZATIONS

1. *Glyoxal Agarose Gel Electrophoresis*

Immediate-early, early, late, and uninfected cellular poly A^+ RNA was denatured with dimethylsulphoxide (DMSO), reacted with glyoxal and electrophoresed through a 1.0% agarose gel. The RNA (up to 7µl), was added to 15µl of a glyoxal solution (50% DMSO, 1-1.4M deionized glyoxal, 10mM Na-phosphate pH6.8), heated to 50°C for one hour, and then loaded onto an agarose gel made in 10mM Na-phosphate pH6.8. The glyoxal solution used in this procedure had been extensively deionized with a mixed-bed ion-exchange resin (Bio Rad AG 501-XB) as outlined by Maniatis *et al.*, (1982); McMaster and Carmichael, (1977); and Thomas, (1983). No loading dye was used except in lanes

Table V

RNA	.105	.210	.315	.420	.525	1.05	1.58	2.10	2.62	3.15
F	7	11.5	17	22	29	56.5	75	106	131	155

The top row indicates the number of µg of yeast RNA that was added to each tube. The bottom row indicates the corresponding reading by the Fluorimeter. The fluorimeter was set up under standard conditions such that 500ng of calf thymus DNA added to a tube with 1.2 mls of pH8 buffer gives a reading of 70.

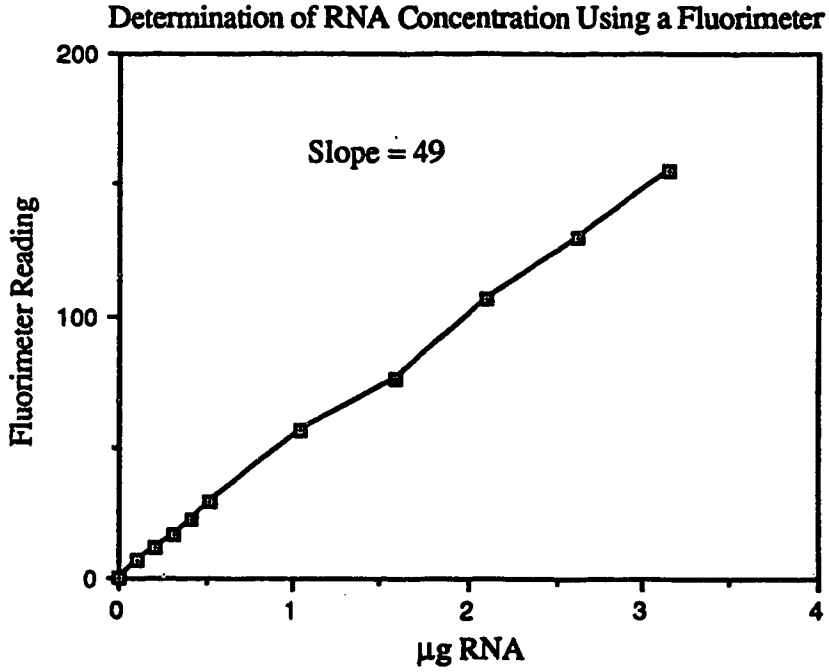


Fig. 2. A graph of the fluorescence due to increasing concentrations of yeast RNA, based on the data found in table V. The slope of a straight line plotted through the points is 49 so that 1µg of RNA gives a reading of 49 fluorescence units.

which did not contain any RNA. Ribosomal RNA from uninfected BGMK cells was run on the outside lanes of the gel, sliced off and stained with ethidium bromide in order to serve as molecular weight markers. The RNA was then blotted to a Pall Biodyne A nylon membrane in 20X SSC, dried and baked in a 80°C oven, according to the manufacturer's recommendations.

2. *Hybridization of Northern Blots with Cloned Viral DNA Probes.*

Cloning of the entire SFV TIR into M13 vectors for sequencing has been presented elsewhere (Upton and McFadden, 1986a and 1986c; Upton *et al.*, 1987). Strand specific probes for both orientations were prepared as described by Hu and Messing (1982). Briefly, 3µl of M13 template DNA (100ng) was mixed with 9.5µl H_2O, 0.5µl of probe primer (1.7ng) and 1µl of 10X sequencing buffer (70mMTris pH7.5, 70mM $MgCl_2$, 500mM NaCl). The primer was annealed to the template DNA by placing the mixture in a 50µl Dade capillary pipet which was then flame sealed at both ends, placed into a 500ml beaker of boiling water, and the beaker with the boiling water and capillary pipet were allowed to cool to room temperature. The DNA solution was transfered to a 1.5ml microtube to which was added: 1µl of dNTP mix (dGTP, dCTP, dTTP, 1mM each), 1µl α-^{32}P dATP (10µCi, 3000 Ci/mmole), and 0.1µl of Klenow DNA polymerase (0.5U). This was incubated at 15°C for 90 minutes and the reaction was stopped by adding 2µl of 0.5M EDTA. The entire reaction was used immediately to probe the blots. The blots were prehybridized and hybridized in 50% formamide, 5X SE (0.9M NaCl and 5mM EDTA), 5x Denhardt's solution, [100X Denhardt's: 2% (w/v) ficoll (400,000 m. wt.), 2% (w/v) polyvinyl-pyrrolidone (360,000 m. wt.), 2% (w/v) bovine serum albumin], 25mM sodium phosphate pH6.8, 250 µg/ml single stranded calf thymus DNA, and 0.3% SDS, at 42°C. The blots were washed in 0.1X SSC and 0.1% SDS at 50°C, then exposed to Kodak XAR-5 film with an intensifying screen at -70°C.

D. S1-NUCLEASE MAPPING OF TRANSCRIPTIONAL START SITES

1. *Spin Dialysis*

For many procedures where a DNA sample was carried through a sequence of manipulations which require different buffers, the sample buffer was changed by spin-dialysis. The sample was first deproteinized by extraction with phenol:chloroform (1:1), and then chloroform. A dialysis system was set up by filling a one-liter beaker containing a small magnetic stir bar with 5mM Tris pH8, and then carefully floating onto the surface of the solution a millipore filter (VMWP 02500), so that the shiny side is facing upwards. On to the surface of this filter was placed the DNA sample, and the magnetic stirrer turned on very slowly so as to gently move the filter across the surface of the solution and not sink the filter. After 20-30 minutes the stirer was turned off and the DNA sample carefully removed. The molecular weight cut off for the filters is not known but nucleotides and restriction enzyme linkers will pass through them.

2. *Preparation of DNA*

Cloned SFV DNA (10µg), was digested with an appropriate restriction enzyme that leaves a 5'-overhang, phenol:chloroform (1:1), and chloroform extracted, and dialysed. The 5'-ends were dephosphorylated by adding one tenth volume of 10X calf intestinal phosphatase (CIP) buffer (0.5M Tris pH9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine), 1µl of CIP (Boehringer Mannheim, 1U/µl) and incubating at 37°C for 1hour. A second 1µl of CIP was added and incubation continued for 1 hour at 45°C. The sample was phenol:chloroform (1:1), and chloroform extracted, and dialysed. The 5'-ends were end-labelled with [γ-^{32}P]ATP by adding one tenth volume of 10X polynucleotide kinase buffer (0.5M Tris pH7.5, 0.1M $MgCl_2$, 50mM DTT, 1mM EDTA, 1mM spermidine), 2µl [γ-^{32}P]ATP (DuPont NEN products, 7000Ci/mmole, 5mCi/0.030mls), and 1µl of T4 polynucleotide kinase (Pharmacia, 10U/µl). This was incubated at 37°C for one hour. This reaction was stopped by adding 2µl of 0.5M EDTA and extracting with phenol:chloroform (1:1), and chloroform. The unincorporated label was separated from

the DNA by passage through a sepharose 6B column (Pharmacia) made in a Pasteur pipet (column buffer: 0.1M NaCl, 10mM Tris pH8, 1mM EDTA). The labeled DNA elutes in the void volume and can be monitored with a hand held Geiger counter. Fractions containing this DNA were pooled and precipitated. The end-labeled DNA was digested with a second restriction enzyme and the desired end-labeled fragment was purified by electrophoresis in low melting point agarose (Langridge *et al.*, 1980).

3. *S1-Mapping*

The Berk and Sharp (1977) S1 mapping technique as modified by Weaver and Weissmann (1979), was carried out as follows. Equal amounts of labeled DNA were ethanol precipitated with 50 µg of either tRNA, immediate-early or late total cellular RNA. The pellet was dissolved in 30 µl of 80% formamide, 0.4M sodium chloride, 0.04M PIPES pH6.4 (Piperazine-N,N'-bis [2-ethane Sulfonic Acid]), and 1mM EDTA. The solution was heated to 85°C for 10 minutes and then hybridization was allowed to occur at 51°C overnight (12-16 hrs). The reaction was stopped by adding 300 µl of ice cold S1 buffer (0.25M NaCl, 30mM KOAc pH4.5, 1mM $ZnSO_4$, and 5% glycerol) containing 300-400 units of S1-nuclease (Miles) and then incubated at 37°C for one hour. The solution was extracted with phenol:chloroform (1:1), chloroform and the S1-resistant material was ethanol precipitated. The material was resuspended in 10mM Tris pH8 and 1mM EDTA and electrophoresed on a nondenaturing 1% agarose or 5% polyacrylamide gel. The gels were dried and exposed to Kodak XAR 5 film with an intensifying screen at -70°C.

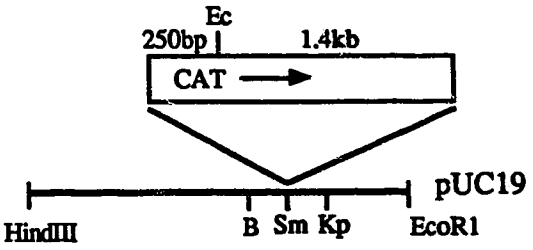
E. PRIMER EXTENSION ANALYSIS

Two seventeen nucleotide antisense oligonucleotide primers dCAGATGCCTTTCGTTGC and dCGGTGGTATATCCAGTG, for the T1 and CAT ORFs, respectively, were purchased from the Regional DNA Synthesis Laboratory at the University of Calgary, and a portion was diluted to a final concentration of 100µg/ml. The

T1 oligo maps between 48 and 65 nucleotides downstream from the initiating AUG of the T1 ORF (Upton *et al.*, 1987), while the CAT oligo maps between 15 and 32 nucleotides downstream from the initiating AUG of the CAT ORF (Gorman, 1985). These were 5'-end-labeled by mixing 1µl of oligo (100ng), 1µl 10X PNK buffer, 0.5-1.0µl [γ-^{32}P]ATP, 7µl H_2O, and 1µl polynucleotide kinase, then incubating at 37°C for 1 hour. The reaction was stopped by heating to 65°C for 10 minutes, diluted to 40µl with H_2O (2.5 ng/µl final concentration), and stored at -20°C. The end-labeled oligo (1.25-5.0ng) was ethanol precipitated with total RNA from virus infected BGMK cells. The nucleic acid was resuspended in 80% formamide, 0.4 M NaCl, and 10mM Pipes pH6.4 to a final concentration of 1mg/ml of RNA. A drop of paraffin oil was added, the mixture was heated at 65°C for 15min and hybridized at 40-41°C overnight. The nucleic acid was ethanol precipitated and then resuspended in 50mM Tris pH 8.3, 100mM KCl, 10mM MgCl, 10mM DTT, and 0.5mM for each of dATP, dGTP, dCTP and dTTP. 25U of AMV reverse transcriptase (BRL, or Life Sciences) was added and the mixture incubated at 42°C for one hour. EDTA was added to 10mM along with 5µl of RNase A (1mg/ml) and the mixture incubated at 50°C for one hour. The nucleic acid was extracted with phenol:chloroform (1:1), chloroform, and then ethanol precipitated. The pellet was resuspended in stop solution (Sequenase kit, United States Biochemical Corp.), boiled and electrophoresed on an 8% urea-acrylamide gel. The gels were dried and exposed to X-ray film. To determine the exact transcriptional start site, the extended product was analyzed in parallel to a sequencing ladder that was produced using the same oligo-primer and a DNA template of the appropriate region in a dideoxy sequencing reaction using the Sequenase kit protocol.

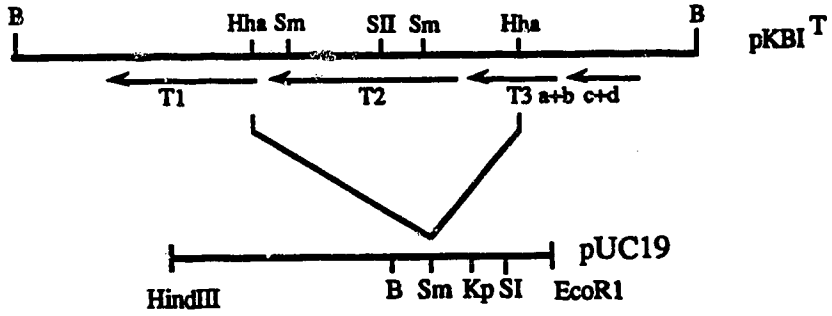
F. PLASMID CONSTRUCTS

1. *pUC19 CAT1 and pUC19 CAT4*



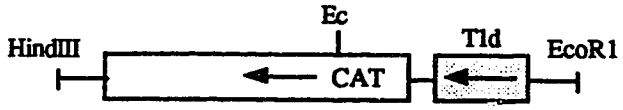
A *Bam*H1-*Hind*III fragment from pSV2CAT (Gorman, 1985; Gorman *et al.*, 1982), containing the bacterial chloramphenicol acetyl transferase gene without the SV40 promoter was blunt-end ligated into the *Sma*I site of pUC19 (Yanisch-Perron *et al.*, 1985). CAT1 is oriented as illustrated while CAT4 is the opposite orientation (for the definition of restriction enzyme notations used in this thesis see the abbreviations section).

2. *pPROT1(a, b, c, and d).*



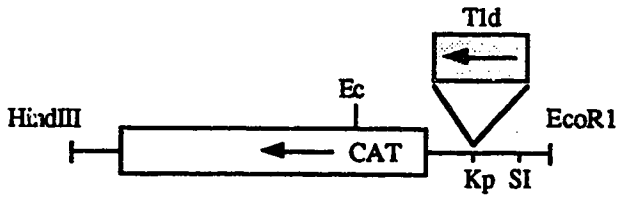
A 1423bp *Hha*I fragment from the terminal *Bam*HI clone pKBI^T of the SFV genome, was blunt-end ligated into the *Sma*I site of pUC19 and this clone is called pPROT1a. An *Sst* II-*Sst* I fragment was removed, the vector resealed by blunt-end ligation, and this is called pPROT1b. A *Sma*I-*Sst* I fragment was removed from pPROT1a, the vector resealed by blunt-end ligation and is called pPROT1d. This leaves a 189bp *Hha*I-*Sma*I viral DNA segment that contains the DNA sequences between ORFs T1 and T2, and it was used to characterize the T1 promoter.

3. *pPROT1d CAT (1 or 2)*



A *Bam*H1-*Hind*III fragment from pSV2CAT (Gorman, 1985; Gorman *et al.*, 1982), containing the bacterial chloramphenicol acetyl transferase gene was blunt-end ligated into the *Bam*HI site of pPROT1d. In pPROT1dCAT1 the CAT gene is oriented to be transcribed by the T1 promoter as illustrated while in pPROT1dCAT2 the CAT gene is in the opposite orientation.

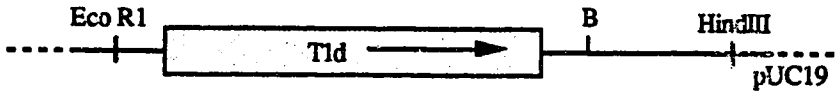
4. *pCATT1d(A or B)*



A BamHI-EcoRI fragment from pPROT1d, containing the T1d promoter sequences was blunt-end ligated into the *Kpn*I site of pUC19CAT4. In pCATT1dA the T1 promoter is oriented to transcribe toward the CAT gene cassette, while in pCATT1dB the T1 promoter transcribes away from the CAT gene cassette.

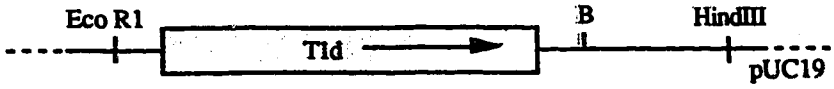
5. *pPROT1d Deletions*

a) 5'-Deletions



1. Eco R1, Bal 31
2. Eco R1 linkers
3. Eco R1, HindIII fragment cloned back into pUC 19.

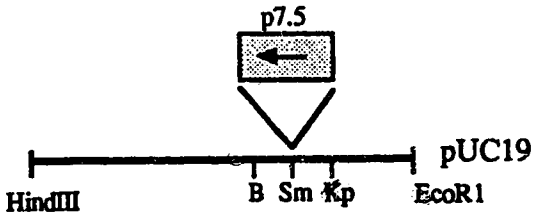
b) 3'-Deletions



1. BamH1, Bal31.
2. BamH1 linkers
3. Eco R1, BamH1 fragment cloned back into pUC19.

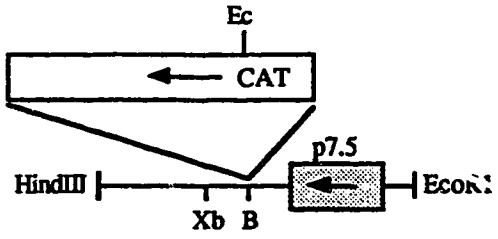
The 5'- and 3'-*Bal* 31 deletions of the T1 promoter were accomplished as follows. pPROT1d (5ug) which had been linearized with either *Bam*H1 or *Eco*R1 for the 3' and 5' deletion series, respectively, was treated with 2.4U of *Bal* 31 exonuclease (BRL), at 30°C in a 100ul reaction. Aliquots (20ul) were removed at 2 minute intervals and the size of the resultant products determined by agarose gel electrophoresis. Appropriate time points were selected, aliquots were blunt ended with T4 polymerase (BRL), *Bam*H1 or *Eco*R1 linkers were ligated (for the 3' and 5' deletions, respectively) and the plasmids recircularized. *Eco*R1-*Bam* HI or *Eco* RI-*Hind* III fragments containing deleted promoter sequences were purified and cloned into *Bam*H1 (*Hind* III) and *Eco*R1 digested pUC19 so that the vector sequences are identical for each deletion. The position of each deletion was determined by Sanger dideoxy sequencing and then the CAT gene cassette was blunt-end ligated into the *Bam*H1 site just downstream of each promoter.

6. *pV7.5(A or B)*



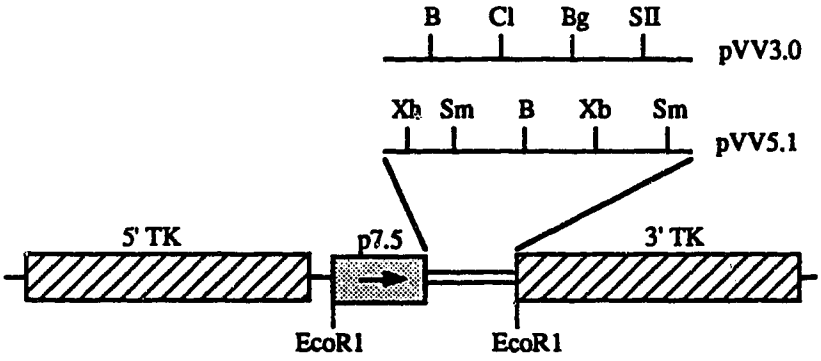
A 289bp *Eco*RI D fragment from pVV5.1 (Franke *et al.*, 1985), containing the vaccinia 7.5-kDa early/late promoter, was blunt-end ligated into the *Sma*I site of pUC19. pV7.5B is illustrated while the 'A' orientation is opposite.

7. *pV7.5CAT(A or B)*



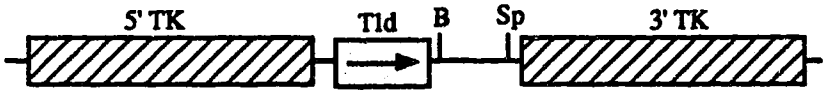
A *Bam*H1-*Hind*III fragment from pSV2CAT (Gorman, 1985; Gorman *et al.*, 1982), containing the bacterial chloramphenicol acetyl transferase gene was blunt-end ligated into the *Bam*HI site of pV7.5B. The 'A' orientation is as illustrated while in the 'B' orientation the CAT gene cassette is reversed with respect to the 7.5-kDa early/late promoter.

8. *pVV5.1 and pVV3.0*



These are vaccinia TK insertion vectors where the vaccinia 7.5-kDa gene early/late promoter and an adjacent multiple cloning sequence were inserted into the EcoRI site of the vaccinia TK gene (Franke *et al.*, 1985). These plasmids were a gift from Dr. D. Hruby (Department of Microbiology, Oregon State University, Corvalis OR).

9. *pVT1d(6 or 8)*

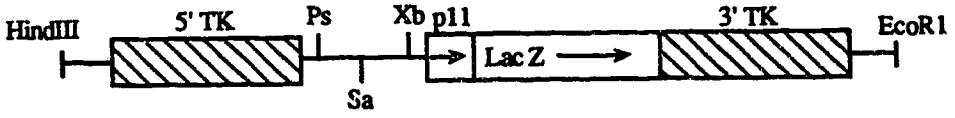


This is a vaccinia TK insertion vector containing the SFV T1d promoter. The 7.5-kDa gene early/late promoter and adjacent multiple cloning site, contained on a *SalI-SstI* fragment from pVV3.0, was replaced with the SFV T1 promoter contained on a *Hind*III-*Eco*RI fragment from pPROT1d, by blunt-end ligation. Clone#8 is as illustrated while clone #6 has the T1d promoter and adjacent multiple cloning site from pUC19 in the opposite orientation with respect to the vaccinia TK sequences.

10. *pVT1dCAT*

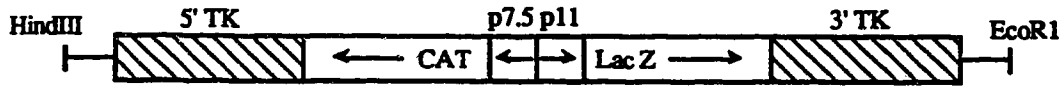
A *Bam*H1-*Hind*III fragment from pSV2CAT (Gorman, 1985; Gorman *et al.*, 1982), containing the bacterial chloramphenicol acetyl transferase gene was blunt-end ligated into the *Bam*HI site of pVT1d6 and a clone was isolated where the CAT gene is in the correct orientation for expression off of the T1 promoter. This plasmid was used to construct the recombinant vaccinia virus: VacT1dCAT.

11. *pSTΔKLac 5*



The plasmid pSTΔKlac5 is a SFV TK insertion vector and was constructed and kindly provided by A. Opgenorth. It was created by : (1) inserting a 436bp fragment containing the 5' portion of the SFV TK gene (Upton and McFadden 1986b), into the *Sph*I site of pUC19. (2) inserting a 736bp fragment containing the 3' portion of the TK gene into the *Kpn*I site of construct #1. (3) Inserting a 3.15kb *Bgl*II fragment from pSC20 which contains the lacZ gene fused to the vaccinia late promoter p11 (Buller *et al.*, 1988a), into the *Bam*HI site of construct #2.

12. *pSTKVCAT*



This SFV TK insertion vector was constructed by cloning the bacterial CAT gene fused to the vaccinia 7.5-kDa gene early/late promoter contained on a SalI fragment from pV7.5CATA into the *SalI* site of pSTΔKLac5. This plasmid was used to construct the recombinant SFV: SFVV7.5CAT.

G. TRANSIENT EXPRESSION ASSAYS

We used a method similar to that described by Cochran *et al.*, (1985a). Semiconfluent BGMK cells in 3.5cm wells of 6-well dishes were adsorbed with SFV (m.o.i.=0.3) for three hours or with vaccinia (m.o.i.=2.5) for 1 hour in a volume of 0.2mls. DME (2.5mls) was added to each well and 0.25mls of calcium phosphate precipitate (Gorman 1985), containing 0.5μg of plasmid DNA was added. The calcium phosphate precipitate was made by first adding the plasmid DNA to 0.125ml of a filter sterilized 2XNNH solution (0.818g NaCl, 0.60g Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma H-3375], 20.1mg $Na_2HPO_4.7H_20$; pH7.1, in 50ml H_20), followed by adding, in a dropwise fashion, 0.125ml of a filter sterilized freshly prepared 250mM $CaCl_2$ solution, while gently vortexing the 2XNNH solution. The 2XNNH solution can be stored for months at 4°C, but it should be at room temperature when it is used. The precipitate was made about 20-30 minutes before it was added to the infected cells and it was left on the cells for the entire period as no effect on expression has been demonstrated by removing the precipitate after a few hours (unpublished observations). The cultures were harvested 20-24 hours later by scraping the infected cells into 1ml of 1X SSC, pelleting in a 1.5ml microtube, and then resuspending them in 0.1ml of 0.25M Tris pH8.0. The cells were freeze-thawed three times by alternating between a dry ice ethanol bath and a 37 °C heat block, and vortexing vigorously each time they were thawed. The

debris was pelleted (12000gs, 30s) and the CAT activity in the lysates was assayed essentially as described by Gorman (1985). A 20µl aliquot of cell extract was mixed with 70µl 0.25M Tris pH8, 35µl H_20, 1µl ^{14}C-chloramphenicol (0.1mCi/ml, 54.2mCi/mmole, DuPont NEN products), that had been diluted 1:5 with 0.25M Tris pH8, and 20µl of freshly made 4mM acetyl CoA (Pharmacia, 27 6200-03). This was incubated at 37 °C for 10-40 minutes and then the sample was extracted with 1.0ml ethyl acetate. The ethyl acetate was placed in a new 1.5ml microtube and the solvent evaporated using a speed vac concentrator (Savant). The residue was resuspended in 20µl of ethyl acetate and the acetylated forms of ^{14}C-labeled chloramphenicol were separated by thin layer chromatography on silica gel coated (0.2mm) plastic sheets (Merck). The entire sample was spotted onto the plates and they were developed in chamber saturated with 95% chloroform, 5% methanol. The plates were air dried, lightly coated with En^{3}Hance spray (DuPont), covered with Saran Wrap, and exposed to X-ray film (X-OMAT AR, Kodak). The amount of chloramphenicol that was acetylated was determined by cutting out of the TLC plate the different acetylated forms of chloramphenicol using the autoradiogram as a template, and then measuring the levels of radioactivity in each form by liquid scintillation counting.

H. CONSTRUCTION OF RECOMBINANT VIRUSES

1. *Construction of SFVV7.5CAT*

Recombinant vaccinia and SFV were made by inserting foreign DNA into the TK genes by homologous recombination essentially as described by Mackett *et al.*, (1985). One six-well dish of BGMX cells was infected with SFV used for transient expression assays (m.o.i.=0.3). Wells #1-3 were transfected with pSTKVCAT (0.5µg/well), and #4-6 were transfected with pSTKVCAT linearized with *Hind* III. After 48 hours the cells from wells #1-3 (called A) were harvested and pooled, as were the cells from wells #4-6 (called B). These cells (A and B) were freeze thawed 3X, sonicated and a portion was

diluted 1:1000 in DME. Fourteen 100mm plates of BGMK cells were infected with virus A and B (7 plates each), using 1ml/plate. After four days the medium was replaced with an agar overlay containing 200µg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), from a stock of 40mg/ml in dimethylformamide. Each plate had 1000-2000 foci, and out of the 7 plates infected with stock A, there was one blue foci and of the 7 plates infected with stock B, there were two blue foci. These were picked, placed in 0.5mls DME, and stored at -70°C. In order to increase the number of blue foci twenty 100mm plates of TK^- T22 cells were infected with 0.5mls of a 1:500 dilution of viruses A and B (10 plates each). The infection was done in the presence of 25µg/ml bromo-deoxyuridine (BUdR). The cells were harvested after 48 hours and the virus stock (A' and B'), was used to infect ten 100mm plates of BGMK cells (5 each for A' and B'). Each plate had 100-200 foci and virus A' (uncut plasmid) did not have any blue foci while virus B' (cut plasmid) had 36 blue foci. Foci (24) were picked and the viruses plaque purified 3X, tested for CAT activity, and the presence of an insert was confirmed by Southern blotting DNA from infected cells that was digested with *Bam* HI and *Bgl* II. All the picked clones were recombinant viruses. One clone was chosen (SFVV7.5CAT), and larger stocks of this virus prepared in BGMK cells.

β-galactosidase activity in infected cells (or tissues) was also assayed using a histological stain (Allen *et al.*, 1988). Monolayers of infected cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS for 5min. at room temperature. The 4% paraformaldehyde was made by first making an 8% paraformaldehyde solution in ddH_2O, heating to 65°C for 20 minutes with periodic vigorous mixing, and then adding 1-2 drops of 1M NaOH and continue mixing. Most of the paraformaldehyde should go into solution. An equal volume of 2x PBS was then added to this mixture. The solution was made fresh for each experiment. After 5 min. the paraformaldehyde was removed, the monolayer was washed with PBS, covered with a staining solution and incubated at 37°C until blue staining was visible. The staining solution was freshly prepared for each use by adding to

PBS, in this order, (1) X-gal (40mg/ml) in DMSO to a final concentration of 1mg/ml; (2) 500mM potassium ferricyanide to a final concentration of 5mM; (3) 500mM potassium ferrocyanide to a final concentration of 5mM; (4) 100mM $MgCl_2$ to a final concentration of 2mM. These stock solutions were stored at -20°C.

2. *Construction of VacT1dCAT*

Two wells of a six-well dish of BGMK cells were infected with vaccinia WR (m.o.i.=0.5) and then transfected with uncut pVT1dCATA (0.2 µg/well). The cells were harvested and the virus titer assayed ($5X10^6$ pfu/ml). This virus stock was used to infect 10 plates of TK^- H143 cells (2000 pfu/plate). After adsorption for 1 hour the innoculum was replaced with an agar overlay containing BUdR (25µg/ml). After 48 hours 1ml of 0.01% neutral red in sterile PBS was placed on top of the agar, and 6 plaques were seen and picked. These were plaque purified 3X on H143 cells in the presence of BUdR (25µg/ml). The DNA of infected cells was isolated, digested with *Hind* III, blotted onto Pall biodyne A membrane and probed with the vaccinia TK probe. Three of the TK^- viruses were recombinants and three were not. One was picked (VacT1dCAT) and larger stocks prepared.

3. *Isolation of Cytoplasmic DNA from Infected Cells for Southern Blots*

In order to characterize the inserts in the recombinant viruses Southern blots of restriction enzyme digested cytoplasmic DNA of infected cells were prepared. The isolation of cytoplasmic DNA was found to be easier than isolation of whole cell DNA and the individual viral bands can be seen on ethidium bromide stained agarose gels. The isolation of cytoplasmic DNA was based on Esposito *et al.*, (1981). Cells from a single 100mm plate were harvested 48 hours after infection using 1X SSC. The cells were pelleted, resuspended in 1ml of TEN (50mM Tris pH8, 10mM EDTA, 150mM NaCl), placed on ice, and to this was added 0.2mls of TEN with 10% NP40, and 2-mercaptoethanol to 0.1M. The cells were incubated on ice for 10 minutes with intermittent vortexing. The nuclei were pelleted at 1000gs for 10 minutes and the supernatant removed

to a new tube. To this supernatant was added 60µl of 10% SDS, 25µl of freshly prepared protease (10mg/ml; Sigma, P-5147), and the sample incubated at 37°C over night. This was extracted twice with 0.5ml phenol, and once with 0.1ml chloroform. There was no high molecular weight cellular DNA, so the extractions were much easier than if total cellular DNA was being prepared. The DNA was ethanol precipitated and resuspended in 10mM Tris pH8, 0.1mM EDTA. I have found that Triton-X100 works as well as NP40, and that a great deal of viral DNA is still associated with the nuclear pellet. This could be due to the extensive cytoskeleton which may be different for each cell type. The action of drugs which disrupt the cytoskeleton has not been investigated. Southern blots were prepared using Hybond-N membranes (Amersham) according to the manufacture's instructions.

4. *^{32}P-labeling of DNA by Nick Translation*

Blots of cytoplasmic DNA were probed with ^{32}P-labeled DNA fragments consisting of λ-phage DNA and LMP agarose purified:

a) *Bam* HI-*Eco* RI fragment of pUC19CAT4 containing part of the CAT cassette.

b) *Bam* HI-*Eco* RI fragment of pSFVTKA containing the SFV TK-gene.

c) *Bam* HI fragment of pkTK-15 (donated by C. Upton) containing the vaccinia TK gene.

The purified DNA fragments were ^{32}P-labeled by nick translation similar to the procedure outlined by Maniatis *et al.*, (1982). DNA fragment (50-500 ng) was mixed with 5µl of 10x nick translation buffer (0.5M Tris pH7.2, 0.1M $MgSO_4$, 1mM DTT, 500µg/ml BSA), 1µl of DNase A (a 1:10,000 dilution of a 0.5µg/ml stock in 50% glycerol), 1.5µl of dNTP mix (3mM each of dCTP, dGTP, and TTP), 5µl of α^{32}P-dATP (ICN, 10mCi/ml, 3000Ci/mmole), and ddH_2O to a total of 50µl. 1µl of *E. coli* DNA polymerase I (D. Morgan) was added and the mixture incubated at 14°C for 2.5hrs. 5µl of 0.5M EDTA was added, the solution extracted with phenol:chloroform (1:1), chloroform and ethanol precipitated. The labeled DNA was pelleted, resuspended in 50µl of 10mM Tris pH8,

1mM EDTA, and the amount of radioactivity was determined by liquid scintillation counting. Generally 10^6 counts per minute of probe were used for each blot.

CHAPTER III
TRANSCRIPTIONAL MAPPING OF THE SFV TERMINAL INVERTED REPEATS

A. INTRODUCTION

As reviewed in chapter I, the sequences in the terminal regions of the poxvi genome are quite plastic and numerous deletions or rearrangements have been identifiec.. Work from many labs indicates that this portion of the genome contains genes which can effect the virulence or growth characteristics of the virus, although they are not essential for virus growth in tissue culture. At the time this thesis was started (spring 1984), the TIRs of vaccinia were one of the few well characterized regions on the poxvirus genome in terms of defining the basic features of the resident transcriptional units. They were found to con[illegible]n four genes, three early genes and one early/late gene (see appendix, I). The 19-kDa early gene was later identified as an epidermal growth factor like protein (Bloomquist *et al.*, 198[illegible] Brown *et al.*, 1985; Reisner, 1985), which is thought to effect the proliferation of the uninfe[illegible]d cells that are adjacent to infected cells (Buller *et al.*, 1988b). Inactivation of the VGF gene by insertional mutagenesis reduces vaccinia virulence as measured by intracranial lethality in mice or skin lesions in rabbits, but has little effect on its growth rate in tissue culture (Buller *et al.*, 1988a). In order to help in the identification of potential growth influencing genes, and to compare transcription of SFV to that of vaccinia, the TIRs of SFV were transcriptionally mapped.

The DNA genome of SFV has been cloned into plasmid vectors and its physical map is shown in Fig. 3 (Cabriac *et al.*, 1985; DeLange *et al.*, 1984b; Wills et al., 1983). *Bam* HI clones representing both the left and right ends of the genome were identified, fine restriction enzyme maps prepared, and M13 subclones of the region were obtained and are indicated in Fig. 4. It is important to note that the orientation of the viral genome in this

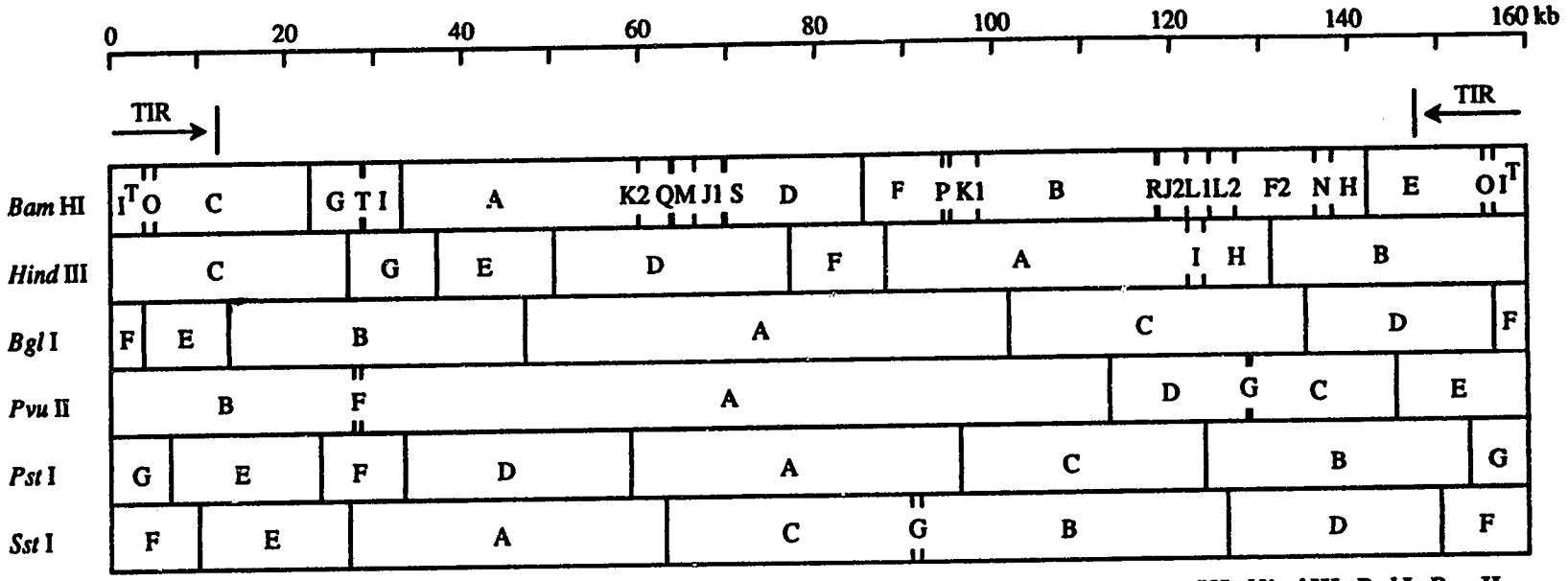


FIGURE 3. A restriction enzyme map of the SFV (Kasza) genome for the enzymes *Bam* HI, *Hind* III, *Bgl* I, *Pvu* II, *Pst* I, and *Sst* I. Based on Fig. 5 in DeLange *et al.*, (1984b).

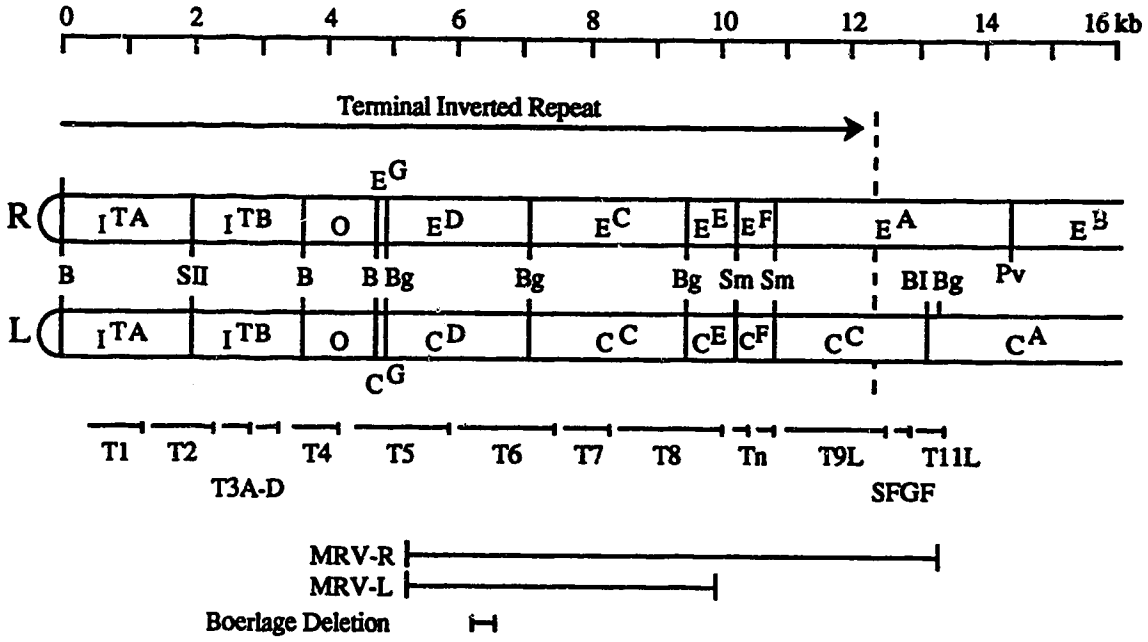


FIGURE 4. A physical map of the right and left TIRs of SFV. The restriction enzyme maps for the *Bam* HI clones I^T, O, E, and C, indicate the segments that were subcloned into M13 vectors for sequencing and to make strand-specific probes. The position of the ORFs are indicated by the horizontal 'T' shaped lines, and all ORFs read towards the hairpin ends. Both the portions of the SFV genome that were transfered to the left and right termini of myxoma virus during the formation of MRV, and the segment that is deleted from the TIR of SFV stain Boerlage, are indicated by the horizontal 'I' shaped lines.

thesis has been reversed compared to its description in all previous publications, including those in which most of this work has been published (Macaulay *et al.*, 1987; Macaulay and McFadden, 1989). In other words, the *Bam* HI E and C fragments which formerly defined the junction between the TIR and the left and right ends of the genome, respectively, now define the right and left ends of the genome as depicted in Fig. 3. This has been done because extensive similarities have been found in the ORF arrangement within the central portion of the SFV and vaccinia virus genomes (C. Upton, personal communication). In order to provide these ORFs in the two viruses with the same relative orientation, the left and right ends of the SFV genome have been reversed. The entire TIR of SFV has been sequenced and the position of the ORFs are indicated in Fig. 4 (Upton *et al.*, 1987a, 1988; Upton and McFadden, 1986a).

Poxvirus gene expression has been separated into two temporal classes as reviewed in chapter I. Transcription of early viral genes occurs as soon as the virus enters the cell using the encapsidated transcriptional machinery and continues until after the start of viral DNA synthesis. Transcription of late viral genes starts after the initiation of viral DNA replication using newly make viral RNA polymerase and results in the shut off of early transcription. In order to analyse the temporal class of a particular gene, the levels of its RNA transcript are measured at different times during the infection and at late times in the presence of an inhibitor of late gene expression using a variety of techniques such as Northern blot analysis, S1-nuclease analysis, and primer extension analysis. The addition of inhibitors, such as arabinosyl cytosine which prevents DNA replication, or cycloheximide which prevents early protein synthesis, at the start of infection, will prevent late gene expression so early gene expression continues and early RNA transcripts accumulate at late times.

In the following work, Northern blot analysis and S1-nuclease analysis are used to determine which areas of the TIR are transcribed. In the Northern blot analysis RNA from cells infected in the presence of cycloheximide, and arabinosyl cytosine was used and is

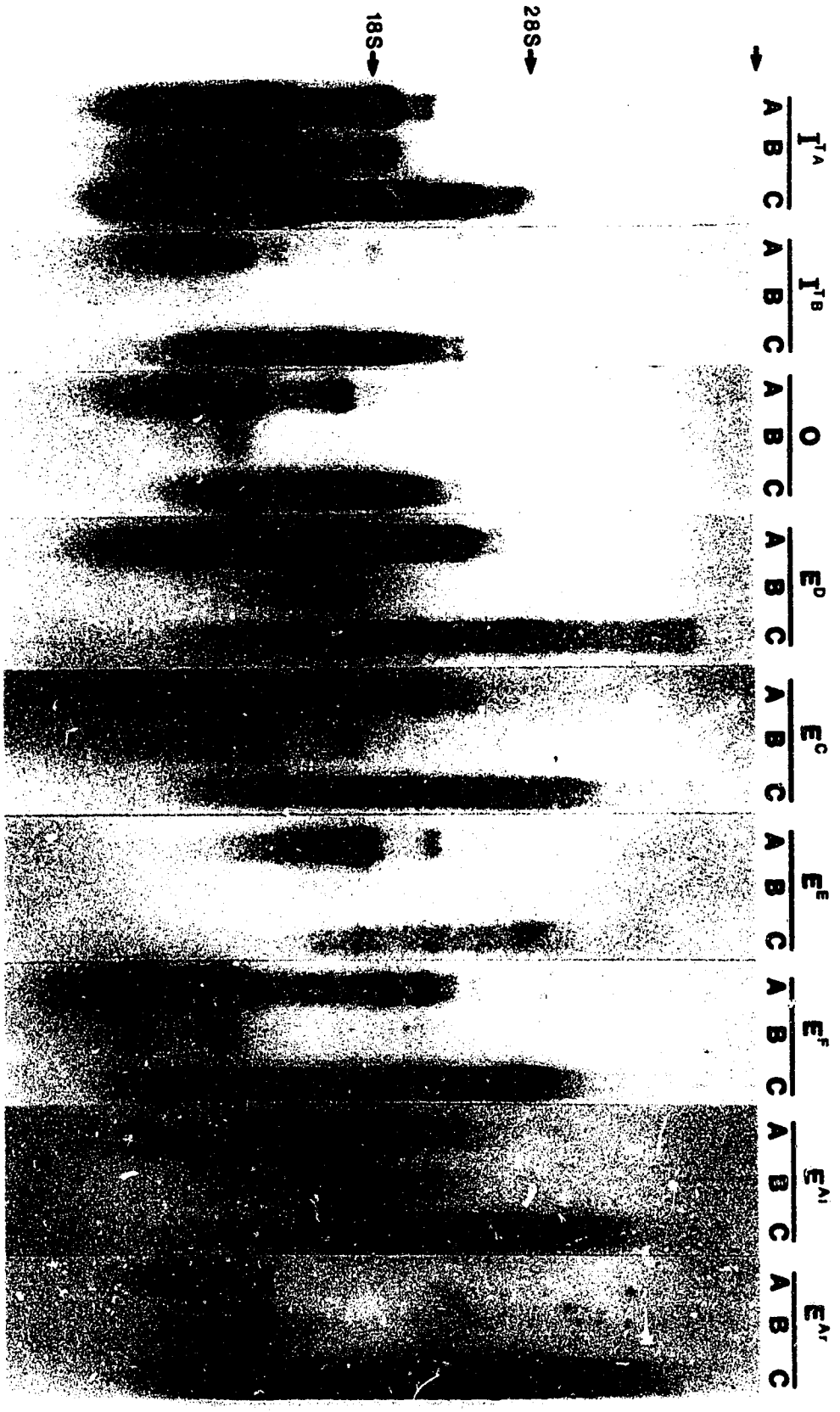
referred to as immediate early and early RNA respectively. No qualitative difference has ever been found in poxviruses between immediate early and early RNA in terms of the genes which are transcribed, so in the rest of the thesis RNA is usually referred to as being either early or late. The early RNA is isolated at early times during the infection or at late times in the presence of any drug which inhibits late gene expression (normally cycloheximide).

The data from the following work indicates that most of the genes are transcribed at early and late times however, it must be cautioned that this data was obtained using RNA isolated from cell infected at a relatively low multiplicity I show in chapter 4 that this low multiplicity can result in the presence of early RNA transcripts at late times during the infection due to the occurrence of a second round of infection. In other words not all the cells are infected during the initial adsorption of the virus and the uninfected cells are later infected at a relatively high multiplicity by the progeny virus from the first round of infection. Thus early transcripts from the second round of infection are present at late times with respect to the start of the first round of infection. This complication was not foreseen when this work was started and based on the results of the work in this thesis I would recommended using high m.o.i. (10 ffu/cell for SFV) in order to temporally classify a particular gene. A second way to test for early promoter activity would be to use an *in vitro* transcription system derived from a crude extract of purified vaccinia virus. This system will only recognized early poxvirus promoters and as I show in chapter 4 poxvirus promoters are functionally conserved among the various poxvirus genera. The reason a high m.o.i. was not used for the following work was because producing high titered stock of SFV is very difficult compared to vaccinia since its yield is usually 10-100 fold lower.

B. NORTHERN BLOT ANALYSIS

In order to determine the approximate number and size of transcripts from the SFV TIR, Northern blots of poly A^+ RNA isolated from SFV-infected cells were used. The

FIGURE 5. Northern blotting analysis of poly(A)$^+$ RNA from SFV-infected cells, hybridized with ^{32}P-labeled strand-specific viral DNA probes. Immediate-early (Cycloheximide, lane A, 16μg), early (Ara C, lane B, 6μg), and late (no drug, lane C, 2μg) poly(A)$^+$ RNA were denatured, reacted with glyoxal, electrophoresed through a 1% agarose gel, blotted, and hybridized with M13 strand-specific DNA probes from the right TIR (see Fig. 4). Probe E^{Ar} is complementary to RNA transcribed away from the hairpin terminus, while all the other probes are complementary to RNA transcribed toward the hairpin terminus. The origin and the rRNA markers from uninfected BGMK cells are indicated by arrows in the left margin.


18S
28S
I'A
I'B
O
ED
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EE
EF
EA'
EA''
A B C

blots were hybridized with cloned strand specific probes spanning the right TIR (Fig. 4), and the profiles with each probe are shown in Fig. 5. From the data it is clear that, unlike the relatively simple profiles observed in the TIRs of vaccinia virus, the transcriptional pattern within the SFV TIR is quite complex (Cooper *et al.*, 1981a; Wittek *et al.*, 1980a, b; Wittek *et al.*, 1981; and Venkatesan *et al.*, 1982). This result was obtained before the entire TIR sequence was known and it is apparent that all of the TIR of SFV is transcribed, and numerous distinct transcripts can be seen with immediate-early or early RNA (lanes A, and B, respectively, in Fig. 5). Because of the complexity of these profiles, the detailed description of the blots, and the assignment of the various transcripts to particular ORFs, will be addressed later (section D). However, two important points can be noted. Firstly, none of the probes hybridized to RNA from uninfected cells (not shown). Secondly, the probes used were strand specific and, with the exception of E^{Ar} (which maps at the TIR/unique sequence junction at the right terminus), only probes complementary to RNA transcribed towards the hairpin terminus hybridized to distinct transcripts. Probes complementary to RNA transcribed away from the hairpin terminus uniformly failed to hybridize to any distinct poly A^+ species (not shown). Probe E^{Ar} appears to hybridize to a single defined transcript directed away from the hairpin terminus, but whether this transcript initiates within or outside of the TIR is not known.

By exposing the Northern blots for different lengths of time it appears that, in general, RNA isolated from cells infected in the presence of ara-C (lanes B, Fig. 5) is similar to RNA isolated from cells infected in the presence of cycloheximide (lanes A, Fig. 5) although the former class often appears in reduced amounts. Others have reported similar findings for immediate-early and early classes of RNA in both SFV and vaccinia (Cabirac *et al.*, 1986; Cooper *et al.*, 1981b). Hybridization of the probes with poly A^+ RNA isolated at late times in the infection (lanes C, Fig. 5) usually displays a heterogeneous smear of high molecular weight transcripts. This is probably caused by 3' heterogeneity due to imprecise termination of late transcripts, as described for vaccinia late

mRNA (Cooper *et al.*, 1981b; Mahr and Roberts, 1984b; Weir and Moss, 1984; Weinrich and Hruby, 1986).

C. S1- NUCLEASE ANALYSIS

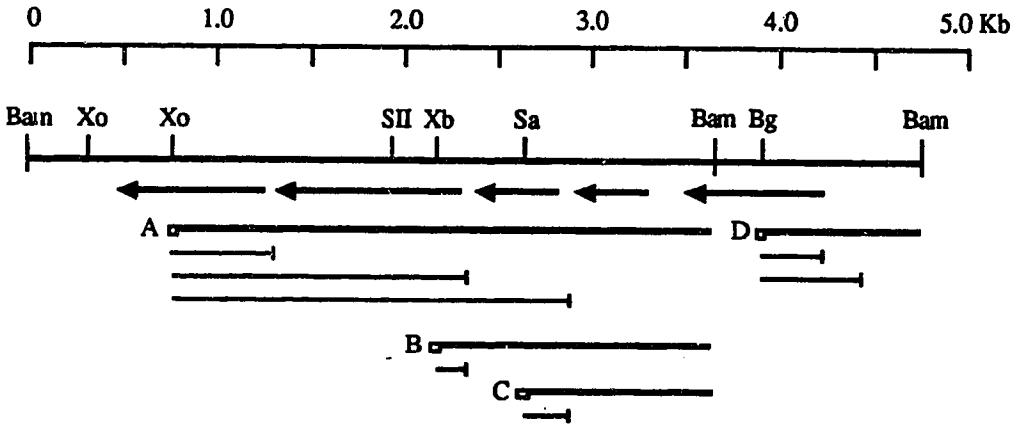
1. *Bam HI fragments I^T and O.*

In order to more clearly define the regions of the SFV TIR that are transcribed during the course of an infection, the location of the initiation sites for RNA transcribed towards the hairpin terminus were mapped by S1-nuclease analysis. Figure 6i shows the probes used to map RNA initiation sites within the terminal 4.7 kb of the TIR and schematically illustrates the results of the S1-protection experiments seen in Figure 6ii. Probe A is 2.9 kb long, spans most of the terminal *Bam*HI clone pKBIT, and is asymmetrically 5' end-labeled at the *Xho*I site. A representative fluorogram is shown in Figure 6ii (panel A). With immediate-early RNA (E), a protected fragment of approximately 0.53 kb is detected but with late RNA (L) this protected fragment is more intense and additional protected fragments of 1.47 and 2.07 kb can be observed. From this data it is not possible to distinguish whether the RNA which gives rise to the 1.47 and 2.07kb protected fragments is transcribed only at late times in the infection or if it is transcribed at both early and late times but at early times the transcripts terminate prior to the 5' end-label at the *Xho*I site. To answer this and to more precisely locate the RNA start sites, probes B and C were constructed.

Probe B is 1474 bp with the 5'-labeled end at the *Xba*I site (Fig. 6i). A 175 bp S1-resistant fragment is detected with both immediate-early and late RNA but there is a high molecular weight smear where the larger protected fragment, indicated by the data from probe A, would be expected. This smear could be caused either by the excess probe needed to detect the 175 bp fragment, or by the relatively low hybridization temperature, which in this experiment was lowered from its usual 51°C to 49°C in order to detect the AT-rich 175 bp fragment. If the hybridization with probe B is carried out at 51°C then the

FIGURE 6. S1-nuclease analysis of the terminal 4.7 kb of the SFV TIR. (i) Schematically illustrates the 5'-end-labeled DNA probes A through D (thick lines), spanning the terminal *Bam* HI clones I^T and O. The labeled 5'-ends are indicated by open squares. The thin lines represent the portion of the 5'-end-labeled probes protected from S1 digestion by RNA (see [ii]), and the small vertical lines indicate the 5'-ends of the RNA. Restriction enzyme notations are given in the list of abbreviations. (ii) Shows a fluorograph of S1-nuclease-resistant 5'-^{32}P-end-labeled DNA:RNA hybrids separated on 1% agarose (probe A), or 5% acrylamide (probes B-D) gels. With probe C, the higher molecular weight protected fragments in lanes E and L map within ORFs 3C/D but are not shown in (i). Molecular weight markers (M) are 5'-end-labeled, *Hind* III-digested lambda DNA (panel A), or 5'-end-labeled *Hin* FI-digested pBR322 DNA (panels B-D). The sizes in panel A are in kilobase pairs and those in panels B-D are in base pairs. Lane P is the unhybridized probe run as a size marker, lanes E, L, and T are the probes hybridized with 50μg of early (cycloheximide) RNA, late RNA, or control tRNA, respectively.

6i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1.0
2.0
3.0
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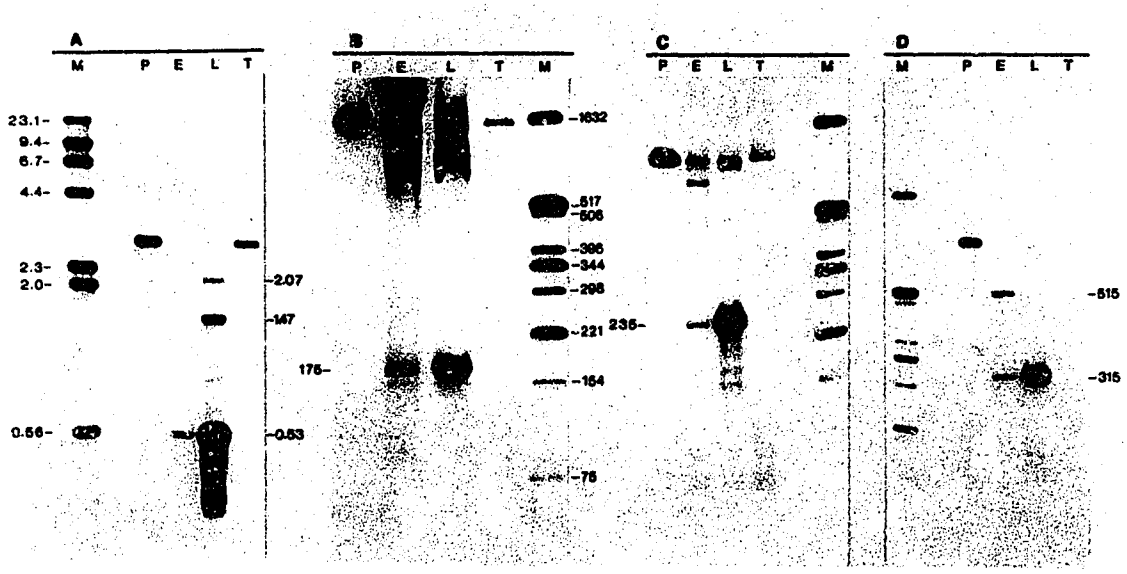
6ii


A
M
P
E
L
T
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
0.56-
2.07
147
0.53
B
P
E
L
T
M
1632
517
506
396
344
298
221
164
75
C
P
E
L
T
M
235-
175-
D
M
P
E
L
T
515
315

high molecular weight protected bands are more distinct (not shown).

Probe C is 1.0 kb with the 5'-labeled end at the *SalI* site 2.6 kb from the viral terminus. A major 235 bp S1-resistant fragment is detected with both immediate-early and late RNA and in addition there are two high molecular weight protected fragments. These results indicate that the three S1-resistant fragments of 0.53, 1.47, and 2.07 kb detected by probe A, which correlate well with the beginnings of open reading frames T1, T2, and T3A/B, respectively (see Fig. 9), are all expressed at both early and late times. However, as described in the introduction to this chapter this interpretation is complicated by the fact that these infections were done using a low multiplicity and we show in chapter IV that the T1 promoter when placed into the vaccinia genome is in fact strictly an early poxvirus promoter. The significance of the less intense high molecular weight protected fragments seen with probe C is not clear, but may represent minor initiations near the two small overlapping ORFs T3C and T3D (see the dashed arrow in Fig. 9).

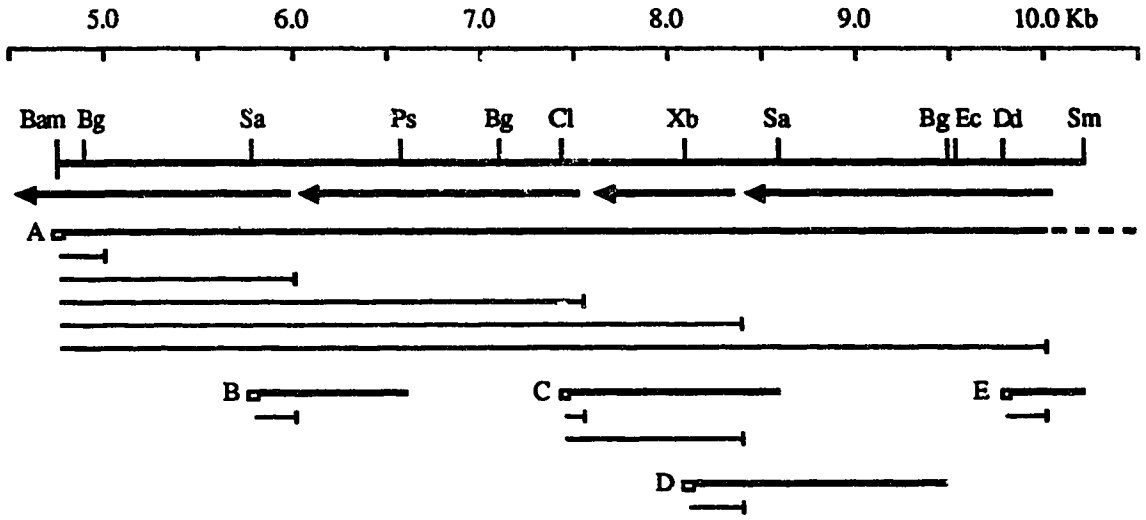
Probe D is 847 bp, with the 5'-labeled end at the *BglII* site 3.9 kb from the viral terminus, and spans most of the 1.2 kb *Bam*HI clone pKBO. As can be seen in panel D two protected fragments are detected using this probe. One of the S1-resistant fragments is 315 bp and is protected with both immediate-early and late RNA. As shown in Figure 6 this corresponds to an initiation site just upstream of ORF T4. A second fragment of 515 bp is detected only with immediate-early RNA and is not detected with late RNA. It is not clear if the larger protected fragment indicates an early specific promoter for T4 or if it is specific for a small (80aa) ORF located just upstream from T4. At this level of analysis this start site does not map precisely to the 5' end of the small ORF, and it should be noted that there is at least four examples of genes in vaccinia virus which have, in tandem, both an early and late promoters (see appendix III).

2. *Bam HI E Fragment Subclones G, D, C, and E.*

Figure 7i illustrates the map locations of the probes which span the next 8.4 kb of

FIGURE 7. S1-nuclease analysis of the TIR of SFV between the *Bam*HI site at 4.7 kb and the *Sma*I site at 10.2 kb from the viral terminus. (i) Schematically illustrates the 5'-end-labeled DNA probes A through E (thick lines) with the 5'-ends indicated by open squares. The thin lines illustrate the portion of the S1-resistant fragments (see [ii]), and the small vertical lines indicate the 5'-ends of the RNA. (ii) Shows representative fluorographs of S1-nuclease-resistant 5'-^{32}P-end-labeled DNA:RNA hybrids, separated on 1% agarose (probe A, panelI), or 5% acrylamide (probes A-E, panel II) gels. Sizes of the protected fragments in panel I (probe A) are in kilobase pairs, whle those in panel II (probes A-E) are in base pairs. In panel II (probes A-E) the signals for early RNA (lane E) are not observed at these exposures. Restriction enzyme notations are provided in the list of abbreviations.

7i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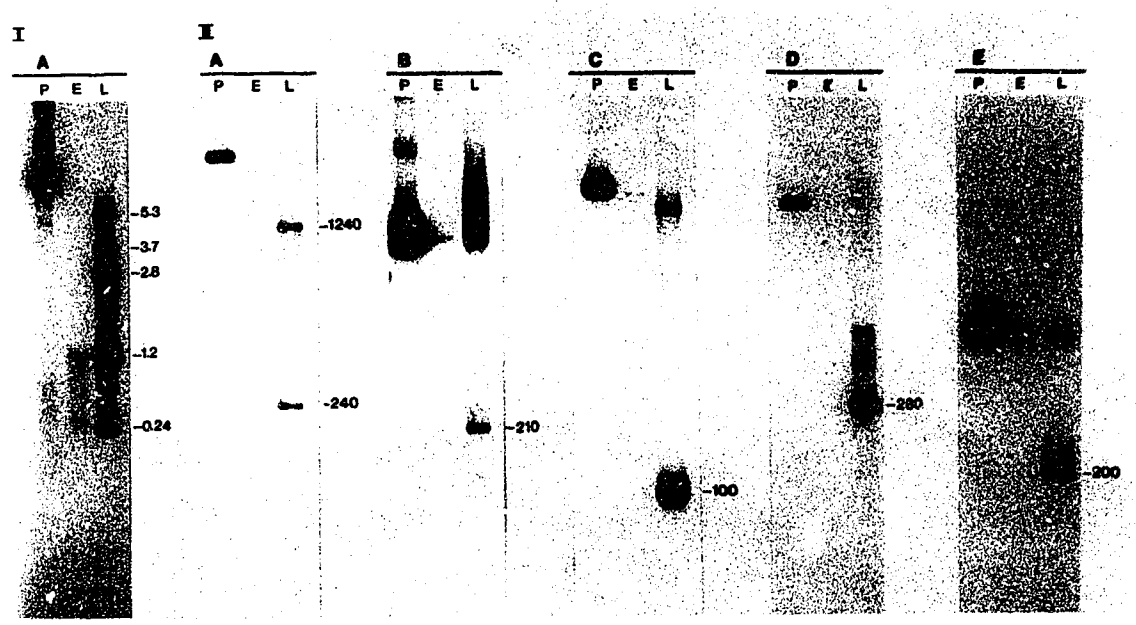
6.0
7.0
8.0
9.0
10.0 Kb
Bam Bg
Sa
Ps
Bg
Cl
Xb
Sa
Bg Ec Dd
Sm
A
B
C
E
D

7ii


I
A
P E L
5.3
3.7
2.8
1.2
0.24
II
A
P E L
1240
240
B
P E L
210
C
P E L
100
D
P E L
280
E
P E L
200

the TIR, and schematically represents the S1-results that are seen in Figure 7ii. Probe A is 8.4 kb with the 5'-end labeled at the *Bam*HI site and was chosen to survey transcription near the TIR boundary. Panel I shows a fluorogram of the S1-resistant fragments derived from probe A. With immediate-early RNA a single S1-resistant fragment of 1.2 kb is detected, while with late RNA at least five major protected fragments of 0.24, 1.2, 2.8, 3.7 and 5.3 kb are detected. In this hybridization an excess of probe was used and the gel was exposed long enough to detect the 1.2 kb fragment protected with immediate-early RNA. As a result, some of the minor bands seen in the hybridization with late RNA may not be actual transcriptional initiation sites, so only those initiation sites which have been confirmed with other shorter probes are indicated here and in Figure 9. For example, in panel II a lesser amount of probe A was used and the S1-resistant material was run out on a 5% acrylamide gel. Protected fragments of 240, and 1240 bp are seen, whereas the intermediate minor fragment of about 700 bp observed in panel I is not. The S1-resistant fragments of 1.2, 2.8, 3.7, and 5.3 kb detected with probe A in panel I correspond well with the presumptive initiating AUG codons of ORFs T5-T8, respectively (Fig. 9). The 240 bp S1-resistant fragment generated with probe A is unusual in that it maps within a major ORF (T5). There are no other significant ORFs in this region except for T5, but two methionine codons in the same reading frame as T5 can be found within 30 nucleotides of this mapped initiation site. This gives rise to the possibility that the C-terminal portion of T5 is expressed as a separate polypeptide and so this initiation site is tentatively labeled as T5C. In vaccinia virus there are examples of overlapping transcripts where the initiation site for one gene is within the upstream transcript indicating that poxvirus promoter regions can themselves be transcribed (Earl *et al.*, 1986; Golini and Kates, 1984; Mahr and Roberts, 1984a). In order to confirm the other start sites seen with probe A, and to more precisely map their positions, probes B through E were constructed.

As shown in Figure 7i probe B is 660 bp long with the 5'-labeled end at the *Sal*I site, 5.8 kb from the viral terminus. The 210bp fragment protected with late RNA

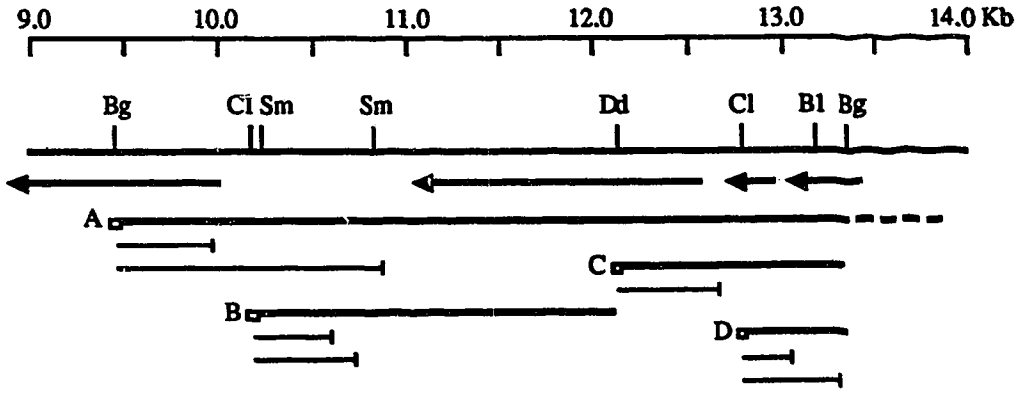
establishes a transcriptional start site at the beginning of ORF T5 (see Fig. 9). Probe C is 1.15 kb long, is 5' end-labeled at the *Cla*I site, and the position of the 100 bp S1-resistant fragment observed with late RNA corresponds to the beginning of ORF T6. There are also two high molecular weight protected fragments detected with late RNA, both of which could potentially encode ORF T7. It is not yet clear why there are two resistant fragments in this region since with probe D, which is 1.4 kb and 5' end-labeled at the *Xba*I site, only a single major S1-resistant fragment of 280 bp is observed at this site. With probe E, 427 bp and 5' end-labeled at the *Dde*I site, a 200 bp fragment is protected with late RNA and corresponds to the start of ORF T8 (see Fig. 9). With longer exposures all the S1-resistant fragments that are observed in Figure 7ii with probes B-E can be detected with early RNA as well, albeit in substantially lesser amounts (not shown). This is also consistent with the data from the Northern blots (Fig. 5) where the distinct transcript sizes observed with immediate-early RNA can be tentatively assigned to each major open reading frame (see section D).

3. *Bam HI C Fragment Subclones E, F, and C.*

Figure 8i illustrates the probes used to span the remainder of the TIR, the adjacent unique internal sequences from the left-hand end of the genome, and the schematic results of the S1-nuclease protection experiments which are shown in Figure 8ii. The 8.8 kb probe A (Fig. 8ii panel A), 5' end-labeled at the *Bgl*II site 9.5 kb from the viral terminus, is from the right end of the genome. A major S1-resistant fragment of 0.49 kb is observed at late times, and corresponds to the start of ORF T8 (see also Fig. 7ii panel E). A second minor protected fragment of 1.36 kb is also found with late RNA. Identical results are obtained if the probe, 5' end-labeled at the same *Bgl*II site, is from either the left or the right end of the viral genome (not shown). Probe B (2.04 kb) was constructed to confirm the position of the 1.36 kb S1-resistant fragment seen with probe A, and is 5' end-labeled at the *Cla*I site 10.2 kb from the hairpin terminus. Using probe B a major S1-resistant fragment of 525 bp

FIGURE 8. S1-nuclease analysis of the junction region between the TIR of SFV and the right-hand unique internal sequences. (i) Schematically illustrates the 5'-end-labeled DNA probes A through D (thick lines) with the labeled 5'-ends indicated by open squares. The thin lines illustrate the portion of the 5'-end-labeled probes protected by RNA (see [ii]), and the small vertical lines indicate the 5'-ends of the RNA. (ii) Shows representative fluorographs of S1-nuclease-resistant 5'-^{32}P-end-labeled DNA:RNA hybrids, separated on 1% agarose (panel A), or 5% acrylamide (panels B-D) gels. Sizes of the protected fragments in panel A are in kilobase pairs; in panels B-D, base pairs. Restriction enzyme notations are provided in the list of abbreviations.

8i


9.0
10.0
11.0
12.0
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14.0 Kb
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Cl Sm
Sm
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Cl
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A
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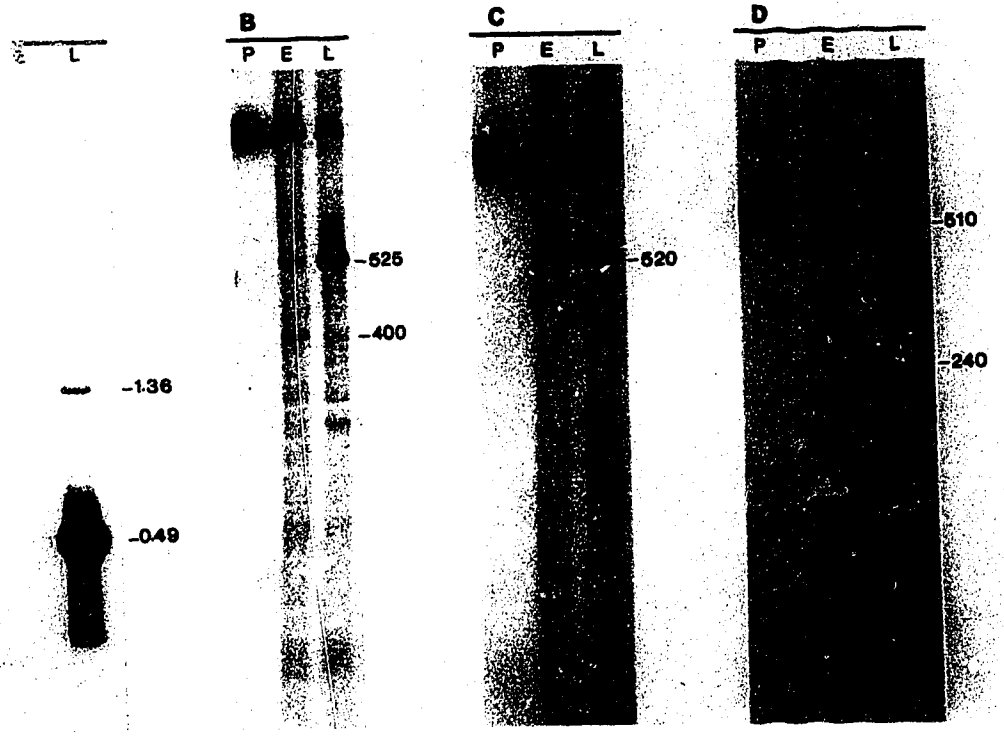
D


L
B
P E L
C
P E L
D
P E L
525
400
620
510
240
1.36
0.49

is seen with both early and late RNA, and a fragment of 400 bp is protected only with early RNA (Fig. 8ii, panel B). Although the start sites determined by probes A and B are slightly different this is most likely due to mobility differences in the two gel systems. These start sites map in an area of the SFV TIR containing four small ORFs (Tn A-D, see below).

Probes C and D were constructed to test for transcripts which map to the open reading frames T9-L at the TIR/unique sequence junction at the left terminus (Upton and McFadden, 1986b), and the SFV growth factor gene (SFGF), (Cheng *et al.*, 1987) in the unique left end sequences. Probe C is 1224 bp, 5' end-labeled at the *Dde*I site near the end of the TIR, and traverses the junction between the TIR and the left-hand unique internal sequences. A minor S1-resistant fragment of 520 bp is detected with late RNA, and thus places the start site about 85 bp upstream from the first AUG of T9-L. This 520 bp fragment was rather difficult to detect and appeared to be under-represented when compared to the other start sites mapped in the TIR. With an equivalent probe end-labeled at the same *Dde*I site, but instead spanning the junction between the TIR and the right-hand unique internal sequences, no protected fragments were observed (not shown). This indicates that, T9-L, but not T9-R, is transcribed, albeit the resulting transcript is present at lower steady state levels than those for the other ORFs in the TIR.

Probe D is 568 bp and 5' end-labeled at the *Cla*I site 12.8 kb from the left SFV hairpin terminus. An S1-resistant fragment of 510 bp is detected with both early and late RNA while a fragment of 240 bp is detected only with late RNA. These start sites are 350bp and 75bp, respectively, upstream from the SFGF ORF, which is homologous to epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha), and vaccinia virus growth factor (VGF) (Cheng *et al.*, 1987). Using primer extension analysis others have found precisely the same start site (75 bp upstream from the AUG), at early times (W. Cheng, personal communication). It is possible that the level of detection in these studies is not sufficiently sensitive to detect the closest start site at early times but this

issue remains to be resolved. SFGF is the first ORF which lies entirely in the unique sequences outside the left TIR. The second ORF is T11-L (Upton *et al.*, 1988), however, the 510bp protected fragment maps to a position within the middle of this ORF.

D. DISCUSSION OF THE COMPOSITE SFV TIR TRANSCRIPTIONAL MAP

These studies indicate that the TIR region of the SFV genome is densely packed with a tandem array of ORFs, all of which are transcribed from the same strand reading towards the hairpin terminus. This efficient utilization of space also continues into the unique right sequences, which includes the SFGF gene and T11-L. This arrangement of tandemly arrayed ORFs may be true outside the left TIR as well because Northern blot analysis with probe E^{Ar} (Fig. 5) indicates that there is at least one transcript in this region directed away from the hairpin terminus. Thus the genetic organization of the TIR of SFV is very different from the TIR of vaccinia virus (see appendix I). On the other hand, sequences outside the left TIR of vaccinia are efficiently utilized for coding (Cooper *et al.*, 1981b), and several other regions of the vaccinia genome have been found to contain tandem arrays of ORFs expressed at early and/or late times (Golini and Kates, 1984; Mahr and Roberts, 1984a and 1984b; Niles *et al.*, 1986; Weinrich and Hruby, 1986; Weir and Moss 1984).

The genomic organization of the TIRs of SFV, including the major ORFs and the transcriptional initiation sites, is illustrated in Figure 9. There is a good correlation between the beginning of each major ORF deduced by DNA sequencing of the TIR, and a transcriptional initiation site determined by S1-analysis. Twelve sites for the initiation of transcription have been mapped within the TIR: ten of these sites are located at the 5' end of the major ORFs (T1, T2, T3A/B, T3C/D, T4, T5, T6, T7, T8, and TnB/C), and the remaining two sites map within ORFs. In fact, at this level of analysis, the transcriptional initiation sites for ORFs T1, T2, T3A/B, T4, T5C, T5, T6, T7, and T8 are all within 30 nucleotides of the presumptive initiating codon.

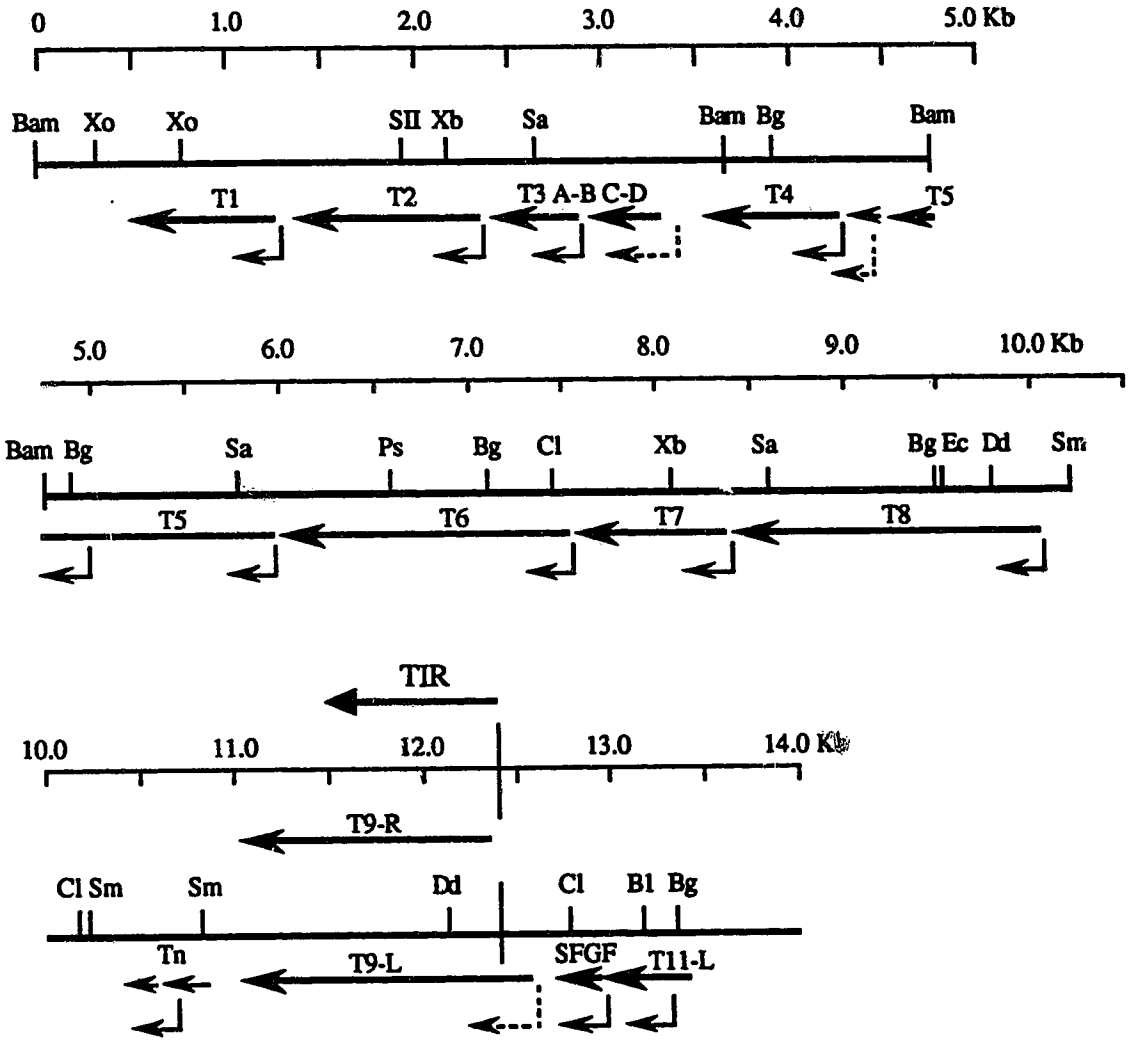


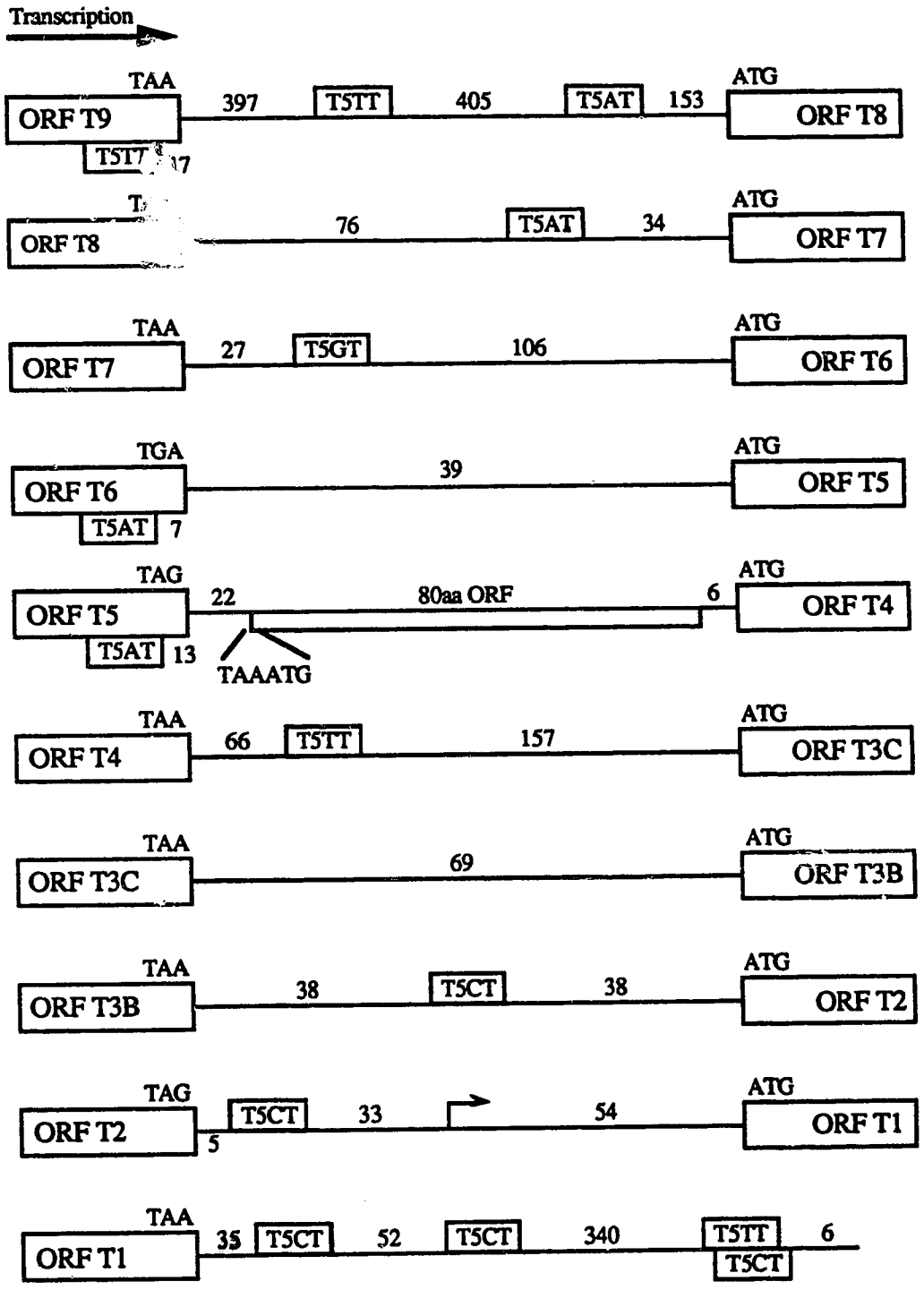
FIGURE 9. The composite transcriptional map of the SFV TIR and flanking regions. The restriction enzyme symbols are provided in the list of abbreviations. The major ORFs T1 through to T11-L were determined by DNA sequencing of the entire TIR plus the flanking unique sequences (see text). T9-L spans the junction between the TIR and the right-hand unique internal sequences, while T9-R is a subset of this ORF found within the left TIR (Upton and McFadden, 1986c). The transcriptional initiation sites, determined by S1-nuclease mapping, are indicated by bent arrows.

Three sites for the initiation of transcription were mapped outside the left TIR. The first site lies just 5' to T9-L (dotted line in Fig. 9), which traverses the junction between the left TIR and the unique internal sequences. The other two start sites map 5' to SFGF, and within the T11-L ORF. The initiation site corresponding to T9-L is 85 nucleotides upstream from the first AUG of the ORF in the unique left-end sequences, and oriented towards the TIR. It is shown as a dotted line in Figure 6 because it was present only as a minor species and it is much less abundant than ORFs T6 and T8, which are both closely related to T9-L in terms of sequence (Upton and McFadden, 1986b). When T9-L, T9-R, and T6 sequences are compared they are very homologous (60% identical amino acids for T9-L and T6) until the junction between the unique portion of at the left end of the genome and the left TIR. At this point the homology between T9-R and T6 breaks down while T9-L and T6 remain closely related. Due to this truncation by the TIR/unique junction T9-R is 67 amino acids shorter than T9-L and it seems likely that during the evolution of the virus termini that T9-R was created by a transposition event from the original copy at the left terminus. As a result, the N-terminal portion of T9-L, including its promoter, was decapitated by the transposition event, leaving the right copy (T9-R) transcriptionally silent (Upton and McFadden, 1986b). If T9-L, T6 and T8 are in fact functionally equivalent then the T9-L transcript may not be required in substantial amounts since ORFs T8 and T6 are both transcribed at high levels. It is interesting to note that in the Boerlage strain of SFV there is a deletion in part of the T6 ORF (Upton *et al.*, 1988; Upton and McFadden, 1986a). Thus at least the entire T6 gene product is not necessary for viability, although it may effect the pathology of the lesions in rabbits (Strayer *et al.*, 1984).

All the major ORFs described here appear to be transcribed constitutively, that is at both early and late times in the infection. It was much easier to detect transcriptional initiation sites with late RNA than with early RNA, and this could be due either to the fact that these ORFs are transcribed at both early and late times and the site of initiation for both transcripts is identical (note that this is relatively rare in vaccinia), or as described in the

introduction to this chapter to the fact that early genes are being expressed during a second round of infection. This latter explanation seems the most likely. Cabriac *et al.*, (1986), have demonstrated by northern blot analysis that the SFV sequences which have been transfered to myxoma virus in the creation of MRV (Fig. 4) are transcribed at early times. Very little RNA is detected at late times and it is in the form of a faint high molecular weight smear. W. Cheng (personal communication), has investigated the expression of the SFGF gene in SFV. At a multiplicity of 10, the SFGF transcripts are detected by northern blot analysis at 2, 4 and 6 hours post-infection which is prior to viral DNA replication but not at 24 hrs post-infection which is after DNA replication. The fact that they detect the same initiation site using primer extension on early RNA as we do using S1 analysis on late RNA would also support that a second round of infection has occured. We initially thought these ORFs were transcribed constitutively (Macaulay *et al.*, 1987), because long end-labeled probes could detect the initiation sites of several ORFs at late times (Fig. 7ii A), as if the lack of termination at late times, which is known to occur in vaccinia, allows for the production of very long transcripts. However, this data can also be interpreted as protection of long stretches of probe by a number of individual overlapping transcripts, from these tandemly arrayed ORFs. These early transcripts are properly terminated, but termination may occur past the site of initiation of transcription for the subsequent downstream ORF. The intergenic regions for the ORFs in the TIR are shown in Fig. 10. Yuen and Moss (1986), and Rohrmann *et al.*, (1986), have both shown that vaccinia early genes terminate about 50 bp downstream from the consensus termination sequence TTTTTTNT and this is found exclusively at the 3' end of all the major SFV TIR ORFs, excluding SFGF, T11-L and T-3C (Fig. 10) (Cheng *et al.*, 1987; Upton *et al.*, 1987). Although the function of this sequence in SFV has not yet been confirmed as an early transcription terminator, its specific location at the 3' end of all the SFV TIR ORFs further strengthens the conclusion that they are all *bona fide* early genes. Also the sequence

FIGURE 10. The intergenic regions between the ORFs within the TIR of SFV. The distance in base pairs between the translational start or stop sequences of the ORFs within the TIR and the consensus early transcriptional stop sequences (T5NT) are shown. Transcription is from left to right and the stop and start codons of each ORFs are indicated. The position of the transcriptional initiation site for ORF T1 is shown as a bent arrow. An 80 amino acid ORF between the major ORFs 3 and 4, along with its consensus late promoter sequence is indicated. The bottom portion of the figure shows the arrangement of the sequences between the T1 ORF and the *Bam* HI site at the hairpin end of I^T.


Transcription
TAA
ORF T9
397
T5TT
405
T5AT
153
ATG
ORF T8
T5TT
17
ATG
ORF T8
76
T5AT
34
ORF T7
TAA
ORF T7
27
T5GT
106
ATG
ORF T6
TGA
ORF T6
39
ATG
ORF T5
T5AT
7
TAG
ORF T5
22
80aa ORF
6
ATG
ORF T4
T5AT
13
TAAATG
TAA
ORF T4
66
T5TT
157
ATG
ORF T3C
TAA
ORF T3C
69
ATG
ORF T3B
TAA
ORF T3B
38
T5CT
38
ATG
ORF T2
TAG
ORF T2
T5CT
33
54
ATG
ORF T1
5
TAA
ORF T1
35
T5CT
52
T5CT
340
T5TT
6
T5CT

TAAAT, which is associated with the transcriptional initiation site of about 70% of the vaccinia late genes, is not found anywhere between the ORFs in the TIR except for at the start of the 80-kDa ORF between T4 and T5. If this is true then early overlapping transcripts may very well be possible and the reason we only see this at late times and not at early times is because at these low multiplicities there is very little RNA from the first round of infection, while for the second round of infection, where presumably the multiplicities could be much higher, then much more early RNA could be made.

The fact that all the major ORFs are expressed at early times is illustrated by the data in lanes A and B on the Northern blots (Fig. 5). Starting at the TIR boundary and moving towards the hairpin terminus in Fig. 4, probe E^{Ar} shows a single transcript directed away from the hairpin terminus. Like other constitutively expressed transcripts, at late times it is associated with a high molecular weight smear, probably due to imprecise termination. Probe E^{Al} detects two major transcripts in this region oriented toward the hairpin terminus. Half of this probe is derived from the viral TIR and half is from the unique internal sequences at the right end, and so the transcripts detected by blotting can not be unambiguously assigned to either T11-L, T9-R or SFGF. Probe E^{F} detects two major small transcripts corresponding to one or more of the Tn ORFs, most likely TnB and TnC since TnA lies entirely within E^{E} and 80bp of TnD lies within E^{A} (Fig. 4, 9, and 10) but neither species was detected by the corresponding probes. Probe E^{E} detects a single major transcript which would correspond to the ORF T8 transcript. Probe E^{C} detects two major transcripts, the smallest of which corresponds to ORF T7 while the larger is most likely made up of two transcripts, almost identical in size, encoding ORFs T6 and T8. Probe E^{D} detects transcripts for ORFs T5 and T6 (484aa and 508aa respectively) which appear to have similar sizes. Probe O identifies three transcripts encoding sequences for the ORFs T4, T5, and T5C. The most intense transcript detected by probe I^{TB} corresponds to a transcript initiating within the T3A-D ORFs, while the minor species corresponds to ORF T4. Probe I^{TB} also detects two very faint species of similar size to the two major bands

seen with probe I^{TA}. This is unexpected in view of the fact that the T2 transcript should be present in both blots since half of the T2 ORF lies in I^{TA} and half in I^{TB}. An alternative explanation is that transcripts corresponding to T1 and T2 (258aa and 325aa, respectively), may be found in the smaller heterogeneous species seen with probe I^{TB}. These tentative assignments remained to be confirmed by *in vitro* translation of hybrid- and size-selected mRNA.

With longer exposures, a number of higher molecular weight species (between 18 and 28S) are seen with early RNA on the Northern blots (I^{TA}, E^{D}, E^{C}, E^{E}, and E^{F}), and similar results have been seen on Northern blots of vaccinia early RNA (Cooper *et al.*, 1981b). There are at least two possible explanations for these transcripts. One is that the high molecular weight transcripts are due to the partial read-through into downstream sequences at early times. Although it is known for vaccinia that at late times imprecise termination results in high molecular weight smears on Northern blots (Wittek and Moss, 1982), at early times termination is thought not to be random, however it may still be only partially efficient. Thus a small fraction of the transcripts may not terminate after the first encoded ORF but instead read-through into the downstream sequences to the next available termination site. This would result in a higher molecular weight but nevertheless distinct transcript size, as opposed to the heterogeneous smear due to imprecise termination seen at late times. A similar phenomenon has been seen with vaccinia transcripts from two separate early gene clusters (Bajszár *et al.*, 1986; Golini and Kates, 1984; Mahr and Roberts, 1984a). Others have also reported read-through transcripts with SFV early RNA (Cabirac *et al.*, 1986).

A second possibility is that these high molecular weight transcripts represent splicing precursors or processing intermediates. No evidence for the splicing of intervening sequences has been found for poxvirus mRNA (see chapter I), however the possibility that S1-nuclease analysis might under some circumstances detect sites for mRNA processing rather than transcriptional initiation can not be rigorously excluded. It

would seem very unlikely that splicing would be involved in expression of these tandemly arrayed ORFs if it was demonstrated that they contain their own promoter sequences.

This chapter has demonstrated that the entire TIR of SFV is transcribed at least at early times and transcriptional initiation sites for each major ORF have been identified. We do not know if any of these ORFs are essential genes though SFGF is a candidate for a gene which may effect virus growth *in vivo*. It is clear that the ORF arrangement in the SFV and vaccinia TIRs are very different. The SFGF gene is homologous to the VGF gene found in the vaccinia (strain WR) TIR but we do not know if vaccinia contains any genes homologous to those found within the TIR of SFV. The fact that early transcription stop signals are found exclusively at the 3' ends of the major ORFs in the SFV TIR not only suggests that these are early genes but also that some of the transcriptional regulatory signals are conserved between these two genera of poxviruses. The following chapters will deal with this later aspect more thoroughly.

CHAPTER IV
ISOLATION AND CHARACTERIZATION OF A PROMOTER FROM SHOPE FIBROMA VIRUS

A. INTRODUCTION

The observation that the consensus sequences which function to terminate early transcription in vaccinia are found exclusively at the 3'-ends of the ORFs within the SFV TIRs was the first evidence that the transcriptional regulatory features might be conserved between these two genera of poxviruses. To explore how conserved the transcriptional machinery is between SFV and vaccinia we have isolated and characterized an early promoter from SFV and show that it functions as an early promoter in both transient expression assays and when imbedded into the genome of a recombinant vaccinia virus.

B. ISOLATION OF THE SFV T1 PROMOTER

When the TIR was being transcriptionally mapped we noticed that transcripts from the T1, T2, T4, T5, T6, T7 and T8 ORFs were much more abundant than from the T3a-d, T9-L, or SFGF ORFs. One reason for this could be that the promoters for the first set of genes are much more active than for the second set. We decided to test the sequences upstream of the T1 ORF for promoter activity. This region was isolated and cloned in plasmid pPROT1d (Fig. 11A). This 189bp *Hha*I-*Sma*I viral DNA fragment contains the first 6bp of ORF T1, the last 84bp of ORF T2, and a 99bp intergenic region. In order to test the ability of this viral sequence to function as a promoter in a transient expression assay, a number of constructs were made and fused to the CAT gene as illustrated in Fig. 11B. In these constructs the CAT gene is always oriented in the opposite direction with respect to the known vector promoter sequences. When the same orientation is used a cryptic poxvirus promoter, which maps somewhere within the pUC19 sequences, drives a

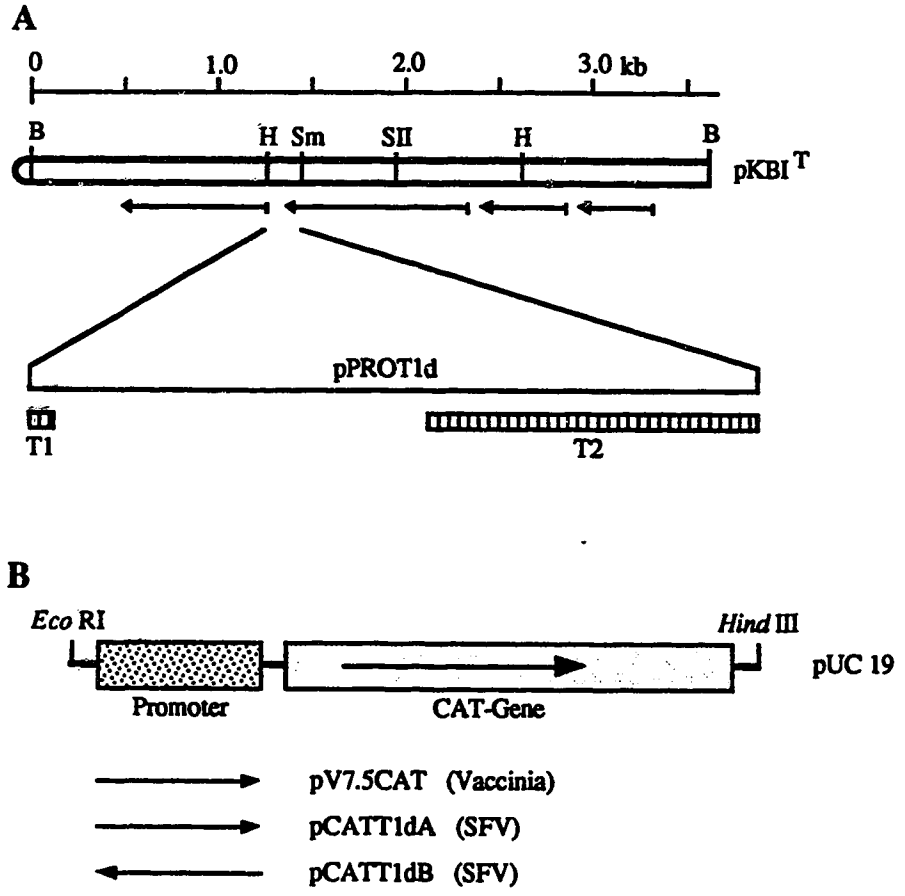


FIGURE 11. (A) Physical map of the terminal *Bam* HI I^T fragment of the SFV genome. The ORFs T1, T2, and T3 A-D (Upton *et al.*, 1987), are indicated by the arrows. The 189-bp *Hha*I-*Sma*I fragment constituting pPROT1d is expanded to show the positions of the T1 and T2 ORFs which are indicated as stippled boxes. Restriction enzyme notations are provided in the list of abbreviations. (B) CAT constructs used to test the pPROT1d fragment in a transient gene expression assay. The orientation of the CAT gene with respect to the pUC19 sequences is such that the vector *lacZ* promoter drives in the opposite direction from the CAT cassette. The directions of transcription for T1d and the vaccinia 7.5-kDa early/late promoter fragments are indicated.

low level of transcription in transfected/infected cells such that promoterless CAT controls give a significant background (our unpublished observations and Miner *et al.*, 1988). pV7.5CAT uses the vaccinia 7.5-kDa early/late gene promoter oriented to transcribe the CAT gene and is used here as a positive control for the system (Cochran *et al.*, 1985a). pCATT1dA uses the ORF T1 upstream sequence oriented so as to transcribe into the CAT gene, while in pCATT1dB it is oriented to transcribe away from the CAT gene. The data from transient expression assays using these constructs is shown in Fig. 12. It is clear from these data that the T1 ORF upstream sequences act in an orientation specific manner to promote the expression of the CAT gene in SFV infected cells (lanes 1-3) but not in uninfected cells (lanes 4-5). Identical results are obtained if the cells are infected with vaccinia virus (lanes 6-8). Thus these SFV sequences upstream of the T1 ORF bestow *bona fide* poxvirus promoter activity, and the cis-acting signals responsible for the activity of SFV T1 and the vaccinia 7.5-kDa early/late promoters in a transient expression assay have been conserved between the two poxvirus genera.

C. STANDARDIZATION OF CONDITIONS

When these assays were first performed it was noticed that if undiluted stocks of SFV were used for the infection then very little CAT activity was detected. Cochran *et al.*, (1985a), had demonstrated that in a transient expression system using vaccinia infected cells, the CAT activity increases with the m.o.i. and plateaus at an m.o.i. of 30-60 pfu/cell when the cells are harvested 12 hours after infection. For convenience and because the SFV replicative cycle is slower than vaccinia, we harvest the infected cells (SFV and vaccinia) about 24 hours after infection. To determine the optimum m.o.i. of virus to use under these conditions serial 1:5 dilutions of stocks of both SFV (2.4×10^7 ffu/ml) and vaccinia WR (6.2×10^8 pfu/ml) were used to infect BGMK cells in 6-well dishes. The results of the transient CAT gene expression assays are graphically presented in Fig. 13A.

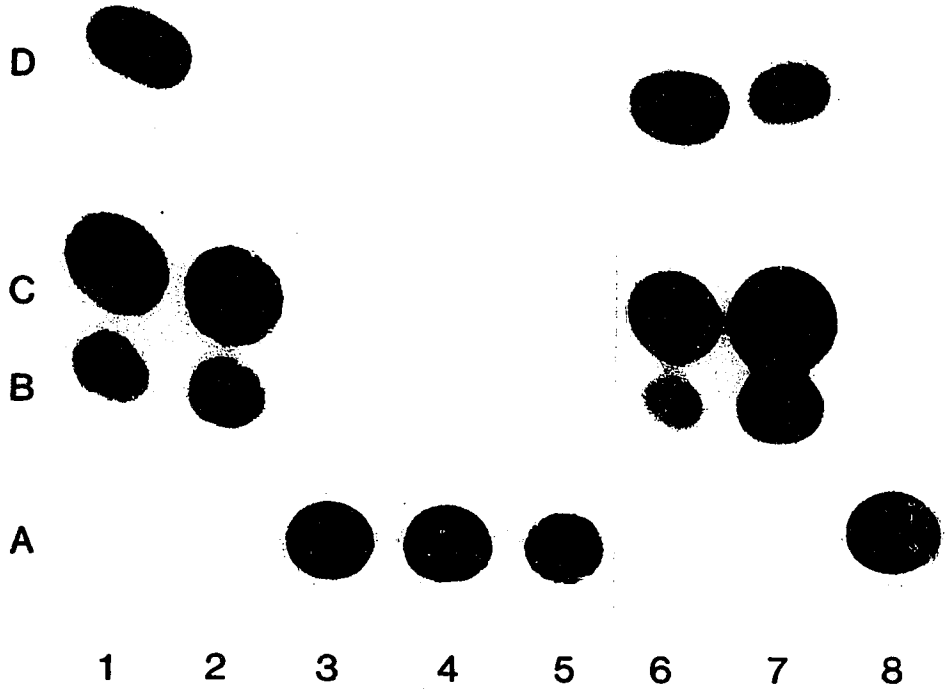
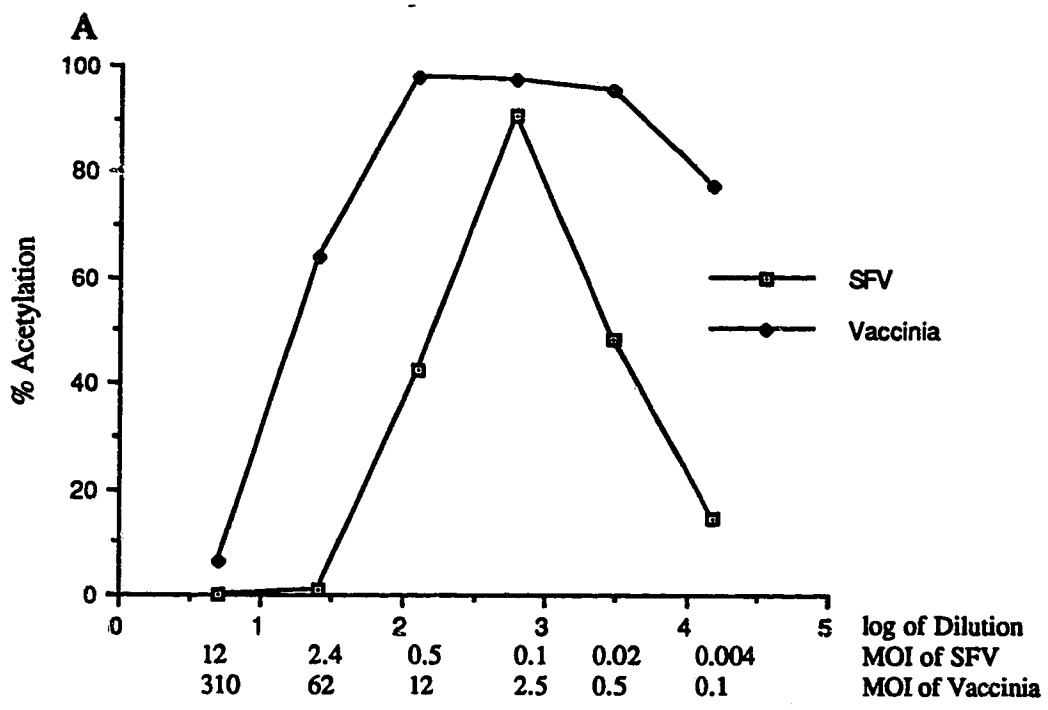
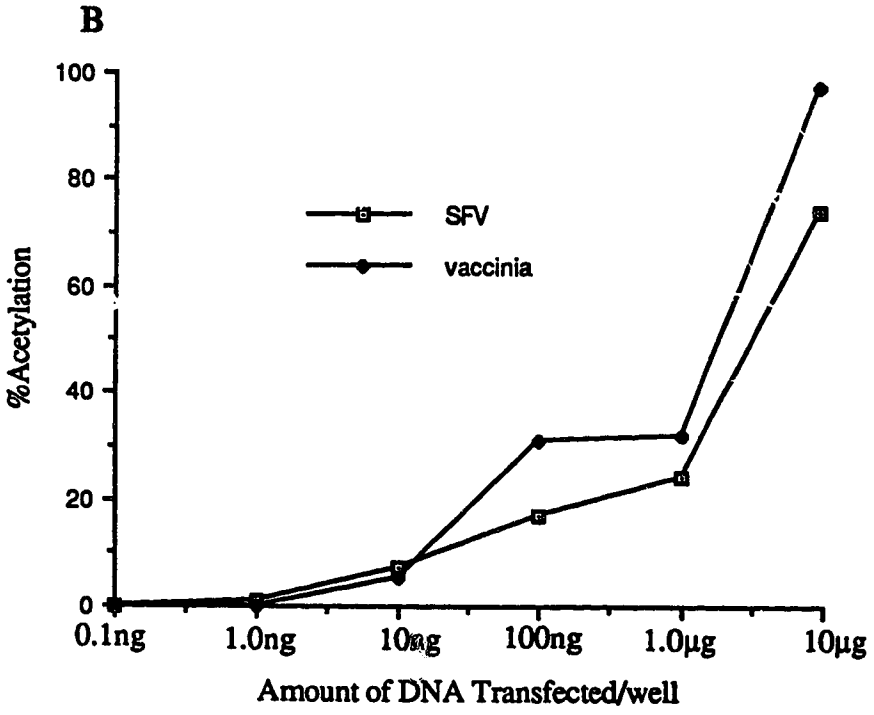


FIGURE 12. Comparison of vaccinia 7.5-kDa and SFV T1d promoters determined by a transient CAT gene expression assay. BGMK cells were infected with SFV (m.o.i. = 0.3, lanes 1-3) or vaccinia (m.o.i. = 2.5, lanes 6-8), or mock-infected (lanes 4 and 5). The plasmid pV7.5CAT (lanes 1, 4, and 6), pCATT1dA (lanes 2, 5, and 7), and pCATT1dB (lanes 3 and 8) were transfected into the cells, and the cell lysates were prepared 20hr later. CAT activity was assayed and a fluorogram of the TLC plate is shown. (A) Chloramphenicol; (B) 1-acetate chloramphenicol; (C) 3-acetate chloramphenicol; (D) 1,3-diacetate chloramphenicol.

FIGURE 13. Optimization of the conditions for a transient CAT gene expression assay. (A) Serial 1:5 dilutions of stock SFV ($2.4x10^7$ ffu/ml) and vaccinia ($6.2x10^8$ pfu/ml) virus were used to infected BGMK cells in 6-well dishes. After virus adsorption 500ng of pPROT1dCAT1 was transfected into each well. The cells were harvest 24 hr post-infection and lysates assayed for CAT activity. The m.o.i was calculated assuming $4x10^5$ cell/well. (B) Serial 1:10 dilutions of the plasmid pPROT1dCAT1 were used to transfect cells that had been infected with either SFV (1:200 dilution of stock virus) or vaccinia (1:625 dilution of stock virus). Cell lysates were assayed for CAT activity 24 hr post-infection. The results in panels A and B are averages of two separate transfections for each point.


A
100
80
60
40
20
0
% Acetylation
SFV
Vaccinia
0
1
2
3
4
5
log of Dilution
12
2.4
0.5
0.1
0.02
0.004
MOI of SFV
310
62
12
2.5
0.5
0.1
MOI of Vaccinia

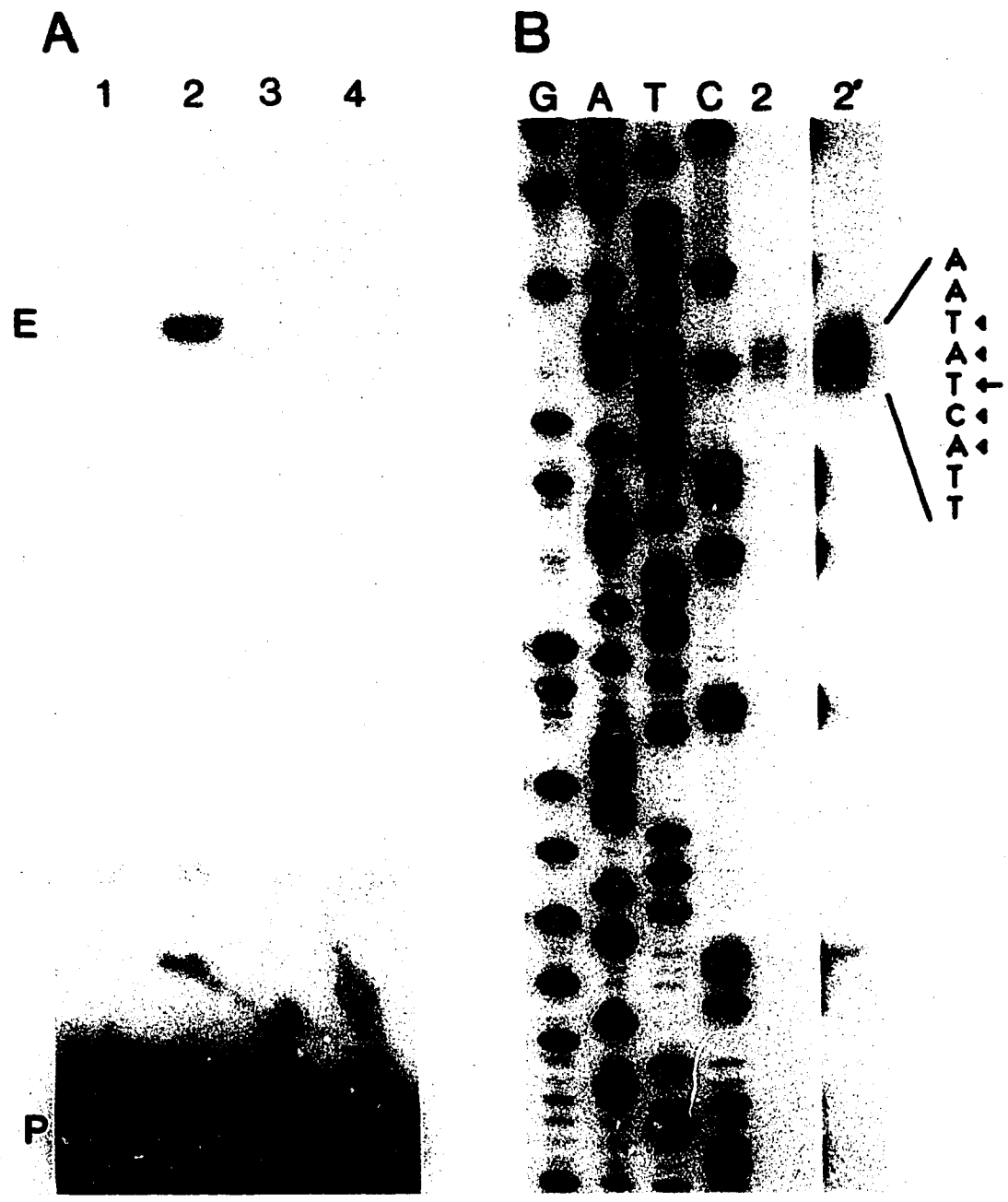

B
100
80
60
40
20
0
%Acetylation
SFV
vaccinia
0.1ng
1.0ng
10ng
100ng
1.0µg
10µg
Amount of DNA Transfected/well

Though the multiplicity of infection, expressed in ffu/cell and pfu/cell for SFV and vaccinia respectively, seems very different for the two viruses we do not know how the number of virus particles/cell ratio compares for the two viruses. All transient CAT assays in this thesis were done using an m.o.i.=0.3 for SFV and an m.o.i.=2.5 for vaccinia. When the amount of transfected plasmid DNA was varied (Fig. 13B), we found that the amount of CAT activity increased with the amount of DNA transfected, similar to that observed in vaccinia infected cells by Cochran *et al.*, (1985a). We therefore chose to transfect 500ng of DNA/well for all the transient assays in this thesis.

D. PRIMER EXTENSION ANALYSIS

To accurately localize the promoter domain for ORF T1 with respect to the sequences that are transcribed, the transcriptional start site for ORF T1 was determined by primer extension analysis. As can be seen in Fig. 14 A, an extended product of 0.12 kb (E) is seen at 20 hours post infection with RNA isolated from infected cells treated with cycloheximide. On a much longer exposure of the gel, the same sized product can be seen with late RNA (lane 3). The faint band which migrates a little more slowly than the end-labeled oligo probe that is present in all the lanes is a contaminant in the probe. When the primer extension reaction products using immediate-early RNA are analysed beside the products of a dideoxy sequencing reaction (panel B), a cluster of 5 extended products centered at a position 54 nucleotides upstream from the AUG of the T1 ORF are observed. The major extended product maps to a T residue on the antisense strand and will be designated the RNA start site. This same start site is also detected at early times and not at late times in an infection with recombinant vaccinia virus (see part F). These results indicate that the ORF T1 transcriptional initiation site lies 54 nucleotides upstream of the presumptive initiating AUG codon and that T1 functions as a typical poxvirus early gene.

FIGURE 14. Determination of the 5'transcriptional start site by primer extension analysis of the T1 ORF. (A) A ^{32}P-end-labeled 17-nucleotide primer was hybridized to 200 μg of total cellular RNA from mock-infected BGMK cells (lane 1), or from BGMK cells infected with SFV (m.o.i.= 0.5-1.0) for 20 hr in the presence (lane 2) or absence (lane 3) of cycloheximide (100 μg/ml). Lane 4 is the labeled primer alone. The products of the reverse transcription reaction were analyzed on an 8% urea-acrylamide gel. The extended product in lane 2 (E), and the labeled oligo primer (P) are indicated to the left of the fluorogram. (B) The extended product from lane 2 in (A) was analyzed beside a dideoxy sequencing reaction produced using the same oligo primer. Lane 2' is from a longer exposure of the sequencing gel. The antisense sequence on the right illustrates the positions to which the extended products correspond (triangles), with the major start site indicated by an arrow.


A
1
2
3
4
E
P
B
G
A
T
C
2
2'
AATATCATT

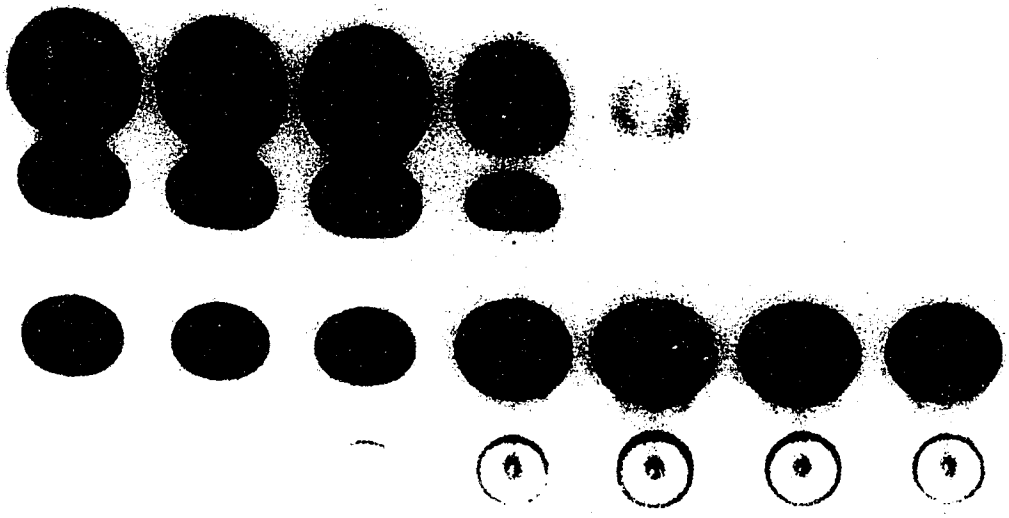
E. DELETION ANALYSIS

To determine the boundaries of the sequences required for a functional promoter in the transient expression system, 5' and 3' deletions were constructed from pPROT1d. The vector sequences are identical in each deletion and only viral sequences have been removed. The position of each deletion and the results of the transient CAT gene expression assays, using vaccinia infected cells, are shown in Figs. 15 and 16. The T1d clone is identical in the two sets of experiments which test the 5' and 3' deletions (Figs. 15A and 15B respectively), but the CAT assays shown were performed to different extents in order to illustrate the fact that the 3' deletions, which remove sequences downstream from the transcriptional start site, are much more efficient at CAT expression than is the full length T1d clone. This pattern of promoter activity was highly reproducible and identical in either SFV or vaccinia infected cells. Fig. 16A shows the approximate position of the deletions and the relative strength of each promoter with the full length T1d clone assigned an arbitrary value of 10. Fig. 16B shows the exact nucleotide position of the relevant deletions in the region around the transcriptional initiation site. These deletions indicate that the domain for the functional T1 promoter can be narrowed down to a stretch of about 28 nucleotides. Removal of the sequences up to the 5'-deletion clone 5-3 reduces promoter activity to 30% of the full length T1d, but the stretch of eight A residues between deletions 5-3 and 5-4 appears to be absolutely critical for detectable promoter activity. However, this 'A' octamer alone is, in and of itself, not enough for promoter activity since the 3'-deletion 3-4, which removes the transcriptional initiation site but retains the 'A' octamer, has only background CAT activity. Therefore, a functional T1 promoter also requires the sequences which include the site of initiation of transcription.

It is important to note that the measurement of CAT activity does not directly measure the transcriptional activity of a particular promoter. Deletions 3-1, 3-2 and 3-3 show a dramatic increase in CAT gene expression compared to the full length clone T1d. I believe that this is due to an increase in the translatability of the CAT ORF and not due to an

FIGURE 15. 5' and 3' deletion analysis of the SFV T1 promoter. (A) Transient CAT assays of the 5' deletions of the T1d promoter. (B) Transient CAT assays of the 3' deletions. T1d indicates the full-length promoter while the positions of each deletion are indicated in Fig. 16. The T1d constructs in (A) and (B) are identical but the CAT assays in the two sets of deletions were performed to different extents (25µl of each lysate in (A) for a 50min. reaction and 5µl of each lysate in (B) for a 15 min. reaction), in order to be within the linear range of enzyme activity for each experiment.

A



B

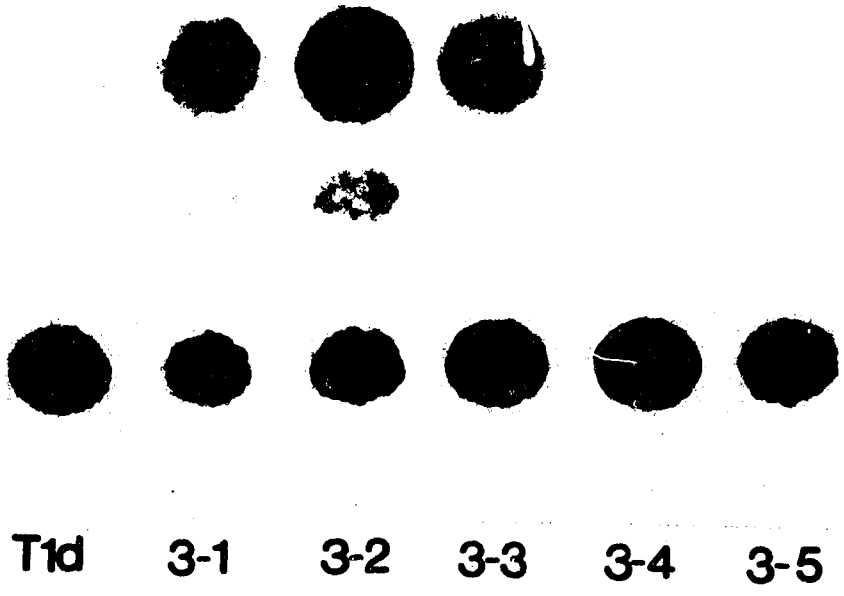
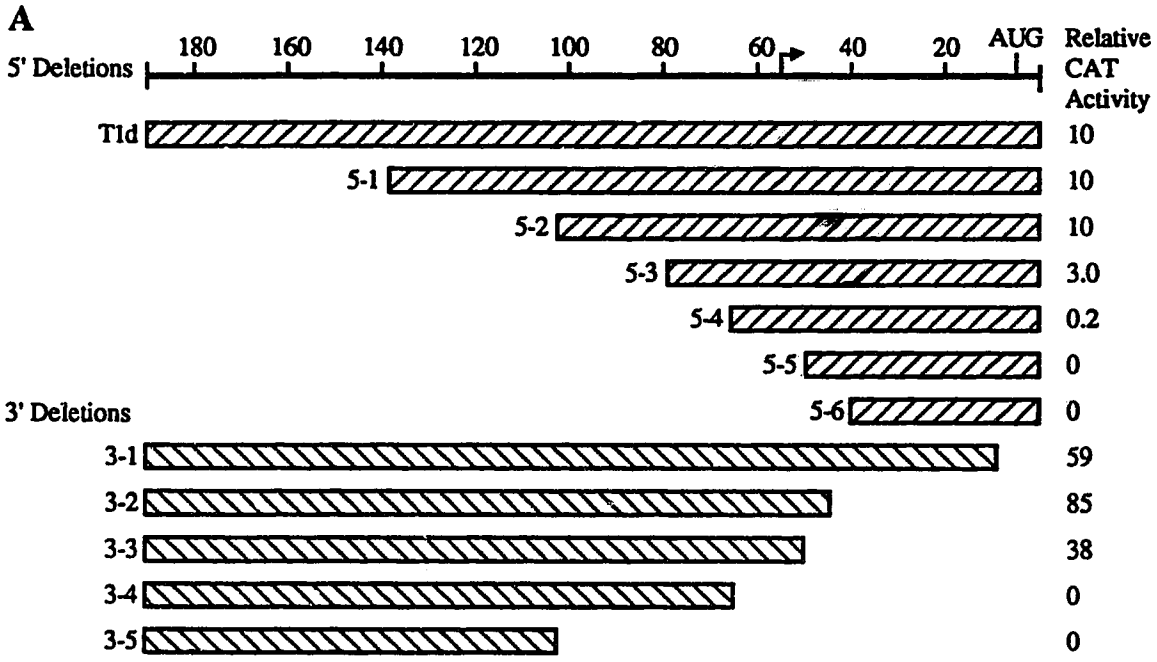
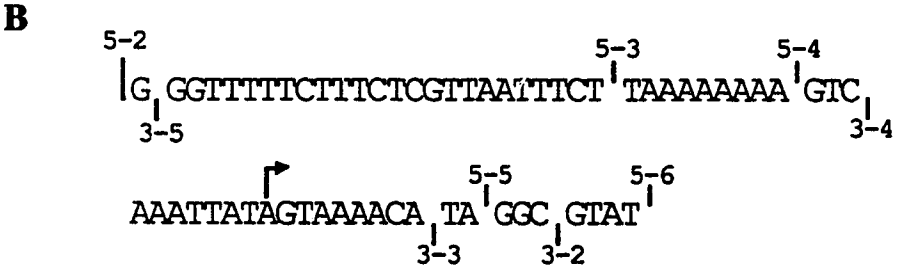


FIGURE 16. Summary of the deletion analysis and primer extension results. (A) The position of each deletion is illustrated with respect to the full-length clone (T1d), the transcriptional start site (arrow), and the AUG of the T1 ORF. The percentage CAT activity was determined from the CAT assays indicated in Fig. 15 and normalized so that the full-length T1d clone would have a value of 10 . The activities for the 5' deletions are an average of two transfection experiments while the 3' deletions are an average of three transfection experiments. The hatched bars indicate the SFV T1 promoter sequences present in each clone. (B) The position of each deletion with respect to the T1 sequence is shown along with the major transcriptional start site. (C) The 12 amino acid mini-ORF which lies between the T1 transcriptional start site and the ATG of the fused CAT gene (see text). This ORF has been deleted of its initiation codon in all 3' deletions.





C

```
GTC ATG AGG GGG GAT CAG CTT GGC GAG ATT TTC AGG AGC TAA GGA AGC TAA A ATG
    Met Arg Gly Asp Gln Leu Gly Glu Ile Phe Arg Ser stop        stop  Met(
```

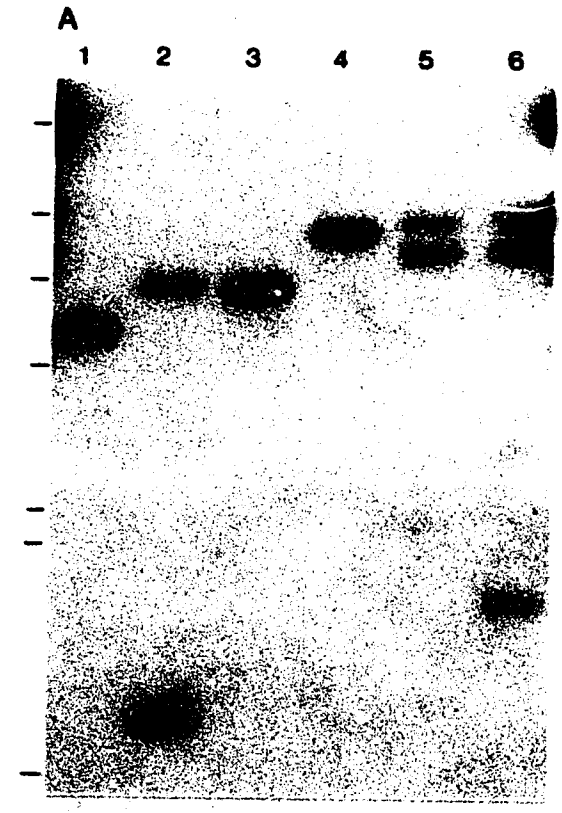
increase in the level of the CAT gene transcript; however, I never measured the levels of the transcripts in each deletion and that would be a more accurate way of determining promoter strength. The increase in CAT activity in deletions 3-1, 3-2 and 3-3 is likely due to the removal of the AUG for T1 ORF which could result in and increase in the translation of the downstream CAT gene. Because of the way the CAT fusions were constructed, a 12 amino acid ORF was created by fusing the first two amino acids of the T1 ORF in frame to a 10 amino acid ORF located in the upstream region of the CAT gene cassette (Fig. 16C). This mini leader ORF is present in the full length T1d clone, all the 5'-deletions, and in the recombinant vaccinia virus (see part F). When the T1 ORF AUG codon is removed in the 3' deletions the first AUG now corresponds to the actual start of the CAT ORF. Others have noted that removal of upstream AUGs improves the expression of downstream genes in recombinant vaccinia virus (King *et al.*, 1987).

F. RECOMBINANT VIRUSES

It has been demonstrated by others that early and late vaccinia promoters function when either on a plasmid introduced into infected cells in the transient expression assay, or inserted directly into the viral genome of a recombinant vaccinia virus (Cochran *et al.*, 1985a). We have noted that both the SFV T1d early promoter and the vaccinia 7.5-kDa early/late promoter function efficiently in the transient expression assay when cells are infected with either SFV or vaccinia. To ensure that this observation holds true when the promoters are recombined within the heterologous poxvirus genome, recombinant viruses were constructed. In one case the SFV T1d early promoter driving the CAT gene was inserted into the TK gene of vaccinia virus to give plasmid pVT1dCAT, and this plasmid was used to generate VacT1dCAT (Fig. 19D). Similarly, the CAT gene driven by the vaccinia 7.5-kDa early/late promoter was inserted into the SFV TK insertion vector pSTΔKLac5, which contains the *lacZ* gene under the control of the vaccinia p11 promoter, to create pSTKVCAT. This latter plasmid was used to generate the recombinant virus

SFVV7.5CAT (Fig. 19C). The recombinant viruses were made as discussed in chapter II, and in order to ensure that the stocks of virus which were prepared did not have any traces of wild type virus and that insertion into the viral genome occured as expected, cytoplasmic DNA from infected cells was analysed by Southern blotting. The results of probing duplicate blots with ^{32}P-labeled vaccinia or SFV TK DNA (panel A, Figs. 17 and 18), or with ^{32}P-labeled CAT DNA (panel B, Figs. 17 and 18) show that there are no traces of wild type virus DNA in these preparations and that the recombinant genomes are organized as diagramed in Fig. 19. Both recombinant viruses efficiently express the CAT gene product and the recombinant SFV virus was selected for its ability to make blue foci and hence expresses β-galactosidase activity (not shown). Total cellular RNA was purified from BGMK cells infected with VacT1dCAT (m.o.i.=10) at 0, 2, 4, and 6 hours after infection and at 6 hours after infection in the presence of cycloheximide. Primer extension analysis was performed using this RNA and an antisense CAT oligo probe (Fig. 20). Panel A shows that there are two major extended products of about 0.13 kb and 0.07 kb (E1 and E2, respectively) which are present at early times (2 and 4 hrs), not at late times (6 hrs) and accumulate to large amounts in the presence of cycloheximide, consistent with the kinetics of early transcripts (Cochran *et al.*, 1985b). When the extended products from the 6Ch lane are analyzed in parallel to a dideoxy sequencing reaction using the same oligo primer (panel B), it is clear that the largest extended product corresponds to precisely the same initiation site that was observed for the native T1 promoter in SFV. When the extended products derived using RNA isolated from BGMK cells infected with SFVV7.5CAT in the presence of cycloheximide are analyzed in parallel to the corresponding dideoxy-sequencing reaction (panel C), it can be seen that the largest major band corresponds precisely with the start site which has previously been described for the native vaccinia 7.5-kDa early promoter (Venkatesan *et al.*, 1981). The smaller extended products (E2, panel A), which are detected when RNA from cells infected by either recombinant virus is tested (panel B and C), map to the junction between the promoter

FIGURE 17. Southern blot analysis to determine the purity and orientation of CAT sequences in the recombinant virus VacT1dCAT. Cytoplasmic DNA from either vaccinia (324ng, lanes 1, 3 and 5), or VacT1dCAT (390ng, lanes 2, 4 and 6) infected cells was digested with *Hind*III (lanes 1 and 2), *Bam*HI (lanes 3 and 4), or *Eco*RI (lanes 5 and 6), electrophoresed in a 0.7% agarose gel and blotted to Hybond-N membrane. Blot (A) was probed with nick translated vaccinia TK DNA, and blot (B) was probed with nick translated CAT gene DNA. The markers to the left of each fluorogram indicate the position of lambda *Hind* III marker DNA. From the top of the gel to the bottom these are: 23kb, 9.4kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb, and 0.5kb.


A
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2
3
4
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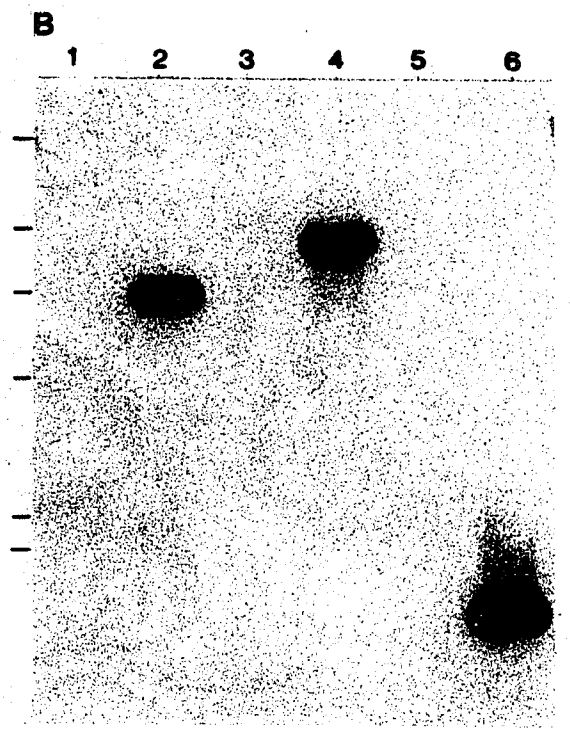
6


B
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6

FIGURE 18. Southern blot analysis to determine the purity and orientation of CAT sequences in the recombinant virus SFVV7.5CAT. Cytoplasmic DNA from either SFV (312ng, lanes 1, 3, 5 and 7), or SFVV7.5CAT (284ng, 2, 4, 6 and 8) infected cells was digested with *Hind*III (lanes 1 and 2), *Bam*HI (lanes 3 and 4), *Hind*III plus *Bam*HI (lanes 5 and 6), or *Eco*RI (lanes 7 and 8), electrophoresed in a 0.7% agarose gel and blotted to Hybond-N membrane. Blot (A) was probed with nick translated vaccinia TK DNA, and blot (B) was probed with nick translated CAT gene DNA. The markers to the left of each fluorogram indicate the position of lambda *Hind* III marker DNA. From the top of the gel to the bottom these are: 23kb, 9.4kb, 6.7kb, 4.3kb, 2.3kb, 2.0kb, and 0.5kb.

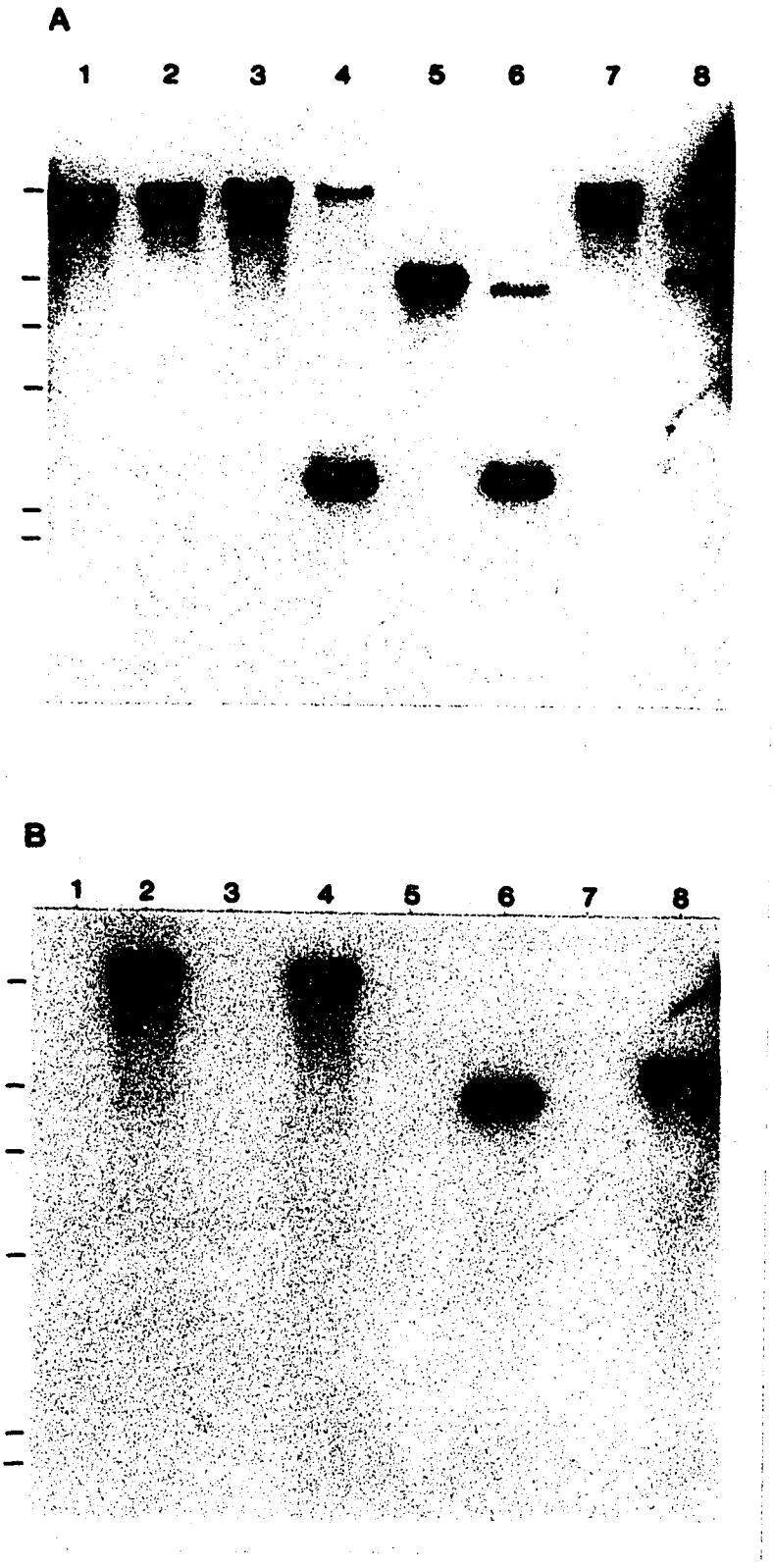
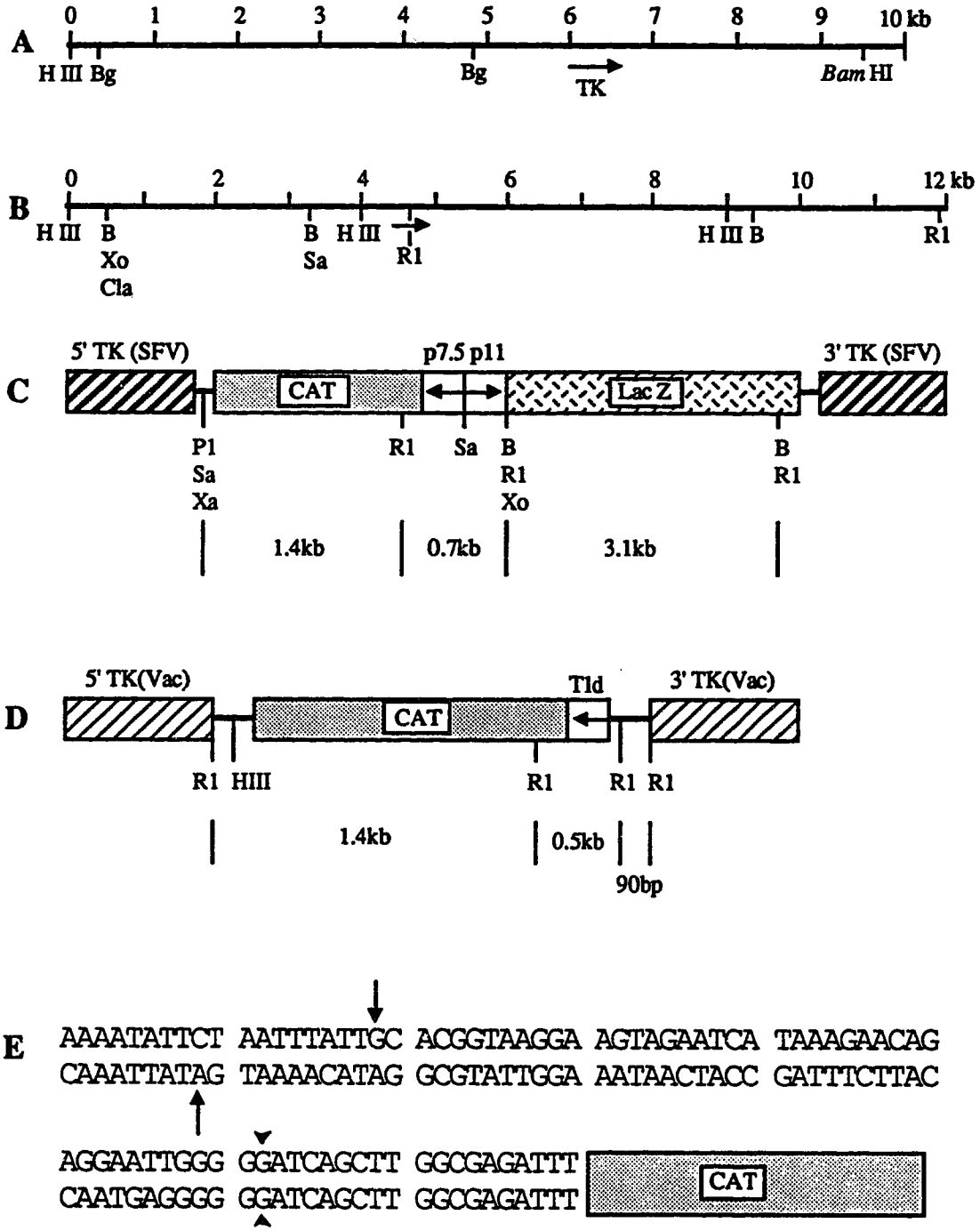

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

FIGURE 19. Recombinant SFV and vaccinia virus. (A) A genomic map of the regions around the SFV TK gene (arrow) (Upton and McFadden, 1986b). (B) A genomic map of the regions around the vaccinia TK gene (arrow) (Bajszar *et. al.*, 1983; Mahr and Roberts, 1984a; Pluciennicak *et. al.*, 1985). (C) An illustration the structure of the recombinant virus SFVV7.5CAT, containing the *lacZ* gene driven by the vaccinia p11 late promoter and the CAT gene driven by the vaccinia p7.5 early/late promoter, inserted into the SFV TK gene. (D) An illustration of the structure of the recombinant virus VacT1dCAT, containing the CAT gene driven by the SFV T1d promoter, inserted into the vaccinia TK gene. (E) A diagram of the results of primer extension analysis (Fig. 20), showing the major transcriptional initiation sites (arrows) which are used in the recombinant SFV (top row) and vaccinia virus (bottom row) by the 7.5-kDa early promoter and the T1d early promoter, respectively. The carets denote presumptive reverse transcriptase pause sites detectd during primer extension (Fig. 20).


A
0 1 2 3 4 5 6 7 8 9 10 kb
H III Bg
Bg
TK
Bam HI
B
0 2 4 6 8 10 12 kb
H III B Xo Cla
B Sa
H III
R1
H III B
R1
C
5' TK (SFV)
p7.5 p11
3' TK (SFV)
CAT
Lac Z
P1 Sa Xa
R1
Sa
B R1 Xo
B R1
1.4kb
0.7kb
3.1kb
D
5' TK(Vac)
Tld
3' TK(Vac)
CAT
R1 HIII
R1
R1 R1
1.4kb
0.5kb
90bp
E
AAAATATTCT AATTTATTGC ACGGTAAGGA AGTAGAATCA TAAAGAACAG
CAAATTATAG TAAAACATAG GCGTATTGGA AATAACTACC GATTTCTTAC
AGGAATTGGG GGATCAGCTT GGCGAGATTT
CAATGAGGGG GGATCAGCTT GGCGAGATTT
CAT

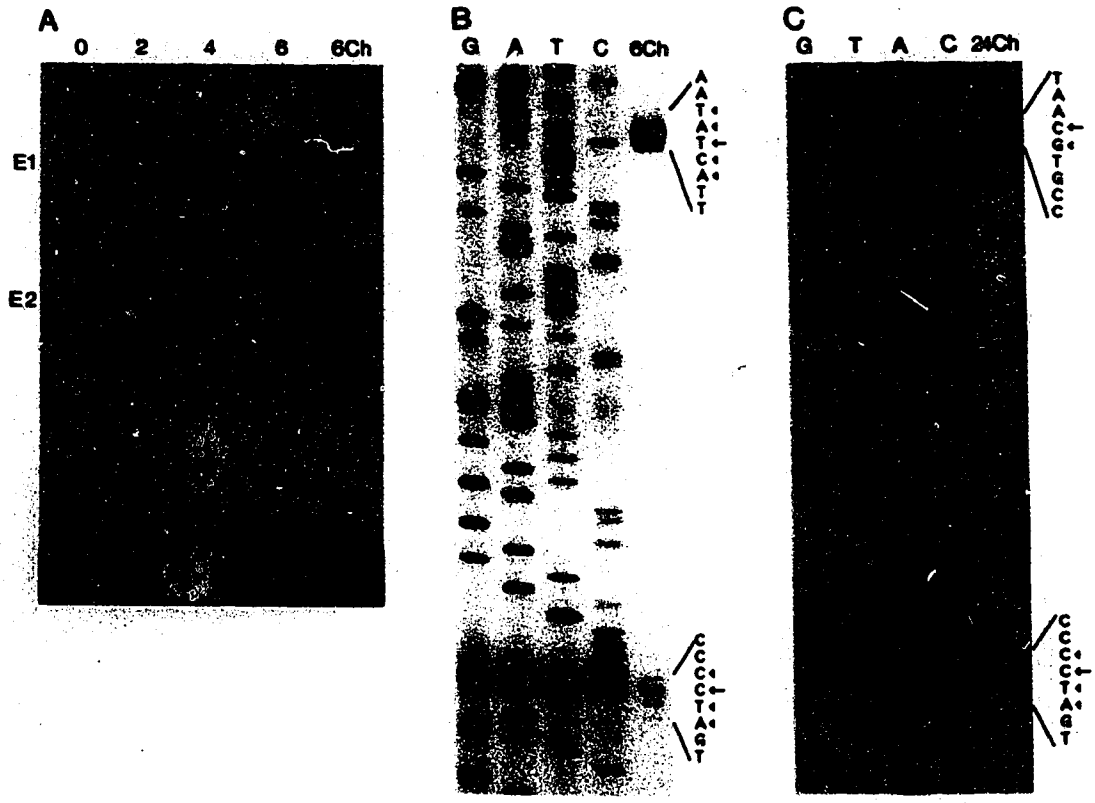


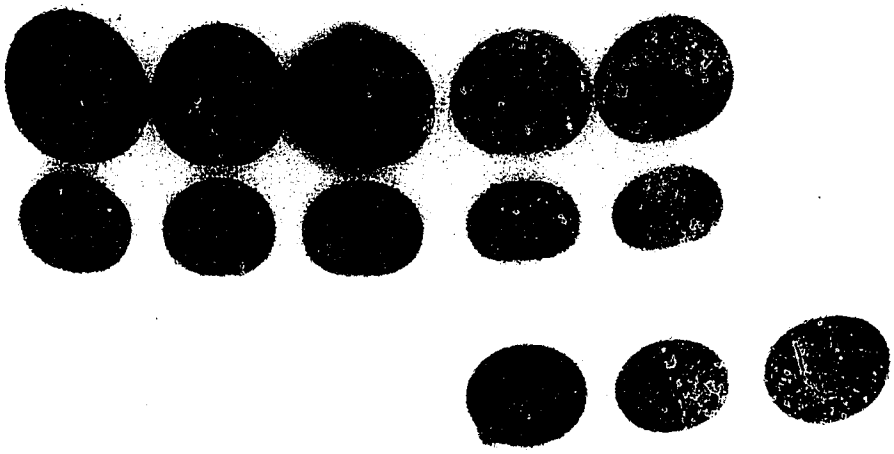
FIGURE 20. Primer extension analysis of the CAT gene in the recombinant poxviruses. (A) A 17-nucleotide ^{32}P-end-labeled antisense CAT oligo was hybridized to 20μg of total cellular RNA harvested from BGMK cells infected with VacT1dCAT (m.o.i.= 10) at 0, 2, 4, or 6hr post-infection, or 6-hr post-infection in the presence of cycloheximide (6Ch). The products of the reverse transcription reaction were electrophoresed on an 8% urea-acrylamide gel and the extended products (E1 and E2) are marked beside the fluorogram. (B) The extended products from lane 6Ch in (A) were analyzed next to the corresponding dideoxy sequencing reaction products. (C) Similarly, the extended products form a primer extension reaction using RNA harvested from BGMK cells infected with SFVV7.5CAT (m.o.i.= 1.0) for 24 hr in the presence of cycloheximide (24Ch) are compared to the corresponding dideoxy sequencing reactions products. The positions of the extended products are shown on the antisense sequences to the right of the fluorograms (B and C) and on the sense strand sequences indicated in Fig. 19E.

sequences and the 5' end of the CAT cassette sequences (carets in Fig. 19E). Whether these are *bona fide* initiation sites or pausing artifacts of the reverse transcription reaction can not be unambiguously determined from this data, but it is noteworthy that the site marks the begining of a stretch of 5-6 continuous G residues. It is nevertheless clear from these results that heterologous poxvirus promoters are recognized in exactly the same fashion, at least in terms of temporal regulation and position of transcriptional initiation, as they are in their native viral environment.

G. EFFECT OF ARA C ON TRANSIENT CAT GENE EXPRESSION

The results of primer extension analysis have shown that the T1 promoter acts as an early promoter in both vaccinia virus and in SFV. Cochran *et al.*, (1985 a, b), have demonstrated that most of the CAT activity observed in a transient gene expression assay using the vaccinia 7.5-kDa early/late promoter comes from the late promoter. In order to be sure that the CAT activity observed in transient expression assays using the T1 promoter was due to early transcription, I measured by how much Ara C, an inhibitor of DNA replication and thus late gene expression, reduced the transient expression from both the T1 and the 7.5-kDa early/late promoter. The CAT assays from SFV infected cells are shown in Fig. 21. The results are summarized in Table VI along with those of Cochran *et al.*, (1985a). Even though these CAT assays are not in the linear range of enzymatic activity and the levels of inhibition by Ara C may be greater, it is nevertheless clear that when the two plasmids are transfected under identical conditions, Ara C inhibits late promoter expression the most (28-kDa), and early promoter expression the least (TK). Cochran *et al.*, (1985a) have shown that expression from the 7.5-kDa early/late promoter in a transient system occurs primarily from the late promoter, even though the early promoter is more active when it is examined in its normal position within the vaccinia genome. My results also indicate that in a transient expression system the 7.5-kDa early/late promoter behaves like a late promoter, and that the SFV T1 promoter behaves like an early promoter.

FIGURE 21. Effect of Ara C on transient expression assays. (A) Transient CAT gene expression assays using pPROT1dCAT (deletion 3-2) (lanes 1, 2, 4 and 5) or p7.5CATA (lanes 3 and 6) were performed either with (lanes 4-6), or without (lanes 1-3) Ara C (40μg/ml). Transfections of both vaccinia and SFV infected cells were done but only the vaccinia infected cells are shown. The percent of CAT acetylated in each lanes 1-6 is 98, 98, 98, 47, 59, and 10% respectively. It is important to note that this experiment was not performed within the linear range of enzymatic activity so the absolute value of inhibition by Ara C could be even greater than what is shown; however, this would not change the comparison between T1 and the 7.5-kDa promoters. (Table VI) Summary of the results showing the percent to which CAT gene expression was inhibited by Ara C. The T1 promoter results are an average of two transfection for both SFV and vaccinia infected cells. These results are compared to those from Cochran *et. al.*, (1985a).



1 2 3 4 5 6

TABLE VI

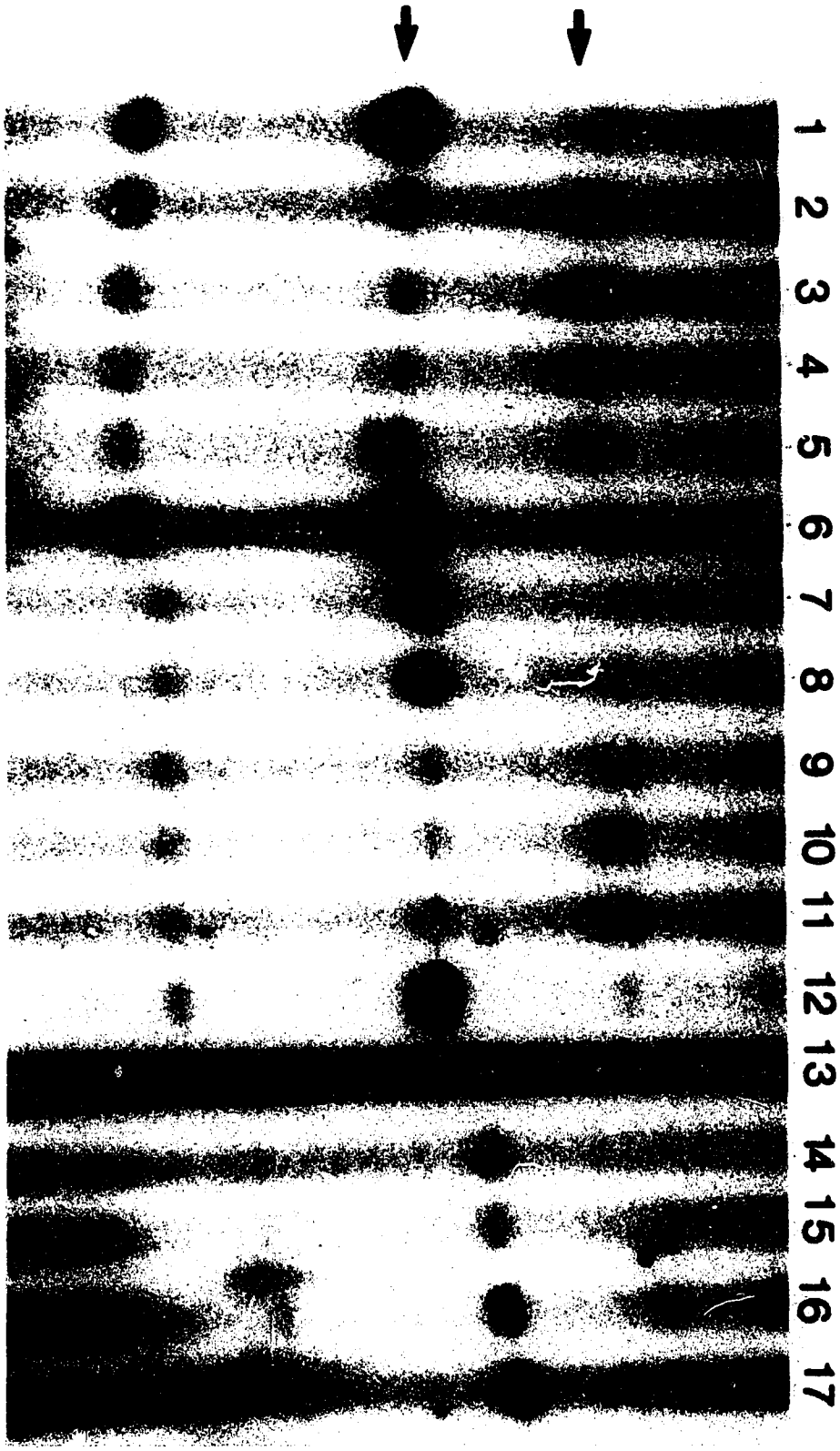
% INHIBITION OF CAT ACTIVITY		
Promoter	Vaccinia Infected Cells	SFV Infected Cells
T1 (3-2)	46%	6.6%
7.5-kDa	90%	82%
*7.5-kDa	74%	
*TK	45%	
*28-kDa	80%	

*From Cochran *et al.*, (1985a).

H. EFFECT OF M.O.I. ON PRIMER EXTENSIONS.

It is clear that the T1 ORF is an early gene and yet the S1 analysis in chapter III indicated the T1 transcript was more easily detected at late times compared to early times. We speculated that this may have been due to the low multiplicity of infection in those studies. In order to look at the effect of the multiplicity of infection on transcription from early promoters in SFV, transcription from a well characterized vaccinia early promoter, the 7.5-kDa promoter, was examined. To accomplish this RNA was isolated from SFVV7.5CAT infected cells at different times after infection using multiplicities of 10, 1, and 0.1 ffu/cell, and the presence of the CAT transcript starting at the 7.5-kDa early promoter was detected by primer extension analysis. The results of primer extension analysis using the CAT antisense oligo primer are shown in Figs. 22, 23 and 24. The second arrow from the top of the fluorogram in Figs. 22 and 23 indicates the major extended product corresponding to the early transcriptional start site for the 7.5-kDa promoter. Unlike most early transcripts in vaccinia, these transcripts are clearly present throughout the infection at all multiplicities. At multiplicities of 10 (lanes 1-6) and 1 (lanes 7-12), these transcripts are more predominant at early times than at late times and they accumulate in the presence of cycloheximide. However, at a multiplicity of 0.1 (lanes 13-17, Figs. 22 and 24) these transcripts are more predominant at late times (lane 16) than they are at early times (lanes 13 and 14). In Fig. 22 lanes 1-6 were first loaded and electrophoresed into the gel, then lanes 7-12 were loaded briefly electrophoresed and finally lanes 13-17 were loaded, so the products in lanes 13-17 which appear to be larger than those in the preceding lanes are actually the same size. Lane 13 in Fig. 22 has a streak running through it so to make sure that this was not a problem with the sample itself two new gels were run, one containing samples 1-12 (Fig. 23), and one containing samples 13-17 (Fig. 24). The first arrow from the top of the fluorograms in Figs. 22 and 24 shows a band that is seen only at late times and in the absence of cycloheximide, and it may correspond to the late 7.5-kDa promoter. These results confirm the S1-nuclease analysis in

FIGURE 22. Effect of the multiplicity of infection on the primer extension analysis of the 7.5-kDa early/late promoter in SFVV7.5CAT. A stock of SFVV7.5CAT ($1.5x10^8$ffu/ml) was diluted to infect 100mm plates of BGMK cells at multiplicities of 10, 1, and 0.1. The RNA was extracted from the cells at different times after infection using five plates of cells for each time point. At an m.o.i.=10 RNA was harvested at 5, 8.5, 12, 16, and 24 hr post-infection (lanes 1-5), and at 16hr post-infection in the presence of cycloheximide (lane 6). At an m.o.i.=1 RNA was harvested at 5, 8.5, 12, 16, and 24 hr post-infection (lanes 7-11), and at 16hr post-infection in the presence of cycloheximide (lane 12). At an m.o.i.=0.1 RNA was harvested at 5, 8, 11.5, and 24 hr post-infection (lanes 13-16), and at 24hr post-infection in the presence of cycloheximide (lane 17). The products of the reverse transcription reaction using the CAT oligo primer were electrophoresed on an 8% urea-arcylamide gel. The two arrows to the left of the fluorogram indicate the major transcripts corresponding to the 7.5-kDa early (bottom arrow), and possibly the late (top arrow) promoters.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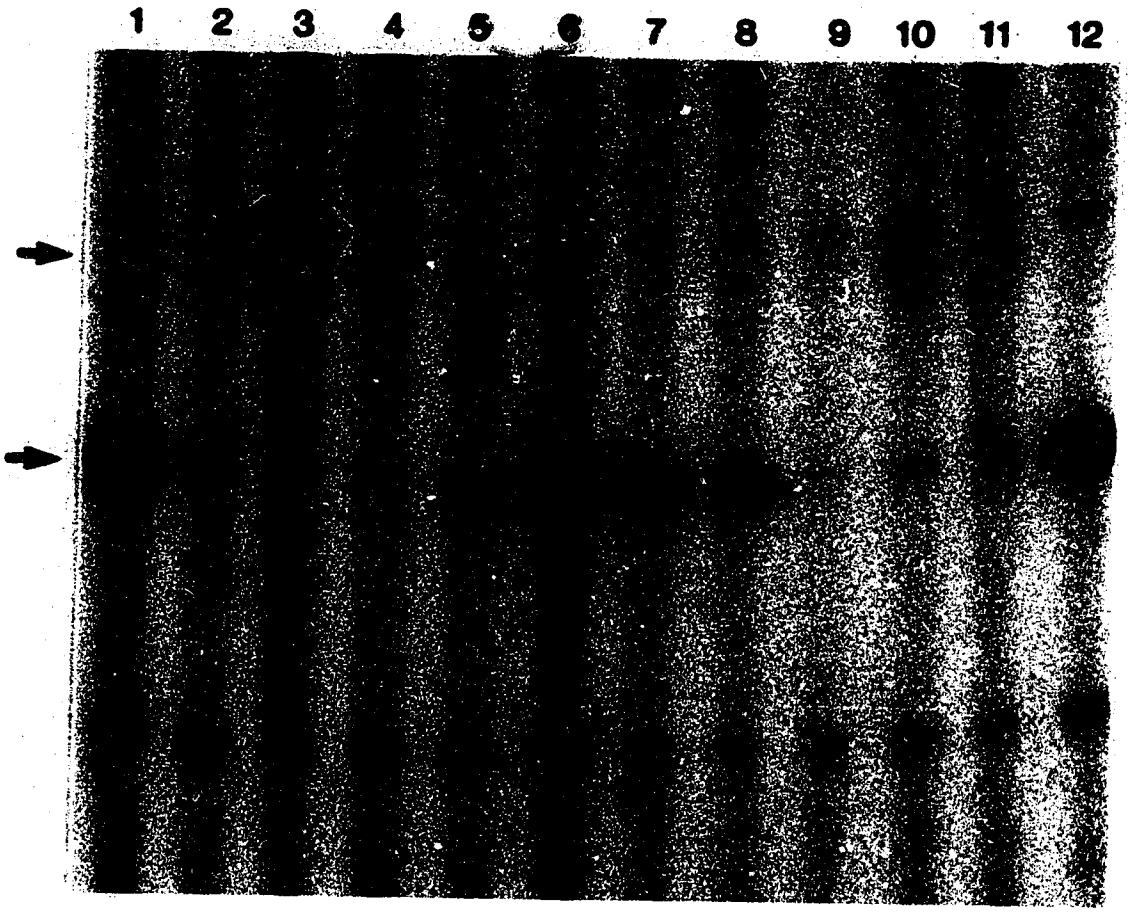

FIGURE 23. The products from lanes 1-12 from Fig. 22, run on a second gel which shows more clearly the late transcription products (top arrow).

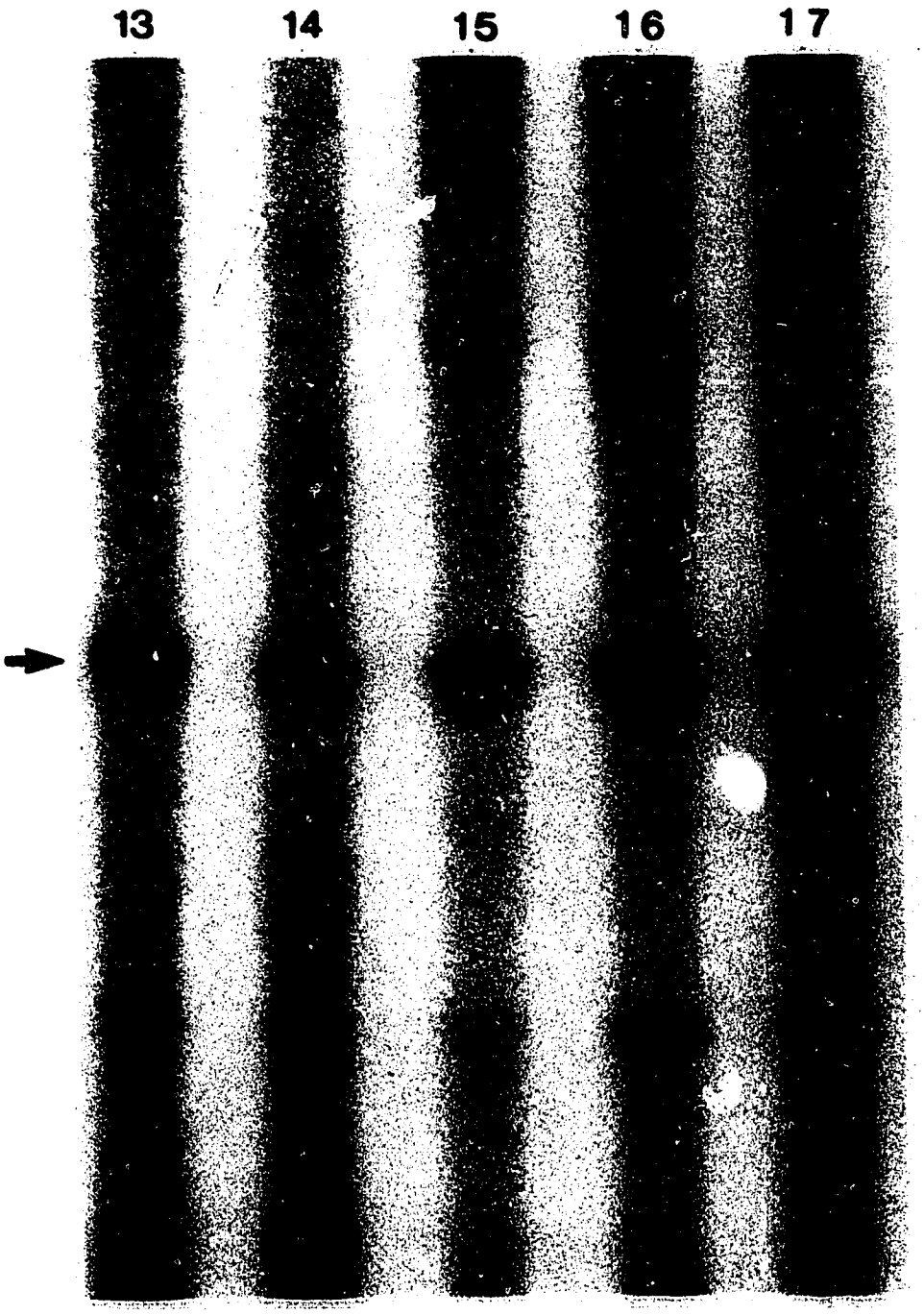

FIGURE 24. The products from lanes 13-17 from Fig. 22 run on a second gel to show more clearly the intensities of the primer extension reaction products corresponding to the 7.5-kDa early promoter (arrow).

chapter III and show that when a low m.o.i. is used the products of early transcription seem more abundant at late times (early times for the second round of infection) than at early times.

I. DISCUSSION

We have used deletion analysis and a transient expression assay to determine the functional sequences required for the early promoter activity of the T1 ORF which maps within the TIR of SFV. Like other early poxvirus promoters the important sequences are contained within approximately 30bp of the transcriptional start site (Cochran *et al.*, 1985b; Weir and Moss, 1987b; Ink and Pickup, 1989), and this stretch of viral DNA is decidedly AT rich. In fact, a series of eight continuous A residues at position -18 to -11 appears critical for T1 promoter function, though upstream sequences do augment promoter activity. This region may correspond to the binding site of a vaccinia early transcription factor which has been shown to protect from DNase 1 the upstream regions of the vaccinia growth factor gene promoter (-14 to -29), and the 7.5-kDa early promoter (-19 to -29) (Yuen *et al.*, 1987; and Broyles *et al.*, 1988). However, the SFV T1 promoter cannot consist of only this element because alone the A octamer is not enough for promoter activity, and sequences which include the transcriptional initiation site are also required.

We have shown that the cis-acting target sequences which constitute a promoter element, and presumably the transcriptional machinery which recognizes these sequences, are functionally conserved between the *lepori-* and the *ortho*-poxviruses. The T1 promoter of SFV behaves as an early promoter and the identical transcriptional initiation site is observed when it is imbedded within either the vaccinia virus or SFV DNA genomes. Similarly, the well characterized vaccinia 7.5-kDa gene early promoter uses the identical start site when it is within either SFV or vaccinia. We are confident that primer extension detects the *bona fide* start position since the early start site that was determined for the 7.5-kDa early promoter (Fig. 7) was identical to that determined by Venkatesan *et al.*, (1981)

using S1 nuclease protection analysis. We cannot verify from this work if a second smaller species seen in the reverse transcription reactions, and which maps to the junction between the T1 or 7.5Kd promoter sequences and the CAT cassette (Figs. 6 and 7), is due to pausing artifacts in the reverse transcription reaction or if they are aberrant transcription initiation sites. We suspect that they are unlikely to represent true sites of initiation of transcription because (1) these sites map to identical positions in the two promoter constructs even though the upstream sequences are very different (Fig. 6), (2) all known early poxvirus promoters are contained within about 30bp of the transcriptional initiation sites and are very AT rich (Bertholet *et al.*, 1986; Cochran *et al.*, 1985b; Hänggi *et al.*, 1986; Ink and Pickup, 1989; Miner *et al.*, 1988; Wier and Moss 1987a, b), but the sequences upstream of these mapped sites are GC rich compared to the average AT content of the two viral genomes, (3) these sites are not observed when the wild type promoters are examined, and (4) if they were true initiation sites of transcription then one would predict that they should be present in all the 5' deletions and thus allow for CAT expression in deletions 5-4 and 5-5. Notwithstanding, the presence of this species does not take away from the fact that an early promoter from SFV functions identically in vaccinia virus with respect to temporal regulation and site of initiation of transcription.

A comparison of the 5' boundaries of the early poxvirus promoters which have been determined by deletion analysis is illustrated in Fig. 24. Cheng *et al.*, (personal communication), has demonstrated that addition of 'A' residues onto a 5' deleted promoter for the SFV growth factor can restore promoter function, and Yuen *et al.*, (1987a) has demonstrated that changing an A nucleotide to a C at position -13 or -26 in the vaccinia 7.5-kDa early promoter will reduce the binding of the vaccinia early transcription factor. Except for this requirement for multiple A residues, it is difficult to assign a consensus promoter sequence, at least among these five early poxvirus promoters that have been rigorously defined by deletion analysis.

SFV-T1	TTTCTCGTTAATTTCTTAAAAAAAAGTCAAATTAT A GTAA
Vac-7.5-kDa	TTCTCGTAAAAGTAGAAAATATATTCTAATTTATT G CACG
Vac-TK	AGTCGAATAAAGTGAACAATAATTAATTCTTTATT G TCAT
CPV-38-kDa	GTTTATACAAGATTGAAAATATATTTCTTTTTATT G AGTG
SFV-GF	TTTATTCAGGAAAAATATAAATATAATACGACATA C TATA

FIGURE 25. A comparison of five early poxvirus promoter sequences. The sequences of the poxvirus early promoters which have been analyzed by 5'-deletion analysis are shown from 35 nucleotides upstream from the major transcriptional start site (*) to 4 nucleotides downstream. The sequences that are underlined indicate the deletion defined position of the sequences at the 5'-boundary that are critical for promoter activity. SFV-T1, the SFV T1 promoter; Vac-7.5-kDa, the vaccinia 7.5-kDa early promoter (Cochran *et al.*, 1985b); Vac-TK, the vaccinia thymidine kinase promoter (Weir and Moss, 1987b); CPV-38-kDa, the cowpox virus 38-kDa promoter (Ink and Pickup, 1989); SFV-GF, the SFV growth factor promoter (Cheng, personal communication).

CHAPTER V
GENERAL DISCUSSION

The objective of this work was to transcriptionally characterize the TIRs of the *leporipoxvirus* SFV, and to compare its transcriptional regulatory system to that of the *orthopoxvirus* vaccinia. This work has demonstrated that transcription within the TIRs of SFV is much more complex than within the vaccinia TIRs, which reflects the different ORF arrangement between these two viruses. I have also shown that the sequences which function as early promoters, and presumably the transcriptional machinery which recognize these sequences, are conserved between these two genera. With the additional findings that the consensus sequences specifying early transcriptional termination in vaccinia are found exclusively at the 3'-ends of the ORFs within the SFV TIRs, and that the bacterial *lac* Z gene driven by the vaccinia late promoter p11 is expressed in a recombinant SFV, it seems likely that the regulation of both early and late gene expression is conserved.

This conservation appears not to be restricted to just the *leporipoxviruses*. The DNA genome of fowlpoxvirus (FPV), an *avipoxvirus*, is not homologous to vaccinia at the level of DNA:DNA hybridizations (Boyle and Coupar, 1986). Nevertheless, several vaccinia promoters (H6, p7.5 and p11) have been used to express foreign genes in recombinant FPV (Boyle and Coupar, 1988b; Taylor *et al.*, 1988a, b). Similarly when the FPV TK gene with its own promoter is placed into a vaccinia insertion vector it will rescue TK^- vaccinia virus under selective conditions implying that the FPV TK promoter is recognized in vaccinia virus. It has also been reported that the vaccinia p7.5 promoter functions in a transient expression assay using *capripoxvirus* KS-1 infected cells (Gershon and Black, 1989). The KS-1 viral DNA is somewhat homologous to vaccinia virus at the level of DNA:DNA hybridizations (Gershon *et al.*, 1989). Sequence analysis of FPV and KS-1 genomes shows that consensus sequences for the termination of early gene

transcription in vaccinia are found exclusively at the 3'-end of some ORFs suggesting that this sequence functions in the same manner in these genera of poxviruses (Gershon and Black, 1989, Tomley *et al.*, 1988).

Another similarity between poxvirus genera is in the replication of their viral genomes. When plasmid DNA is transfected into SFV or vaccinia infected cells it is replicated in a sequence independent fashion (DeLange and McFadden, 1986; Merchlinsky and Moss, 1986). If these plasmids contain the replicative intermediate forms of the hairpin termini from either vaccinia or SFV they are resolved into normal hairpin termini structures resulting in a linear plasmid molecule with two hairpin ends (DeLange *et al.*, 1986; DeLange and McFadden, 1987; Merchlinsky and Moss, 1986). This occurs if the cells are infected by either vaccinia or a *leporipoxvirus* so it seems that, as with transcription, the target sequences and machinery which resolves replicated poxvirus genomes into daughter genomes is conserved between these two poxvirus genera (DeLange and McFadden, 1989; McFadden *et al.*, 1988; Merchlinsky and Moss, 1989a).

Some of the immediate practical implications of such findings are that all the poxvirus promoters that have been isolated and characterized can be used without modification to express genes in other poxvirus infected cells either transiently or in the form of a recombinant virus. There is no evidence that homologous promoters work better than heterologous promoters; however, it is possible that the optimum promoter sequences for different genera of poxvirus could be different and this is something that can be tested. Since promoter functions are conserved this makes the productions of intertypic recombinant poxviruses much easier. A gene from one poxvirus can be inserted directly into a different genera of poxvirus without having to engineer a new promoter sequence upstream of this gene. For example, it is known that a segment of DNA from SFV containing the TK gene with its own promoter, can rescue TK^- vaccinia virus when placed into a vaccinia insertion vector and transfected into vaccinia infected cells (C. Upton, personal communication). The generation of intertypic recombinants is potentially a very

powerful tool in the elucidation of poxvirus gene function. It would permit the mapping of viral proteins for which a genus specific reagent, such as a non cross-reacting antibody, is available. Thus by inserting sequences from one genera (eg. *orthopoxvirus*) into the other genera (eg. *leporipoxvirus*), one could screen for the expression of novel antigenic phenotypes in recombinant virus infected cells. It is not yet known if an essential gene or a subunit from a multisubunit enzyme where protein:protein interactions may be important, from one genera of poxvirus can replace the equivalent gene in a different genera. If this is possible then it would assist in the creation and screening of conditional lethal mutations in essential poxviral genes. In the case where the genomes of the two poxvirus genera do not cross hybridize, an essential viral gene from one genus could be mutagenized *in vitro* and then recombined into the other genus so as to replace the endogenous wildtype gene. Recombinant virus could be screened for by use of a genus specific DNA probe, and then tested for a conditional lethal phenotype. Likewise a gene containing a conditional lethal mutation from one genera of poxvirus could replace the wildtype homologue in a different genera to create a new virus blocked at the same point in its replicative cycle. Defined intertypic recombinants constructed in this fashion could prove useful in dissecting the functions of viral genes essential for poxvirus replication and morphogenesis.

Another use for intertypic recombinants is to screen for viral genes which influence the biological differences seen between the various poxvirus genera. For example, why does SFV replicate more slowly, induce less cytopathology, and have a more restricted host range? Are there specific genes which influence these properties? SFV, unlike the related *leporipoxviruses* MRV and myxoma, does not grow in lymphocytes from adult rabbits. The claim has been made that a single *Bam* HI fragment from MRV DNA inserted into the SFV genome allows SFV to grow in lymphocytes (Strayer *et al.*, 1988). The sequences involved in this change in host range have not yet been characterized so it is not known if the MRV sequences are adding a new gene or replacing a SFV gene with its MRV counterpart. Nevertheless, such a change in host range may be very important in the virus

pathology for the rabbit as it may allow the recombinant virus to disseminate through the animal in a fashion analogous to the pathogenic profile of MRV. Host range genes of vaccinia have not yet been transfered to SFV so it is not known if SFV could be given the same broad host range as found among many *orthopoxviruses*. These sort of experiments may reveal a great deal about the molecular basis for host range in poxviruses.

Although this work has found many similarities in molecular biology between the *lepori-* and *orthopoxviruses* there are some differences. Primer extension analysis has demonstrated that early transcripts appear to be more abundant at late times in SFV than in vaccinia infected cells. CAT transcripts, as detected by primer extension analysis, were no longer apparent by six hours post-infection in VacT1dCAT infected cells, but CAT RNA can still be detected at late times in SFVV7.5CAT infected cells. The promoters in these two cases are different and identical transcripts have not been tested in the two viruses but it is known that early 7.5-kDa gene transcripts are not seen at late times in vaccinia infected cells (Cochran *et al.*, 1985b), and early T1 gene transcripts can be seen at late times in SFV infected cells. It is not known if the early RNA seen at late times in SFV infected cells is functional, but it is distinctly possible that overall, viral RNA is more stable at late times in SFV than in vaccinia infected cells.

A second difference in the molecular biology of the two viruses was first observed in transient transfection studies used to study replication, telomere resolution, and also recombination between the homologous portions of transfected plasmid DNA (Evans *et al.*, 1988; Merchlinsky, 1989). By using conditional lethal RNA polymerase assembly mutants it has been shown that both replication and recombination of transfected DNA requires early virus gene expression while resolution requires late virus gene expression (DeLange, 1989; Merchlinsky, 1989; Merchlinsky and Moss, 1989b). Both replication and recombination occur to much greater extents in *leporipoxvirus* infected cells than in vaccinia infected cells (DeLange *et al.*, 1986; Evans *et al.*, 1988). This may reflect differences in the amount of the enzymes involved, their activity, the stability of the cognate transcripts,

or the accessibility of the enzymes to the target sequences. How these differences in the molecular biology of poxvirus genera are related to the biological differences will depend on an understanding of the virus genes involved. Relatively little is known about the differences between poxvirus genera at the molecular level but it seems very likely that the important sequences which regulate the expression, and resolution of the poxvirus genome are conserved throughout the *chordopoxviruses*.

The data presented in this thesis is consistent with the hypothesis that all poxviruses have evolved from a common ancestor. The various genera are relatively conserved in the central regions of the genome where most of the essential genes are located, but have diverged considerably near the termini in terms of their coding repertoire, but not in their regulatory sequences. Thus a careful analysis of the inter-generic conserved features of the molecular machinery for such essential processes as transcription and replication should be invaluable in dissecting the structure/function relationships of the requisite viral proteins involved.

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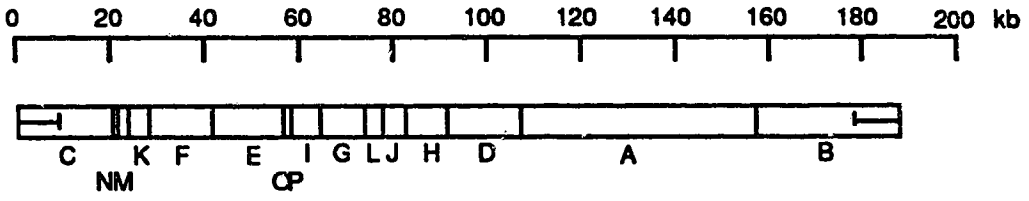
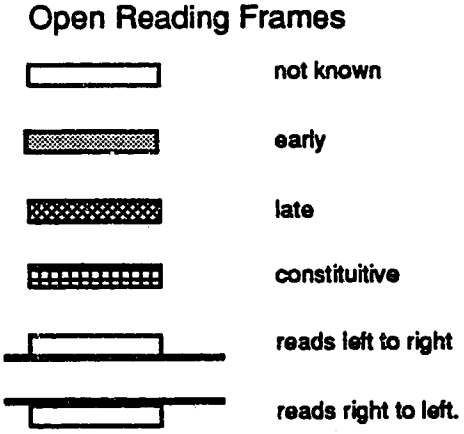
APPENDIX I
A PHYSICAL MAP OF THE VACCINIA VIRUS GENOME

A. INTRODUCTION

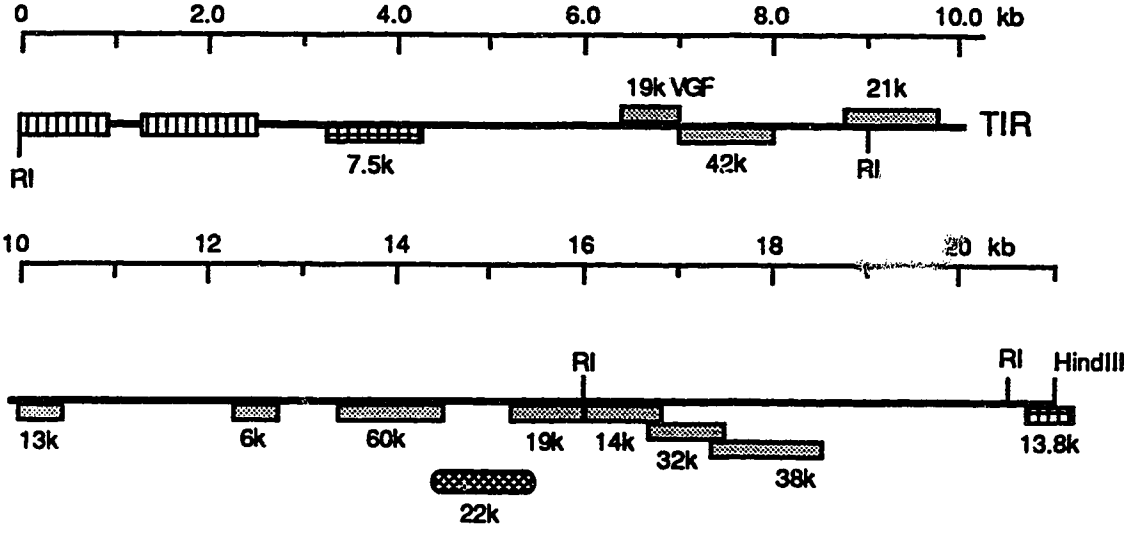
This places onto the *Hind* III map of the vaccinia genome, the positions of the open reading frames and their transcriptional class where it is known.

A Hind III map of the vaccinia WR genome





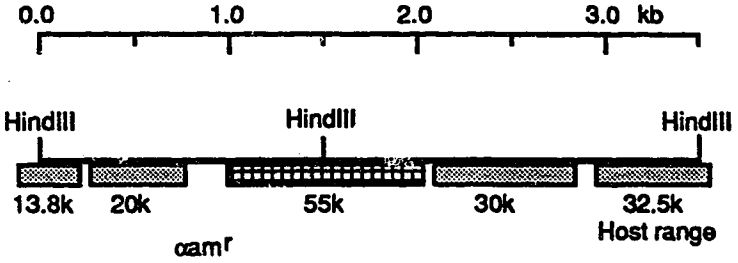
B. THE INVERTED TERMINAL REPEATS AND *HIND* III C



By nicking the viral DNA with S1 nuclease and then self-annealing the separated strands of viral DNA Garon*et al.*,(1978), visualized the TIRs as a characteristic panhandle shaped molecule. The 9 kb terminal *Eco* RI fragment, which shows 'snap-back' reannealing kinetics, was cloned by Wittek *et al.*,(1980a), and shown to hybrid select early viral RNA which translates *in vitro* into 42, 19, and 7.5-kDa polypeptides. Using hybrid selection, these polypeptides were mapped to specific areas on the terminal EcoRI fragment (Cooper *et al.*,1981a), and the exact position of the transcripts were determined by S1-analysis (Venkateson *et al.*,1981, 1982; Venkateson and Moss, 1981). Sequences for the 19-kDa and 42-kDa early genes have been published (Venkateson *et al.*,1982). The transcriptional and translational maps for the 6.6 kbp and 5.5 kbp *Eco* RI fragments were determined by Wittek *et al.*,(1981), and Cooper *et al.*,(1981b). The start site for the 22-kDa late gene has not been located, but the approximate map position was determined by translation of hybrid selected mRNA. An attenuated vaccinia mutant 6/2 is missing most of *Hind* III C and does not secrete at least two polypeptides (35-kDa and 13.8-kDa) elaborated by wildtype vaccinia. The 35-kDa protein has been sequenced and it shows homology to a C4b-binding protein which functions in the complement control pathway. The 35-kDa protein may correspond to the 38-kDa protein on this map (Kotwal and Moss, 1988). The 13.8-kDa protein has been sequenced and was found to be transcribed at early and late times. When inactivated by insertional mutagenesis the virus is somewhat attenuated but not as much as the 6/2 mutant strain (Kotwal and Moss, 1989). The 19-kDa gene encodes

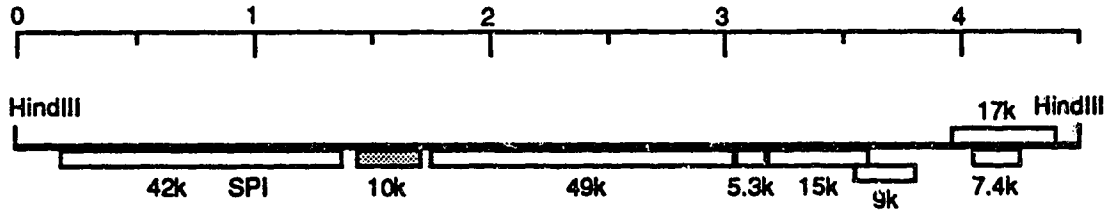
a nonessential EGF-like growth factor which also affects virus virulence (Blomquist *et al*, 1984; Brown *et al.*,1985; Buller *et al.*,1988a, b; Reisner, 1985; Twardzik *et al.*,1985).

C. *HIND* III N AND M



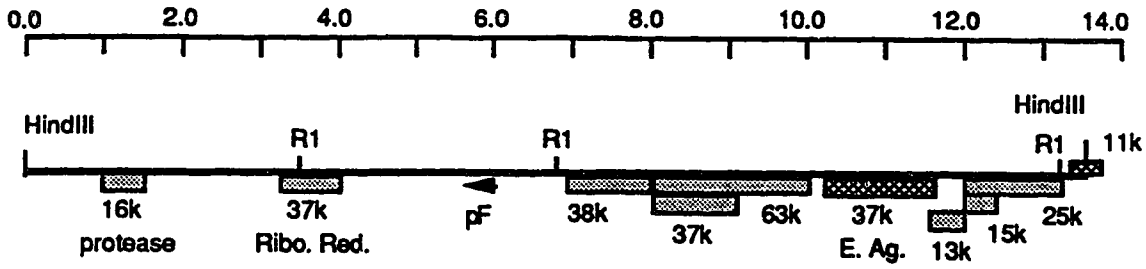
A transcriptional map for this region and part of *Hind* III K has been produced (Morgan and Roberts, 1984). The sequence for *Hind* III N and half of M has been presented and the 55-kDa gene may be the α-amanitin resistance locus (Tamin *et al.*,1988; Villarreal and Hruby, 1986). The 32.5-kDa gene has been sequenced and it has been shown to be a host range gene that allows vaccinia to grow on some human cell lines (Gillard *et al.*,1985, 1986).

D. *HIND* III K



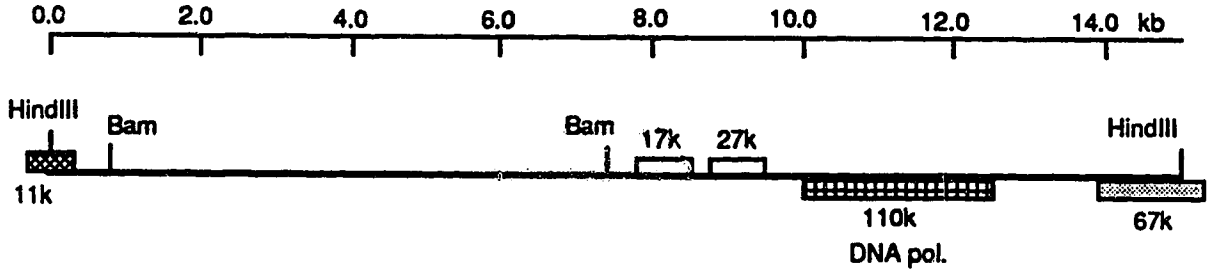
The *Hind* III K region has been sequenced and a transcriptional map for the left portion only detects the 10-kDa ORF (Boursnell *et al.*,1988; Morgan and Roberts, 1984). The 42-kDa gene is one of the three vaccinia genes related to the serine protease inhibitor superfamily of genes (serpins). The other two genes (SPI-1 and SPI-2) are in *Hind* III B fragment. The 49-kDa gene is homologous to a 37-kDa gene encoding a major envelope antigen in *Hind* III F. Belle Isle *et al.*,(1981) could only detect early gene products by in vitro translation of RNA hybrid selected by this fragment.

E. *HIND* III F



Transcriptional-translational analysis has concentrated on the right hand portion of this fragment (Golini and Kates, 1984). The 37-kDa late gene is a major envelope antigen (E. Ag.) found only on extracellular virus and not on intracellular virus (Hirt *et al.*,1986). The early promoter pF has been used to drive the expression of heterologous genes in transient expression analysis and in recombinant virus, and has been characterized by 3'-deletion analysis (Coupar *et al.*,1987; Panicali *et al.*,1983; Panicali and Paoletti, 1982; and Shepard *et al.*,1987). The gene which this promoter drives is apparently nonessential as shown by insertional mutagenesis (Panicali and Paoletti, 1982). The 37-kDa early gene is the small subunit of ribonucleotide reductase (Ribo. Red.) and has been sequenced and transcriptionally mapped (Slabaugh *et al.*,1988). The 16.2-kDa early gene has been sequenced and transcriptionally mapped and it shows homology with a retroviral protease-like gene (Slabaugh and Roseman, 1989).

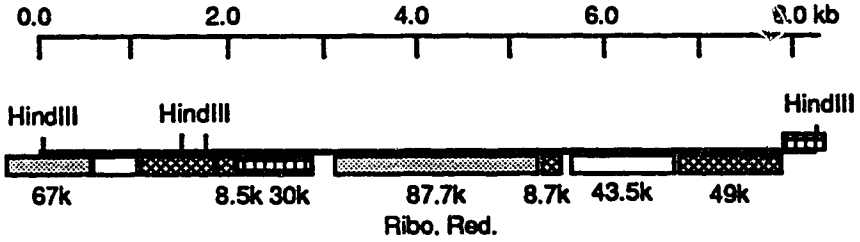
F. *HIND* III E



The sequence of 5.4 kb to the right of the *Bam* HI site is known, and the 110-kDa ORF codes for the DNA polymerase for which PAA resistance, aphidicolin resistance, and ts mutants are available (Challberg *et al.*,1979; DeFilippes, 1989; Earl *et al.*,1986; Jones and Moss, 1984, 1985; Moss and Cooper, 1982; Sridhar and Condit, 1983; Traktman *et al.*,1984). Originally it was considered strictly an early gene (Jones and Moss, 1984; Traktman *et al.*,1984), but recently Kunzi and Traktman (1989), have found separate early

and late start sites. The 11-kDa gene has been transcriptionally mapped and encodes a very basic, phosphorylated major late polypeptide found on the virus surface (Hiller and Weber, 1982; Kleiman and Moss, 1975a, b; Wittek *et al.*,1984). Others have identified an 11-kDa basic phosphoprotein which is associated with viral DNA in isolated factories and in virions (Pogo *et al.*,1975; Kao *et al.*,1981; Kao and Bauer, 1987), but it is not yet known if these two proteins are the same. *In vitro* translation of early RNA hybrid selected with this fragment gives the 36-kDa and 31-kDa RNA polymerase subunits (Jones *et al.*,1987).

G. *HIND* III O, P, AND I

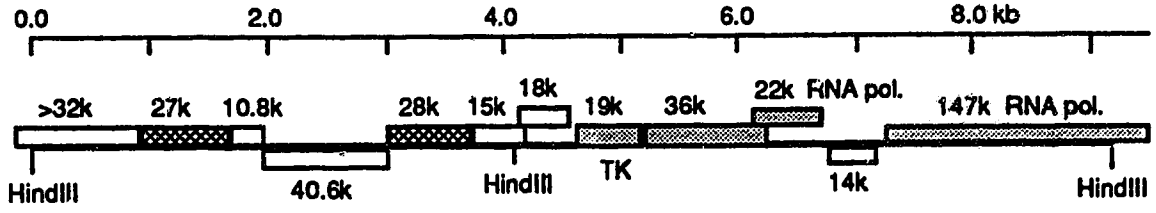


The *Hind* III P fragment was recently found by two groups, but they place it in different positions (Schmitt and Stunnenberg, 1988; and Tengelsen *et al.*,1988). The position seen here as given by Tengelsen *et al.*,(1988), is apparently correct since they have sequenced a *Kpn* I fragment which contains this region (not published). Tengelsen *et al.*,(1988), have sequenced the 87.7-kDa gene, and it is the large subunit of ribonucleotide reductase (Ribo. Red.). Schmitt and Stunnenberg (1988), have sequenced and transcriptionally mapped, the entire *Hind* III I fragment.

H. *HIND* III G

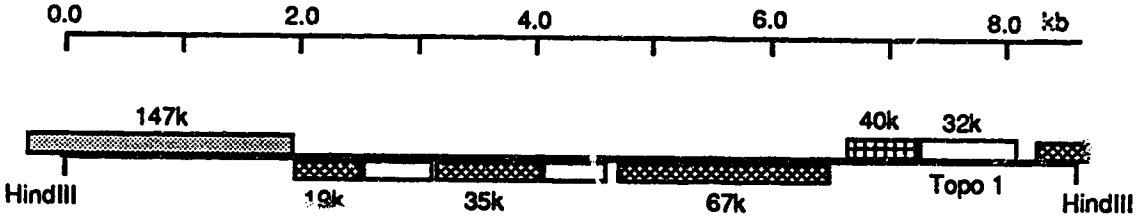
Very little information is available on this region of the genome. The right half apparently contains an IBT^{D} mutation (Condit and Niles, 1989; Fathi *et al.*,1986).

I. *HIND* III L AND J



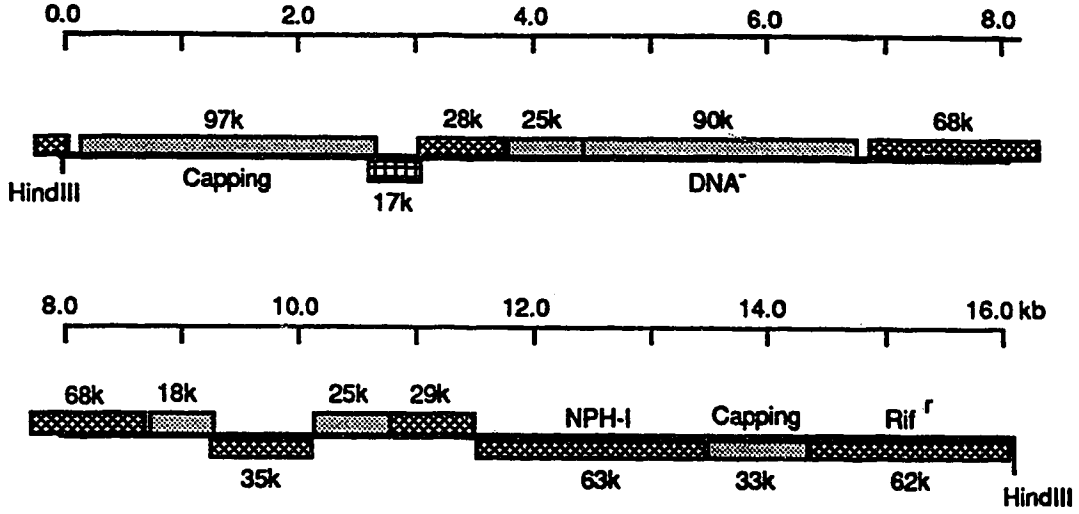
The transcriptional map and sequence of this region is known (Broyles and Moss, 1986; Mahr and Roberts 1984 a, b; Pluciennicza*l et al.*,1985). The 22-kDa and 147-kDa early genes are subunits of the viral RNA polymerase (RNA pol.), for which ts mutants are available (Broyles and Moss, 1986; Ensinger, 1987; Hooda-Dingra *et al.*, 1989; Thompson, *et al.*, 1989a). Other RNA polymerase subunits have been mapped to the genome by immunoprecipitation of *in vitro* translation reactions primed with hybrid selected RNA (Jones *et al.*,1987; Morrison *et al.*,1985) The promoters for the 147-kDa and 36-kDa genes have been tested in recombinant vaccinia virus (Barbeyron *et al.*,1987). The 28-kDa late gene is a major late protein and its promoter has been used to express heterologous genes (Wier and Moss, 1984). The 19-kDa early gene encodes the thymidine kinase (TK) gene (Bajszar *et al.*,1983; Weir and Moss, 1983; Weir *et al.*,1982; Hruby and Ball, 1982).

J. *HIND* III H



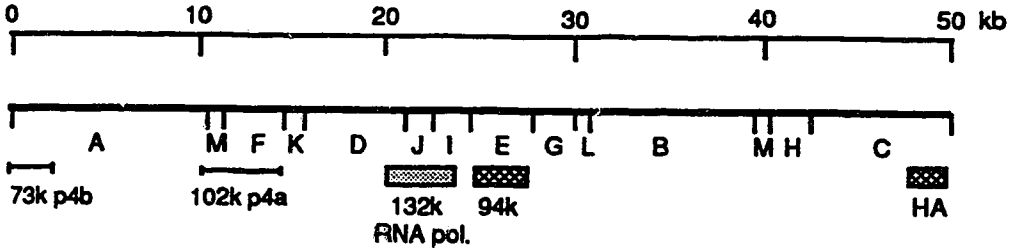
The entire fragment has been sequenced, transcriptionally and translationally mapped (Rosel *et al.*,1986). The 35-kDa late gene has some homology to the hydrophobic region of the spike glycoprotein precursor of VSV (Rosel *et al.*,1986), while the 32-kDa essential gene encodes the viral type-I topoisomerase (Topo I) (Shuman and Moss, 1987; Shuman *et al.*,1989).

K. *HIND* III D



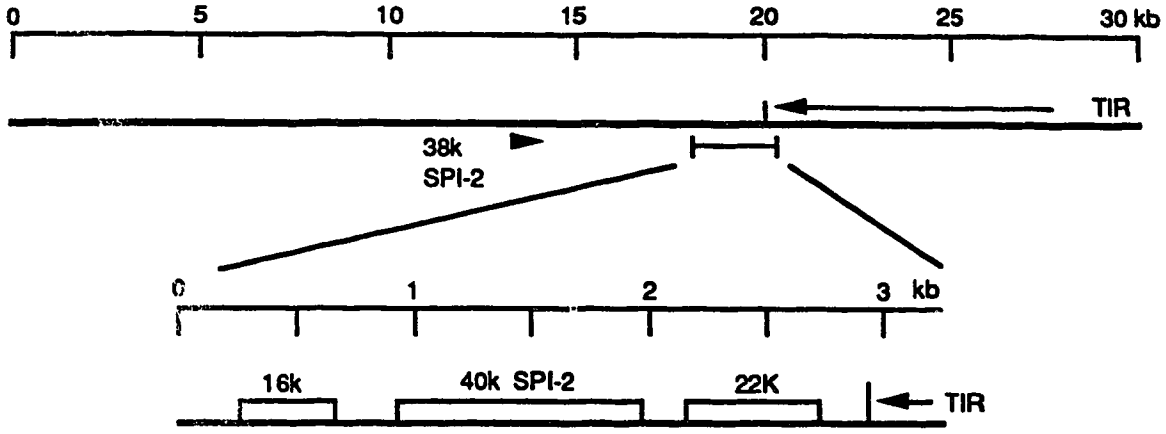
The entire region has been sequenced and transcriptionally and translationally mapped (Niles *et al.*,1986; Lee-Chen *et al.*,1988; Lee-Chen and Niles, 1988a, b; Weinrich and Hruby, 1986). The 97-kDa and 33-kDa essential early genes are subunits of the capping enzyme complex (Morgan, *et al.*,1984; Niles *et al.*, 1989). The 35-kDa late gene is homologous to carbonic anhydrase from horse and is a nonessential transmembrane protein in the intracellular form of the virus (Niles and Seto, 1988). Rifampicin resistance mutations are located in the 62-kDa late gene, also referred to as L65. This gene and its promoter has been extensively characterized (Miner *et al.*,1988; Miner and Hruby 1989a, b; Tartaglia and Paoletti, 1985, 1986; Weinrich *et al.*,1985; Weinrich and Hruby, 1986, 1987). The 72-kDa gene encodes the nucleoside triphosphate phosphohydrolase I (NPH-I or ATPase-I), a DNA dependent ATPase packaged within the virion (Broyles and Moss, 1987a). Kunzi and Traktman (1989), have shown that ts mutations in this gene are of the delayed late phenotype so it may have a role in late transcription. The 90-kDa gene shows some homology to a protein kinase, is essential for DNA replication, and contains a number of temperature sensitive mutations (C17, C24, E69, and 6389) which are of the DNA$^-$ phenotype (Condit and Niles, 1989; Evans and Traktman, 1987; Roseman and Hruby, 1987; Traktman, 1989). *In vitro* translation of early RNA hybrid selected with this fragment gives the 20-kDa RNA polymerase subunit (Jones *et al.*,1987).

L. *HIND* III A



The leftmost 3 kb have been sequenced (Rosel and Moss, 1985; Weinrich and Hruby, 1986). This region encodes the major virion structural protein p4b. About 7.5 kb to the right of p4b maps a second major structural protein p4a (Wittek *et al.*,1984b), and 6 kb around this gene have been sequenced (Meir and Wittek, 1988). Between p4a and p4b is a 39-kDa late virion core protein that is immunodominant in vaccinia infected rabbits (Maa and Esteban, 1987). The cowpox genes encoding the second largest subunit of the viral RNA polymerase complex (RPO 132), and the A-type inclusion body protein (p160), have been mapped, and their vaccinia equivalents (rpo 132-kDa and 94-kDa), are located in an analogous position on the vaccinia virus genome (Patel *et al.*,1988; Patel and Pickup, 1989). There are ts mutants of the delayed late phenotype for the rpo132 gene (Condit and Niles, 1989), and the 94-kDa gene is not essential as shown by insertional mutagenesis (Patel *et al.*,1988). The central portion of the *Hind* III A region contains the 14-kDa gene encoding a viral envelope protein which affects virus penetration, plaque size, and elicits neutralizing antibodies (Dallo *et al.*,1987; Rodriguez *et al.*,1987; Rodriguez and Esteban, 1987). The mutations ts22 and IBT^r which are both involved in regulating the cellular 2-5A dependent RNAse are located in the *Hind* III A fragment. Both have the same abortive late phenotype though it is not yet known if they map to the same ORF (Cohrs *et al.*,1989; Condit and Niles,1989; Pacha and Condit, 1985; Thompson and Condit, 1986) The sequence of the vaccinia hemagglutinin gene (HA) has been determined (Shida, 1986). *In vitro* translation of early RNA hybrid selected with this *Hind* III fragment gives the 132-kDa, 34-kDa, 23-kDa, 21-kDa, and 17-kDa RNA polymerase subunit (Jones *et al.*,1987; Morrison *et al.*,1985).

M. *HIND* III B



The location and sequence of two serpin type proteins (SPI 1 or B13R, and SPI 2 OR B24R), are known (Kotwal and Moss, 1989; Smith *et al.*, 1989). The sequence by Smith *et al.*, (1989) is apparently more correct than that given by Kotwol and Moss (1989). All three serpin genes in vaccinia are early genes (Smith *et al.*, 1989).

APPENDIX II
POXVIRUS ENZYMES

A. INTRODUCTION

The poxvirus replicative cycle is localized exclusively to the cytoplasm of the infected cell and many of the enzymes required for the expression and replication of the poxvirus genome appear to be of viral origin. Various enzyme activities have been found to be induced in virus infected cells, or they are associated with the purified virus particle itself. The encapsidated enzymes have been the most thoroughly characterized since purification of the virus particle away from the cell is easily achieved and this removes many contaminating cellular activities. It should be remembered that encapsidation of an enzyme does not prove it is of viral origin. Below are listed some of the enzyme activities that are induced upon infection or are associated with purified virus particles.

B. DNA LIGASE

A DNA ligase activity is seen to be induced in cells infected with vaccinia virus and this induction is inhibited by puromycin but it is not affected by AraC or rifampicin. However no differences have yet been detected between the ligase activity seen in infected and uninfected cells (Sambrook and Shatkin 1969; Spadari 1976). An ORF with homology to known ligases maps to L1 (Geof Smith, personal communication).

C. ALKALINE PROTEASE

This is an endoproteolytic activity detected when purified virions are disrupted at pH 10.6, but it has not been purified or very well characterized (Arzoglou *et. al.* 1979).

D. PROTEIN KINASE

A protein kinase and two major phosphate acceptor proteins have been purified from vaccinia virions. The purified protein kinase is about 62-kDa and shows maximum activity at alkaline pH values. The two major phosphate acceptor proteins are solublized by Np40 and DTT so they are not tightly core associated. One protein is 37.5-kDa, phosphorylated predominantly on serine residues, while the other protein is 11.7-kDa phosphorylated mostly on threonine residues and maps to the *Hind* III site between fragments E and F (Downer *et al.*,1973; Kleiman and Moss 1973, 1975a, b; Paoletti and Moss 1972a).

E. ENDORIBONUCLEASE

This is a core associated activity which will cleave the virion associated high molecular weight RNA into 8-12s RNA. ATP or GTP are not required and 8-12s RNA is not degraded when added to the reaction so there is some specificity. The enzyme has not been purified and the reaction not well characterized so its role in gene expression remains unknown (Paoletti and Lipinskas, 1978).

F. DEOXYRIBONUCLEASES

Three deoxyribonuclease activities are induced by poxvirus infection and they have been characterized with respect to their pH optimum and substrate specificity (McAuslan and Kates, 1967).

1. Acid DNase.

This DNase activity is induced in infected cells only if DNA replication is allowed to occur so it is considered a late enzyme (McAuslan and Kates, 1967). It has been purified from viral cores, and is a 50-kDa polypeptide which exists as a dimer in solution. It has a pH optimum of 4.5, a pI of 4.5 and is active in the presence of EDTA (Roseman-Hornbeak *et. al.* 1974; Roseman-Hornbeak and Moss, 1974; Pogo and Dales, 1969; Pogo

and O'Shea, 1977). It functions primarily as an exonuclease on ssDNA though some endonucleolytic activity has been reported (Roseman-Hornbeak and Moss, 1974).

2. Neutral DNase.

This DNase has also been isolated from purified virus cores and behaves as an early gene in infected cells. The purified enzyme has a pH optimum of 7.8, a pI of 3.7, a molecular weight of 50-kDa, and acts as an endonuclease on ssDNA (McAuslan and Kates, 1967; Pogo and Dales, 1969; Pogo and O'Shea, 1977).

3. Alkaline DNase.

This is the least well characterized of the DNases. It has been reported only in infected cells, acts on native DNA and has a pH optimum of 9.2 (McAuslan and Kates, 1967; Jungwirth *et al.*,1969).

G. NUCLEOTIDE PHOSPHOHYDROLASES

The vaccinia virion contains two nucleotide phosphohdrolase activities (NPH-I and NPH-II), which hydrolyze nucleotide triphosphate to nucleotide diphosphate plus inorganic phosphate. Both enzymes require Mg^{2+} and ssDNA as a cofactor, though NPH-I is stimulated somewhat by RNA (Broyles and Moss, 1987a; Gold and Dales, 1968; Munyon *et al.*,1968; Paoletti *et al.*,1974a, b; Paoletti and Moss, 1972b, 1974). NPH-I hydrolysis ATP or dATP only, while NPH-II works best on ATP though it acts on all NTPs (Munyon *et al.*,1968; Paoletti and Moss, 1974). NPH-I is 61-kDa and NPH-II is 68-kDa, they are immunologically distinct enzymes and NPH-I has been assigned to ORF D11 (Broyles and Moss, 1987). Kunzi and Traktman (1989), have recently shown that a ts mutant which maps to NPH-I has a defective late phenotype, and may be involved in late transcription.

H. POLY(A) POLYMERASE

Vaccinia RNA made *in vivo* or *in vitro* has 5' caps and 3' poly(A) tails. A distinct

viral poly(A) polymerase has been isolated from both infected cells (Brakel and Kates, 1974a; Nevins and Joklik, 1977b), and purified viral cores (Brakel and Kates, (1974b; Brown *et al.*,1973; Moss *et al.*,1973, 1975; Moss and Rosenblum, 1974; Sheldon and Kates, 1974; Shuman and Moss, 1988b). Analysis of primer and nucleotide specificities has indicated that poly (A) is added in a template independent fashion and is added to any polyribonucleotide primer though poly A and C work best (Shuman and Moss, 1988b). The purified enzyme has a molecular weight of 80-kDa, and consists of two subunits of 57 and 37-kDa, which distinguishes it from its host cell counterpart (Moss *et al.*,1975; Nevins and Joklik, 1977b; Shuman and Moss, 1988b).

I. TOPOISOMERASE

A type I topoisomerase activity is found both in vaccinia infected cells and vaccinia virions (Bauer *et al.*,1977; Pogo and Bauer, 1986; Shaffer and Traktman, 1987). This virally encoded 32-kDa polypeptide is inhibited by novobiocin, coumermycin and benerril which are prokaryotic topoisomerase II inhibitors, but not by camptothecin a prokaryotic topoisomerase I inhibitor (Fogleson and Bauer, 1984; Shaffer and Traktman, 1987; Shuman *et al.*,1988; Shuman and Moss, 1987). The role of topoisomerase in replication or transcription is unknown but its inhibition by novobiocin is similar to the inhibition by novobiocin of *in vitro* transcription. The topoisomerase does not cosediment with RNA polymerase on glycerol gradients (Broyles and Moss, 1987b), nevertheless the RNA polymerase and associated ATPase activities are inhibited by novobiocin. No ts mutants exist in the gene and attempts to make insertion mutants have failed (Shuman *et al.*,1989). It has been cloned and over expressed in *E. coli* where it is active, so the study of its biophysical properties should be possible (Shuman 1989; Shuman *et al.*,1988).

J. THE CAPPING ENZYMES

Vaccinia mRNA produced *in vivo* or *in vitro* possess 5' caps of the structure $m^7G(5')ppp(5')N_1{}^m pN_2 p$, or $m^7G(5')ppp(5')N_1{}^m pN_2{}^m p$ which are type I and type II caps respectively and where N_1 is adenosine or guanosine (Boone and Moss, 1977; Keith *et al.*,1980; Moss *et al.*,1976; Wei and Moss, 1974).

Cap formation occurs in vitro by the following series of reactions:

1. $pppN_1\ pN_2\ p \longrightarrow ppN_1\ pN_2\ p + P_i$

2. $GTP + ppN_1\ pN_2\ p \longrightarrow G(5')ppp(5')N_1\ pN_2\ p + PP_i$

3. $G(5')ppp(5')N_1\ pN_2\ p + SAM \longrightarrow m^7G(5')ppp(5')N_1\ pN_2\ p + SAH$

4. $m^7G(5')ppp(5')N_1\ pN_2\ p + SAM \longrightarrow m^7G(5')ppp(5')N_1{}^m pN_2\ p + SAH$

Where reaction one is catalyzed by a polynucleotide triphosphatase, reaction two by guanylyltransferase, reaction three by (guanine-7)-methyl transferase, and reaction four by an mRNA (nucleoside-2')-methyl transferase. SAM and SAH indicate S-adenosyl methionine and S-adenosyl homocysteine respectively. These enzyme activities have been isolated and characterized from purified vaccinia virus cores.

The polynucleotide triphosphatase, guanylyltransferase, and (guanine-7)-methyl transferase copurify as a 127-kDa complex consisting of a 95-kDa and a 31.4-kDa subunits (Martin *et al.*,1975; Martin and Moss, 1975; Shuman, 1989; Shuman and Hurtwitz, 1981; Tutas and Paoletti, 1977; Venkatesan *et al.*,1980). The substrate on which this complex acts must be a polyribonucleotide with two or three 5' terminal phosphate groups. Poly (A) with a single 5' terminal phosphate does not act as a CAP acceptor (Ensinger *et al.*,1975; Martin and Moss, 1975). This is a consistent finding among most groups and

implies that if a polycistronic mRNA were to be cleaved into monocistronic fragments then these fragments could not be capped and therefore would not be translated. However, Spencer *et al.*,(1978), have reported a 5'-phosphate-polyribonucleotide kinase that phosphorylates a 5'-monophosphate to a 5'-di or tri- phosphate species and allows the capping of 5'-monophosphate RNA species. This enzyme has not been purified or well characterized and the significance of this observation is unknown. Only GTP or dGTP act as donors for the guanylyltransferase, and only G(5')ppp(5')N1 pN2p act as acceptors for the guanine methyl transferase (Martin and Moss, 1976). Guanine residues with a phosphate in the 2' or 3' positions, or in a 3'-5' linkage, are not methylated thus internal guanine residues are not methylated by this enzyme. The 95-kDa subunit of this enzyme complex is reported to contain a GTP pyrophosphate exchange activity (Shuman *et al.*,1980b), and can be labeled with α-^{32}P-GTP as it forms a covalently linked intermediate with GTP (Shuman and Hurwitz, 1981). Shuman (1989), has determined that a 59-kDa tryptic fragment of this subunit still has guanylyltransferase and RNA triphosphatase activities and a 21-kDa tryptic fragment of the small 31.4-kDa subunit contains residual methyltransferase activities. Thus the enzymatic activities of this complex have been assigned to individual polypeptides and these two genes have been mapped onto the viral genome as D1 and D12 (Morgan *et al.*,1984; Niles *et al.*,1989).

The mRNA (nucleoside-2')- methyl transferase has been purified and is a single polypeptide of 38-kDa. Only m^7G(5')ppp(5')N$_1$ pN$_2$ p- acts as an acceptor molecule thus, 2'-O methylation is the final step in CAP formation (Barbosa and Moss, 1978a,b). The capping enzyme complex cosediments with the viral RNA polymerase on glycerol gradients (Broyles and Moss, 1987b), and it acts to terminate early transcription *in vitro* (Shuman and Moss, 1988a).

K. DNA POLYMERASE

A 110-kDa single subunit DNA polymerase, distinct from the cellular enzyme and containing a 3'-5' exonuclease activity has been purified and characterized from vaccinia infected cells (Challberg and Englend, 1979). A number of drug resistant and ts mutants are available and were used to map the gene onto the genome (DeFilipes, 1989; Jones and Moss, 1984; Moss and Cooper, 1982; Sridhar and Condit, 1983; Traktman *et al.*,1984), and transcriptional mapping indicated that it was strictly an early gene (Jones and Moss, 1985; Traktman *et al.*,1984), but recently Kunzi and Traktman (1989), detected separate early and late transcripts indicating there is an early and a late promoter.

L. THYMIDINE KINASE

Thymidine kinase is one of the first viral enzymes to be detected in infected cells (Kit *et al.*,1962), and when purified from infected cells it was found to be 40-42-kDa and function as a dimer (Kit *et al.*,1977). Mutations in this gene were one of the first to be selected for as a drug resistance phenotype (to BUdR, Dubbs and Kit, 1964). It is thus a nonessential gene and because both TK^+ and TK^- phenotypes can be selected for, it was important in the development of marker rescue techniques used to map the gene onto the genome, and then to make recombinant viruses (Hruby and Ball, 1982; Weir *et al.*,1982). Transcriptionally mapping and sequencing show that it is strictly an early gene, in the *Hind* III J fragment (Bajszar *et al.*. 1983; Mahr and Roberts, 1984; Weir and Moss, 1983).

M. RIBONUCLEOTIDE REDUCTASE

A ribonucleotide reductase activity is induced in infected cells and can be distinguished from the host cell enzyme (Slabaugh *et al.*,1984; Slabaugh and Mathews, 1984). The viral enzyme is composed of two subunits one of 37-kDa which maps to *Hind* III F fragment and one of 86-kDa which maps to the *Hind* III I fragment (Slabaugh *et al.*,1988; Tenglesen *et al.*,1988). This viral gene is responsible for the sensitivity of the

virus to hydroxyurea (HU) and HU resistant mutants in the 37-kDa subunit have been isolated. These mutants are interesting because they overcome the HU block by amplifying the gene for the 37-kDa subunit as a tandomly repeated structure which is unstable and reverts easily when HU is removed (Slabaugh *et al.*,1988; Slabaugh and Mathews, 1986).

N. RNA POLYMERASE

A viral RNA polymerase has been isolated from vaccinia infected cells and from purified virions. It is similar in composition to other eukaryotic RNA polymerases consisting of two large subunits (147-kDa and 132-kDa), and a number of smaller subunits. A detailed description of the characteristics of this enzyme can be found in chapter I.

O. NICKING-JOINING ENZYME

This is a DNase that has been purified from vaccinia cores, does not require ATP, and acts on supercoiled plasmid substrates but not relaxed plasmid substrates *in vitro* (Lakaritz *et al.*, 1985; [illegible], 1989). This enzyme first nicks the supercoiled plasmid, and then linearizes it. In a small proportion of the linearized substrate one of the ends is cross-linked in a manner resembling the hairpin ends of the viral genome. The purified enzyme is 50-kDa but trypsinization to create a 44-kDa fragment activates the enzyme and increases the optimum NaCl concentration, so it may initially exist as a proenzyme. The *in vivo* role of the enzyme is not known and the nature of the cross-linked sequences has not yet been reported.

APPENDIX III
POXVIRUS PROMOTER SEQUENCES

A. INTRODUCTION

Deletion analysis of poxvirus promoters indicates that the minimal sequence requirement for a poxvirus promoter includes the transcriptional initiation site and up to 36 bp upstream. These sequences for the poxvirus genes which have been transcriptionally mapped are listed in this appendix. In these sequences the initiating ATG of the gene is underlined, and the transcriptional initiation sites which have been mapped by S1 or primer extension analysis are indicated by an '*'. The promoters are separated into early and late classes and it should be noted that the 7.5-kDa, D2, I3, and H6 genes contain separate early and late start sites, while the H6 and CPV 132-kDa RPO genes have transcripts which map to the same start site at both early and late times. A letter in brackets after the name of a vaccinia gene in the references, indicates the *Hind*III fragment where that gene is found if it is not already indicated by the name of the gene.

B. EARLY PROMOTERS

1. AGGGTTTTTC TTTCTCGTTA ATTTCTTAAA AAAAAGTCAA ATTATA*GTAA N45 GTCATGA
2. CAAGGTGATA GGATTTATTC AGGAAAAATA TAATACGACA TAC*TA*TAATA N67 TAAAATGG
3. TACGTAGACT GTTTATACAA GATTGAAAAT ATATTTCTTT TTATT*GAGTG N65 GCCATGGA
4. AATAATTAAT TTCTCGTAAA AGTAGAAAAT ATATTCTAAT TTATT*GCACG N43 TCATGAAA
5. TATATTATTA GTTTATATTA CTGAATTAAT AATATAAAAT TCCCAA*TCTT N44 CAAAAATG
6. TATAATAAAG CAACGTAAAA CACATAAAAA TAAGCGTAAC TAATAAG*ACA ATGGATATT
7. GGATATATTA AAGTCGAATA AAGTGAACAA TAATTAATTC TTTATT*GTCA CATGAACGGC
8. CCAATGTTTG GGATTCATTT AAATGAAAAT ATATTTCTAA ATTCTA*TAAA N72 GAAATGG
9. AAATCAGACG CTGTAAATTA TGAAAAAAAG ATGTACTACC TTAATA*AGAT N186 TCATGAA
10. CGTCATGATA AAAATTTAAA GTGTAAATAT AACTATTATT TTTATA*GTTG N56 TAAAAATG
11. TATTATTTTT ATAGTTGTAA TAAAAAGGGA AATTTGATTG TATACT*TCGG N23 TAAAAATG

12. GCTTGTTAAT AAGTAAATGA AAAAAAACTA GTCGTTTATA ATAAAACACG ATATGGATGC
13. ACTTCGTTAT CGTCGTTATC TACTTTGGGA TACTTATTAT CCTTTACTAT AAAAATGTCC
14. GCTTGGTTTA ACGGCGTTTC GGAAAATGAA AAGGTACTAG ATACGTATAA N17 TATAATGA
15. CTCAAGGGTT TATTTATTAA TGCTTTAGTG AAATTTTAAC TTGTGTTCTA AATGGATGCG
16. AATATAACGT GGTATCTTCT CCATAAAACT GATGAAATAT ATAAAGAAAT AAATGTCGAG
17. TTGTTGCGGC ATAGTAAAAA TGAAATGATA ACTCTGTTAA AAATAGCTCT AGTATGGGAA
18. GAGATAATAA CTAATAACTA ATAATAATGA AAACAAACTA TAGAGTTGTA AATGGGATGAA
19. ATGATTATTT TTAACAAAAT AACATAAAAA TAATATATTT TTTTAGGATT CGATCATGAC
20. AAATTGTTGC AAATATACAG AAATAGAAAT AGAAATATTA ATTTTTATAC N79 GAATATG
21. AATATATGGG ATGGTAAATA ATTTTGAAAT AAAATATTAG TTTTATGTTC AACATGAATA
22. CAAGTACAGG CATATAGATA ACTGAAAAAA AATTTATTGT TATTGTTATT TTAGTTATGGA
23. TCATTACTAA TCGTATATTA TAATTGAAAA TGGATTAGTT TAATATGACG CTCTGTCATGG
24. TACGTCAAAT CCCTATTAAT GAAAAGTTAA ATAATTTTTT TATTACACCA ACAAAAATGTT
25. TTATTCTCAA ATGAGATAAA GTGAAAATAT ATATCATTAT ATTACAAAGT N18 TCATGAG
26. GATCGTCTAA AAGAATTGCT TCTAAAATGA AAAAAAACAC TGATTCAGAA ATGGATCAAC
27. CTTTATTCTA TACTTAAAAA ATGAAAATAA ATACAAAGGT TCTTGAGGGT N70 TAATGG
28. AATTATAAAA AATGAAAAAA TATACACTAA TTAGCGTCTC GTTTCAGAC ATGGATCTG
29. ATCGGGATTT TATTTTGAAA TAGCCAGAAT TGAAAACGAA GATCAATAGG CAGATA
30. ATTTTTATGT TTTATTGATA ATTGTAATAA CATACAATTA AATCAATATA GAGGAAGGAG
31. GTTAAAGAAG TCCATTTAAG GCTTTAAAAT TGAATTGCGA TTATAAGATT AAATGGCAGA
32. ACTAAATTAA TTTGATAATA AATCAAAAAT GGAAAACTAA GGTCGTTAGT AGGGAGGAGA
33. AAATATAAAA TATAAAATAT AAAATAAAAT ATACAATTTC AATACTCACA TAATTCAA
34. CTAGCCACAG TAAATCGTTA AAAATTTAAA AAATAGAAAA TAGAAACGTA TAGAACGCCA
35. AATTGTATGT TTTTACAATT ATCAATAAAA CATAAAAATA ATATGATCTT GGTGGCGTGA
36. TTTTTATAAT CGATAATCGA TACAAAACAT AAAAAACAAC TCGTTATTAC ATAGTAGGCA

C. LATE PROMOTERS

1. ATATGTATGT AAAAATATAG TAGAATTTCA TTTTGTTTTT TTCTATGCTA TAAATGAATT
2. GTTTGTAACA TCGGTACGGG TATTCATTTA TCACAAAAAA AACTTCTCTA AATGAGTCTA
3. AAGATTGGAT ATTAAAATCA CGCTTTCGAG TAAAAACTAC GAATATAAAT AATGGAAGCC
4. TAAATCGTTT TGTATATCCG TCACTGGTAC GGTCGTCATT TATTACTAAA TAAATGATGC
5. TCACTAATTC CAAACCCACC CGCTTTTTAT AGTAAGTTTT TCACCCATAA ATAATAAATAC
6. TAAATTATAT GTGTTTACGA TAAAGTAGTT TTATCCATTT GTACGTTATA AATGGATAAG
7. GAAAATATAA CTCGTATTAA AGAGTTGTAT ATGATTAATT TCAATAACTA AATGGCGGCG
8. CTAAAAAAAT TATACATCAT AAACCAATTT CCTAGTTGTT TGTAACTTTA AATGGACTCT

9. TCTACGGATG GATGATATAG ATCTTTACAC AAATAATTAC AAAACCGATA AATGGAAATG
10. TAATTTTGTT AATTTTAATC TGATAAAGTC ATTGTGATTT CCTAAAAGTA N54 TAAAAATG
11. GGAATAAAAT ACTACTGTTG AGTAAATCAG TTATTTTTTT TATATCGATA TTGATGGACA
12. ATATATTCAT AGAGGATATT TCATCTCCGT AAATATATGC TCATATATTT N19 TAAATGAA
13. GTAATTCCCA TACTAAGAGC TATTTTTAAA CAGTTATCAT TTCATTTTTA CTATGCCGCA
14. ACGATAAATA CGAATATCTG TCTTATATTT ATAATATGCT AGTTAATAGT AAATGAACTT
15. TAGTCACGTT CTTACTCCTC CAGAACGTAG ATATGTAAAC GTGCACTTTA TAATGGCTAG
16. CTAGTTAAAC TACTGCTGTG ATTTTTAAAA CATAGTTATT ACTTATCACT CATAAATGAG
17. ATAGATATTT CTCTACGGAG TTTATTGTAA GCTTTTTCCA TTTTAAATAG AAAATGAATA
18. TATGAAGATA AGGAAAAGGT CATAAATACA ATAAACTTAC TAAGAGATAA AGACGGCGTT
19. GAGGTTTTCT ACTTGCTCAT TAGAAGTATA AAAAAATAGT TCCGTAATTA AATGGCTAAG
20. TACAGGATTT TCTGGCAACG TCTAGAAATA AAATGTTTTT ATATAAAATA N25 TAACATGA
21. CATATATTAG CGCTAGACAT ATTACAGAAC TATTTTAGAT TATGATATTT AAATGAGTTG
22. CGAGAATATG ATAGAAATGA TGAAGGAAAG ATCGACTATT TTAAATAGCA N70 GAAATGG
23. CTTATAATTA CACGATTGTA GTTAAGTTTT GAATAAAATT TTTTTATAAT AAATGGAGGT
24. ATATTCTAGA TCGTTGATAG AACAGGATGT ATAAGTTTTT ATGTTAACTA AATGTGGCCA
25. TCTACTACTA TTGATATATT TGTATTTAAA AGTTGTTTGG TGAACTTAAA TGGCGGAATT
26. TGATGATGAA TATGATGAAA GCGATAAAGA AAAGCCAATA TTCAATGTAT AAATGGATAA
27. TATACAACTA GGACTTTGTC ACATATTCTT TGATCTAATT TTTAGATATA AATGGTGGAT
28. TTAACGCAGT TTGGAAAAAA GAAGATATCT GGTAAATTCT TTTCCATGAT AAATGGAAAG
29. TCTGTACATC ATGCAGTGGT TAAACAAAAA CATTTTTATT CTCAAATGAG N53 TCATGAGT
30. CAGTGCCTTC TTGACATAAA AGAACTAAGT TATGATATTT GTAAATTGTT N125 TATGC
31. ACAAAGGTTC TTGAGGGTTG TGTTAAATTG AAAGCGAGAA ATAATCATAA AT N38 ATG

D. REFERENCES

1. *Early Promoters*

1.	SFV T1	Macaulay and McFadden, 1989.
2.	SFV SFGF	W. Cheng, personal communication.
3.	CPV 38-kDa	Ink and Pickup, 1989.
4.	7.5-kDa (C)	Cochran *et al.*,1985b; Venkatesan *et al.*,1981.
5.	VGF (C)	Venkatesan *et al.*,1982.
6.	42-kDa (C)	Venkatesan *et al.*,1982.
7.	TK (J2)	Weir and Moss, 1983.

8.	DNA pol. (E)	Earl *et al.*,1986.
9.	22-kDa RNA pol. (J4)	Broyles and Moss, 1986.
10.	147-kDa RNA pol. (J6)	Broyles and Moss, 1986. (major start site)
11.	147-kDa RNA pol. (J6)	Broyles and Moss, 1986. (minor start site)
12.	D1	Lee-Chen *et al.*,1988.
13.	D2	Lee-Chen *et al.*,1988.
14.	D4	Lee-Chen *et al.*,1988.
15.	D5	Lee-Chen *et al.*,1988.
16.	D7	Lee-Chen *et al.*,1988.
17.	D9	Lee-Chen *et al.*,1988.
18.	D12	Lee-Chen *et al.*,1988.
19.	N2	Tamin *et al.*,1988.
20.	M1	Tamin *et al.*,1988.
21.	Protease (F2).	Slabaugh and Roseman, 1989.
22.	Ribo. Red. (37-kDa) (F)	Slabaugh *et al.*, 1988.
23.	pF	Coupar *et al.*,1987.
24.	Ribo. Red. (87.7-kDa) (I)	Tengelsen *et al.*,1988.
25.	I3	Schmitt and Stunnenberg, 1988.
26.	RPO 132 (A)	Patel and Pickup, 1989.
27.	H6	Rosel *et al.*,1986.
28.	K1	Mars and Beaud, 1987.
29.	H3 (C-terminus)	Rosel *et al.*,1986.
30.	pT15	Mars and Beaud, 1987.
31.	pT20	Mars and Beaud, 1987.
32.	pT25	Mars and Beaud, 1987.
33.	pT28	Mars and Beaud, 1987.
34.	pT35	Mars and Beaud, 1987.
35.	pT38	Mars and Beaud, 1987.
36.	pT56	Mars and Beaud, 1987.

2. *Late Promoters*

1.	p11 (F)	Bertholet *et al.*,1985.
2.	28-kDa (L)	Weir and Moss, 1984.
3.	p4b (A)	Rosel and Moss, 1985.

4.	p4a (A)	Meir and Wittek, 1988.
5.	7.5-kDa (C)	Cochran *et al.*,1985b.
6.	H1	Rosel *et al.*,1986.
7.	H3	Rosel *et al.*,1986.
8.	H5	Rosel *et al.*,1986.
9.	H8	Rosel *et al.*,1986.
10.	D2	Lee-Chen and Niles, 1988.
11.	D3	Lee-Chen and Niles, 1988.
12.	D6	Lee-Chen and Niles, 1988.
13.	D8	Lee-Chen and Niles, 1988.
14.	D10	Lee-Chen and Niles, 1988.
15.	D11a	Lee-Chen and Niles, 1988.
16.	D11	Lee-Chen and Niles, 1988.
17.	D13 (proximal)	Weinrich and Hruby, 1986.
18.	D13 (distal)	Weinrich and Hruby, 1987; Miner *et al.*,1988, 1989b
19.	A1	Weinrich and Hruby, 1986.
20.	A2	Weinrich and Hruby, 1986.
21.	A3	Weinrich and Hruby, 1986.
22.	RPO (132-kDa)	Patel and Pickup, 1989.
23.	ATI (160-kDa)	Patel and Pickup, 1987.
24.	37-kDa E. Ag. (F)	Hirt *et al.*,1986.
25.	I1	Schmitt and Stunnenberg, 1988.
26.	I2	Schmitt and Stunnenberg, 1988.
27.	I5	Schmitt and Stunnenberg, 1988.
28.	I7	Schmitt and Stunnenberg, 1988.
29.	I3	Schmitt and Stunnenberg, 1988.
30.	M1	Tamin *et al.*,1988.
31.	H6	Rosel *et al.*,1986.