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Vaccinia virus gene expression is characterized by tight temporal regulation. Viral early transcription begins within the cytoplasm upon infection; three hours later, early genes are repressed, DNA replication occurs and late gene expression commences. As an approach toward understanding the mechanisms governing vaccinia late gene expression, I have undertaken an in vivo analysis of six vaccinia late promoters. The promoters were identified by abutting putative vaccinia promoter sequences to a reporter gene and assaying for reporter gene activity during transient expression. Once biologically active promoter sequences were identified, the L65 and ORF A1 promoters were subjected to 5' deletion mutagenesis to define active regions. At this point, attention focused on the L65 promoter due to the added complexity of two differentially regulated RNA start sites. Additional 5' deletion mutagenesis pinpointed regions responsible for transcription from these sites. The availability of these cloned small promoter fragments facilitated assays for vaccinia late promoter specific binding factors. The
discovery of a protein factor which binds specifically to the L65 promoter suggests that this DNA binding protein may also be a transcription factor.

This promoter drives the expression of a major late polypeptide of unknown function (L65); to determine if post-transcriptional regulatory mechanisms affect L65 expression and to localize L65 during the infection cycle, L65 specific anti-sera was prepared against a trpE/L65 fusion protein expressed in E. coli. This reagent, together with the information obtained regarding the cis-acting elements and trans-acting factors involved in the transcriptional regulation of the L65 promoter will allow a directed approach toward understanding the regulation of L65 gene expression and perhaps the function of L65.
THE REGULATION OF A VACCINIA VIRUS LATE GENE REQUIRED FOR MORPHOGENESIS

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CHAPTER I

INTRODUCTION

The development of multi-cellular organisms relies in part on temporally and spatially ordered gene expression. Currently, a variety of experimental systems exist with which developmental processes can be studied. One group of these, the large DNA viruses, have played an important role as models for more complex systems. Infection by these viruses involves an interaction between the virus and the host cell, providing insights into host functions. The degree of virus/host cell interaction varies, smaller DNA viruses such as SV40 and bovine papilloma virus (Livingston and Bikel, 1985) utilize much of the host regulatory machinery, selectively modifying it for viral functions. The larger DNA viruses like herpesvirus (Roizman and Batterson, 1985), and baculovirus (Miller, 1988) encode more of their own regulatory proteins. At perhaps the far end of this scale are the poxviruses like vaccinia, which replicate within the cytoplasm of infected cells in relative independence of host nuclear regulatory factors (Moss, 1985).
Vaccinia Virus

Historically, vaccinia virus (VV) was developed as the vaccine which was successfully used to eradicate smallpox. In the late 1700's, Edward Jenner discovered that extracts from cowpox lesions would induce immunity to variola virus (smallpox) in humans without causing serious disease. Later, it was shown that a closely-related orthopoxvirus, vaccinia virus, is serologically related to variola and hence can confer immunity. From this somewhat applied beginning, vaccinia research has since provided fundamental biological information in a number of areas:

1) Vaccinia was the first animal virus to be accurately titered, physically purified and chemically analyzed (Moss, 1985). It has set the stage for current efforts to understand various modes of viral DNA replication and host/viral interactions.

2) Vaccinia has properties which make it an excellent vector for the expression of foreign genes. Current uses of vaccinia virus engineering technology include the production of protective antigens for use as recombinant vaccines in the veterinary and medical sciences (Macket et al., 1982; Moss and Flexner, 1987), as well as the expression of proteins used in basic biological research (Hruby and Thomas, 1987).

3) Vaccinia's cytoplasmic site of replication and relative independence from host nuclear factors means that fundamental processes such as RNA and DNA metabolism, gene expression and intracellular localization can be studied in the absence of complicating host functions. As a model for fundamental eukaryotic genetic processes, vaccinia seems particularly appropriate because strong homologies have
been noted between VV DNA polymerase, several subunits of VV RNA polymerase and the corresponding mammalian proteins (Broyles and Moss, 1986; Earl et al., 1986). This conservation of structure and function implies that basic biochemical mechanisms may be conserved between the virus and host. Using this assumption as a starting point, I will first present an overview of the replication cycle of the virus, then focus briefly on the regulation of the viral genes expressed before DNA replication and finally highlight recent developments in the regulation of vaccinia virus late gene expression.

Vaccinia Replication Cycle

Vaccinia virus infection normally occurs in mammalian host cells; however, tissue culture cells from a variety of organisms can accommodate VV replication, including mosquito, and fish cells (Hruby et al., 1980; Franke and Hruby, 1985; D. Hruby, unpublished data). Vaccinia particles themselves appear as non-icosahedral, brick-shaped structures covered with a mass of wormlike surface tubular elements. When thin sections of viral particles are viewed by electron microscopy, the core can be seen as a biconcave structure which contains the viral DNA genome in a nucleoprotein complex consisting of a variety of DNA binding proteins, one of which, the 11K bears remarkable biochemical and sequence similarity to histone H1 (Kao et al., 1981; Kao and Bauer, 1987; J. Miner unpublished results). In addition, the concavities of the core contain structures of unknown function called lateral bodies which girdle the thinnest part of the core (Figure I.1) (Moss, 1985).
VACCINIA VIRUS REPLICATION CYCLE

Figure I.1
The virus initiates infection of a susceptible host cell by associating with the plasma membrane. Vaccinia enters the cell by either membrane fusion or by endocytosis (Moss, 1985). Once inside, and after the removal of the outermost of the two membrane layers surrounding the core particle (uncoating I), the immediate early genes are expressed. Products from the immediate early genes are responsible for a second uncoating event (uncoating II) and the expression of the delayed early genes, a hypothetical gene class currently defined as being transcribed after uncoating II and prior to viral DNA replication, which may produce products necessary for DNA replication (Kates and McAuslan, 1967). This second uncoating event leads to the release of the viral nucleoprotein complex into the cytoplasm, the beginning of viral DNA synthesis, and the expression of late genes once replication is underway. The replicating DNA aggregates into large cytoplasmic inclusion bodies called virosomes or viral factories (Morgan, 1976; Moss, 1985). It is within these structures that morphogenesis of progeny virions takes place. Gently curved lipid membranes form and coalesce into spheres which surround a portion of the virosome. This membrane-enclosed viroplasm migrates away from the virosome, organizes and condenses into both the biconcave core and the lateral bodies (Morgan, 1976). Biochemically, the morphogenic process is characterized by the association of L65 (a major late vaccinia protein) with the virosome, followed by proteolytic cleavage of the major core precursors p4a and p4b (Katz and Moss, 1970a; Katz and Moss, 1970b; Van Slyke et al., 1989; Miner and Hruby, 1989a). Newly formed mature particles can be found as early as 6 hpi. These virus particles migrate away from the virosome and collect within the cytoplasm. The continual
spread of infection can occur by both cell-cell fusion, or the generation of infectious, extracellular, enveloped virus (Moss, 1985).

**Vaccinia Early Gene Transcription: Initiation**

The mature vaccinia virion packages and encodes a large array of enzymes, some of which function during immediate early gene transcription. These include a multi-subunit RNA polymerase (Jones et al., 1987; Thompson et al., 1989), topoisomerase (Shaffer and Traktman, 1987; Fogglesong and Bauer, 1984; Shuman and Moss, 1987), poly A-polymerase (Brakel and Kates, 1974; Moss et al., 1975), nucleoside triphosphate phosphohydrolase (Broyles and Moss, 1987), and several RNA methyl transferases (Martin et al., 1975; Monroy et al., 1978; Morgan et al., 1984). These enzymes actively transcribe about half the 180 kilobase (Kb) genome (~100 genes) at early times post infection (Belle Isle et al., 1981; Morgan and Roberts, 1984; Golini and Kates, 1984; Mahr and Roberts, 1984; Lee-Chen, 1988). Transcripts produced from these early genes are structurally similar to eukaryotic messages, containing a 3' poly A-tail and a cap I structure which consists of a 5' terminal 7-methylguanosine connected via a triphosphate bridge to a 2' O-methyl nucleoside (Gershowitz and Moss, 1979; Boone and Moss, 1987). Except for the poly A-tail, these RNA's are colinear with their corresponding gene. Thus far, no evidence for RNA splicing has been found in vaccinia virus, consistent with its cytoplasmic site of replication.

The cis-acting signals responsible for specific transcriptional regulation of early genes have been analyzed in detail for a number of early genes including the thymidine kinase gene and the 7.5 Kd gene
(Cochran et al., 1985; Weir and Moss, 1987). Putative promoter regions were excised, abutted to a reporter gene and recombined back into the virus at the non-essential thymidine kinase locus. Sequential deletion of promoter fragments identified sequences that were necessary and sufficient for early gene transcription. These active regions are short, A-T rich segments located just 5' of the RNA transcription start site; they do not closely resemble eukaryotic or procaryotic promoters (Vassef, 1987). These early promoters are not generally functional at late times post-infection, though the existence of constitutive genes has been reported (Tamin et al., 1988; Weinrich and Hruby, 1987).

To analyze the mechanisms responsible for early gene expression, in vitro transcription systems have been derived from purified vaccinia cores which specifically initiate and terminate transcription in a vaccinia promoter and terminator dependent manner (Rohrmann et al., 1986; Spencer et al., 1980; Nevins and Joklik, 1977). These core extracts contain a VV-encoded DNA dependent RNA polymerase activity found in a high molecular weight complex which, even when purified 2500 fold, still contains mRNA guanylyltransferase (capping enzyme) and nucleoside triphosphatase I (DNA dependent ATPase). The specific initiation of this complex is insensitive to α-amanitin (a drug which inhibits cellular RNA polymerases) and is sensitive to non-hydrolyzable ATP analogs, suggesting a role for an ATPase during mRNA synthesis (Broyles and Moss, 1987). Further purification of this complex results in the loss of the promoter specific transcriptase activity, implying the separation of a promoter specific transcription factor. This activity could be reconstituted by mixing purified VV RNA polymerase with certain protein fractions.
Purification of this activity revealed a heterodimeric protein with associated DNA dependent ATPase activity consisting of two polypeptides (MW 77,000 and 82,000) which stimulated transcription 20-30 fold from a VGF promoter in vitro (vaccinia early transcription factor VETF) (Broyles and Moss, 1988). This factor has also been shown to bind specifically to the VGF and 7.5 K promoter sequences (Yuen et al., 1987). This protein complex is remarkably similar to a heterodimeric protein (MW 68,000 and 70,000) isolated in our lab, which binds specifically to the promoter region of the thymidine kinase and 7.5 K early promoters (Wilson et al., 1988). It is interesting that no clear consensus sequence has been identified which binds this factor; rather, it appears to bind to a variety of A-T rich sequences involved in the regulation of early transcriptional initiation.

**Early Gene Transcription: Termination**

The lack of a strong consensus for early initiation is in contrast to the highly conserved cis-acting signals required for termination (Yuen and Moss, 1986; Yuen and Moss, 1987). The DNA sequence TTTTTNT, or UUUUUNU in the cognate RNA, is found 50 nucleotides upstream from the transcription termination site of many early genes (Yuen and Moss, 1986 and 1987). In vitro transcription systems which accurately mimic in vivo termination have been used to analyze both the cis-acting sequences and the trans-acting factor involved in this reaction (Rohrmann et al., 1986). Purified VV RNA polymerase will not terminate in response to this sequence, termination requires the activity of an ancillary factor termed vaccinia termination factor (VTF) (Shuman et al., 1987). This factor was
purified on the basis of its ability to render VV RNA polymerase susceptible to the termination signal and may mediate its effects by recognizing the termination signal in nascent RNA (Shuman and Moss, 1988). VTF may be associated with the RNA polymerase itself, an idea that became more tenable with the surprising discovery that this termination factor is identical to the vaccinia mRNA capping enzyme (Morgan et al., 1984; Shuman and Moss, 1988). Termination does not require the presence of a 5' methyl cap structure, thus it is unclear if events occurring at the initiation of transcription are independent of events occurring at the termination of transcription. Transcriptional termination is a regulated process during viral infection, because at late times post infection, the early consensus termination signal is not recognized, generating large readthrough transcripts.

The switch between early and late modes of transcription is striking. Early gene products are responsible for the expression of the delayed early gene class and thus, the onset of viral DNA replication. Concomitant with DNA replication, expression of the late genes begins. Recently, preliminary evidence for the existence of another replication dependent gene class (intermediate) has been presented (Vos and Stunnenberg, 1988). While the existence of other temporal gene classes has been anticipated (Weinrich et al., 1985; Moss, 1985), the preliminary nature of the data regarding intermediate genes precludes discussion of their regulation. It has been suggested that a subset of this class of genes functions as regulatory factors for the activation of the late genes.
Late Gene Transcription: Cis-Acting elements

Late genes as a class have been mapped to regions throughout the genome, and sequencing efforts have usually revealed tightly packed, tandemly oriented, arrays of late genes (Chipchase et al., 1980; Mahr and Roberts, 1984; Wittek et al., 1984; Weinrich and Hruby, 1986; Lee-Chen and Niles, 1988). These late genes encode a variety of structural proteins and enzymes which function at late times post infection, or are packaged within the maturing virion for use at early times during the next infection cycle.

Transcription of late genes begins with the onset of DNA replication and exhibits marked differences from early transcription. Not only are late promoter sequences different than early promoters, the structure of the resultant message is also quite different. Experiments studying vaccinia virus late RNA are complicated by a lack of discrete 3' termination, generating large heterogeneous transcripts. An elegant set of experiments by Mahr and Roberts (1984) demonstrated the nature of late RNA and its complexity. Size-fractionated RNA translated in vitro showed that the messages encoding a particular gene product ranged in size from 1 Kb to 5 Kb. In spite of the fact that these long non-terminated transcripts read through downstream early and late genes, no strong evidence of translation of these downstream open reading frames (ORF) has been obtained. The significance of this 3' heterogeneity is unknown, however it is possible that RNA-RNA duplexes, formed as a consequence of antisense transcription are used in regulating expression. The size heterogeneity of late transcripts is not solely attributable to the lack of termination at late times. Primer extention experiments suggested that
the 5' end of the RNA was not colinear with the DNA sequence (Weinrich and Hruby, 1987; Rosel et al., 1986). This remained an enigma until two labs made a concerted effort to understand the structure of late messages from a highly expressed vaccinia late gene (11K) (Schwer et al., 1987; Bertholet et al., 1987). They demonstrated the existence of a capped 5' poly(A) head or leader sequence not encoded by the DNA, beginning at the S1 mapped start site. This was later shown to be true for a strongly expressed cowpox gene (Patel and Pickup, 1987), the 7.5Kd late promoter and the L65 late promoter (J. Miner unpublished results). Some ambiguity remains as to the length of the poly(A) leader; it appears to be heterogeneous from 30 to 100 nucleotides. The addition of this poly(A) leader probably occurs by an RNA polymerase slippage mechanism first discovered in T4 phage late transcription initiation (Kassavetis et al., 1986). Vaccinia virus derived in vitro transcription systems have been shown to produce RNA's with a similar poly(A) leader structure. These transcription systems have been used to show that the presence of the TAAAT motif, found at the start of many late genes, causes VV RNA polymerase to slip during initiation, adding a string of adenosine residues (Wright and Moss, 1987; Magistris and Stunnenberg, 1988; Ahn and Moss, 1989). The TAAAT sequence is also an important cis-acting element involved in vaccinia late transcription (Hanggi et al., 1986). Many vaccinia late genes have the consensus sequence TAAAT(G) located at the S1 mapped RNA start site where the ATG is the translational start signal (Rosel et al., 1986). Detailed analysis of 11K, 28K (a major late vaccinia protein) and ORF A1 late promoters suggested that 40 nucleotides upstream of the initiation site was both necessary and
sufficient to confer late gene regulation on the expression of a reporter
gene (Hanggi et al., 1986; Miner et al., 1988; Weir and Moss, 1984;
Bertholet et al., 1985 and 1986; Weir and Moss, 1987). In addition, site
directed mutagenesis revealed that the TAAAT motif plays a role similar
to that of the TATA box found in other eukaryotic promoters (Hanggi et
al., 1986). Point mutations within this signal changed the position of the
start site but not necessarily the level of transcription; however, deletion of
this signal entirely abolished transcription. Sequences which modulate
the level of RNA from a particular TAAAT motif are located further
upstream between -40 and -10 from an initiation site at +1 (Miner et al.,
1988; Bertholet et al., 1986; Weir and Moss, 1987). Within this region of
both the 28 K and the ORF A1 promoter, a string of 8 adenosine residues
can be found which, when deleted or mutagenized, eliminates all
promoter activity (Miner et al., 1988; Weir and Moss, 1987). An analysis of
the information content of late gene promoters suggests there is little (if
any) conservation of sequences between promoters, except for the
TAAA(G) (T) motif, yet all these late promoters regulate expression at late
times post infection (Mars and Beaud, 1987). It is possible that the
primary sequence is not the only component of late promoters and that
secondary DNA structure plays a role.

**Late Gene Transcription: Transcription Factors**

The trans-activation of these promoters in VV infected cells implies
that diffusible factors are involved in recognition of, and transcription
from, these sequences. The analysis and purification of the protein
factors involved in vaccinia late transcription has proceeded along two
fronts. The first approach uses a VV late gene specific in vitro transcription extract in fractionation protocols to purify the components of the transcription complex (Wright and Moss, 1987; Fogglesong, 1985). These transcription systems have previously been used primarily to characterize the mechanics of the poly (A) leader addition reaction, however work is beginning on the purification of components necessary for transcription initiation.

Another approach to study factors involved in late gene regulation is to use DNA sequences that are known to activate transcription as probes for DNA binding proteins in gel retention assays. Results obtained using this assay in other labs are unpublished and will not be discussed, however, chapter III describes the use of this assay to isolate a protein from infected cells which binds to the L65 promoter (Miner and Hruby, 1989b).

**Conclusion**

This review has focused primarily on transcriptional regulation of vaccinia virus gene expression. The biochemical mechanisms behind this process are in the process of being described for vaccinia and thus far, appear to correspond with transcription reactions occurring in higher organisms. This similarity suggests that analysis of vaccinia regulation may yield fundamental insights into general regulatory mechanisms. I have chosen a late gene ORF D13 and its protein product, L65, as a candidate for an indepth analysis of vaccinia virus late gene regulation. My objectives were: I. Characterize cis-acting elements responsible for late transcriptional regulation; II. Isolate protein factors
which bind to these cis-acting sequences; III. Using an immunological approach, analyze L65 expression and distribution. I have described in the chapters that follow, my attempts to accomplish these goals. Off shoots of the research discussed here are currently being pursued in the Hruby lab and should yield additional information regarding the regulation of vaccinia gene expression.
CHAPTER II

MOLECULAR DISSECTION OF CIS-ACTING REGULATORY ELEMENTS FROM 5' PROXIMAL REGIONS OF A VACCINIA VIRUS LATE GENE CLUSTER

Authors: Jeffrey N. Miner, Scott L. Weinrich and Dennis E. Hruby
SUMMARY

Promoter elements responsible for directing the transcription of six tightly-clustered vaccinia virus (VV) late genes (ORFs D11, D12, D13, A1, A2 and A3) from the HindIII D/A region of the viral genome were identified within the upstream sequences proximal to each individual locus. These regions were identified as promoters by excising them from the VV genome, abutting them to the bacterial CAT (chloramphenicol acetyl transferase) gene, and demonstrating their ability to drive expression of the reporter gene in transient expression assays in an orientation-specific manner. A series of 5' deletions of the ORF A1 promoter from -114 to -24 showed no reduction in promoter activity while additional deletion of the sequences from -24 to +2 resulted in the complete loss of activity. Deletion of the ORF A1 fragment from -114 to -104 resulted in a 24% increase in activity suggesting the presence of a negative regulatory region. Deletions designed to define the 5' boundary of the ORF D13 promoter identified two positive regulatory regions; the first between -235 and -170 and the second between -123 and -106. Significantly, this places the ORF D13 regulatory regions within the upstream coding sequences of ORF A1. In addition, sequence homologies between temporally-related promoter elements support the notion of kinetic subclasses of late genes.
INTRODUCTION

Vaccinia virus (VV), the prototype of the poxvirus family, is a large double-stranded DNA-containing animal virus which replicates in the cytoplasm of infected cells (Moss, 1985). The relative independence of VV replication from host cell nuclear functions (Moss, 1974; Pennington and Follet, 1974; Silver and Dales, 1982; Silver et al., 1979), coupled with its complex assembly process (Wittek, 1982), necessitates that the virus be capable of modulating the temporal expression of the approximately 200 genes encoded within its 185 Kb genome. Penetration of VV into susceptible host cells is followed by expression of early viral functions which are responsible for the shut-off of host-directed macromolecular syntheses and the initiation of viral DNA replication (Moss, 1985; Moss, 1974; Moss, 1978; Moss and Salzman, 1968; Salzman and Sebring, 1967). Concomitant with DNA replication, viral late genes are transcribed and translated, producing both structural proteins and viral enzymes necessary for morphogenesis and the production of infectious progeny virions (Moss, 1978; Moss, 1985; Dales and Pogo, 1981). Early genes are not expressed at late times during infection. The mechanisms governing the switch between the early and late modes of viral gene expression are not yet known. Although only a limited number of VV late genes have been analyzed in detail, previous studies suggest that transcription of late genes is regulated primarily by the 5' flanking sequences (Bertholet et al., 1985; Bertholet et al., 1986). Deletion analyses have indicated that promoter sequences are within approximately 30 base-pairs of the
transcriptional start site. These elements do not contain obvious homologs of the usual eukaryotic or prokaryotic consensus signals (Bertholet et al., 1986; Hanggi et al., 1986; Weir and Moss, 1987). Since many of the VV late genes encode essential viral functions, it is not possible to study and manipulate these loci in their normal genomic context. Therefore to circumvent this problem, VV late promoters have been studied using either transient expression assays (Cochran et al., 1985), or by recombination of promoter elements into alternate locations within the viral genome (Bertholet et al., 1986; Hanggi et al., 1986; Weir and Moss, 1987). The results of both approaches have been similar. The VV transient expression system employs plasmids containing chimeric viral promoter:reporter gene cassettes, which are transfected into susceptible host cells, followed by superinfection with VV to provide a source of VV RNA polymerase and potential trans-acting factors. Measurement of reporter gene activity gives relative values for VV promoter function. Transient expression assays have been used extensively in other viral systems such as SV40 and herpesvirus to define the sequences and trans-acting factors involved in the regulation of gene expression (Alwine, 1985; Keller and Alwine, 1985; McKnight and Tjian, 1986; Coen et al., 1986). This approach affords the advantage of rapid assay of native and mutated promoter sequences. For example, Cochran et al. have shown that the 7.5 Kd VV promoter, when linked to the coding portion of the bacterial chloramphenicol acetyltransferase gene (CAT) in a recombinant plasmid, will catalyze CAT expression only in cells transfected with this plasmid and superinfected with VV. Transcription was initiated correctly and had kinetics of expression consistent with the
late gene promoter element within the 7.5 Kd promoter (Cochran et al., 1985). In our efforts to understand VV late gene expression, we have focused on a region of the genome that is expressed primarily at late times. We previously mapped a 65,000 molecular weight polypeptide-encoding late gene near the Hind III D/A junction (Weinrich et al., 1985). Designated L65, this protein was shown to be a major viral product late in infection that was not associated with the mature virion. Tartaglia et al. mapped rifampicin resistance to an A-T to G-C transition mutation within the coding sequences of L65 (Tartaglia and Paoletti, 1985; Tartaglia et al., 1986). In the presence of rifampicin, both VV morphogenesis and cleavage of precursor polypeptides to mature viral structural proteins are inhibited. Hence the late protein L65 may play a role involved in virion assembly. Sequence analysis of the genomic region surrounding the L65 locus indicated the presence of six densely-packed, tandemly-oriented ORFs, of which L65 was the fourth ORF of the cluster (Weinrich and Hruby, 1986). [Using the new VV genetic nomenclature proposed by Rosel et al. (Rosel et al., 1986), L65 is now synonymous with ORF D13]. All of these ORFs are transcribed late in infection in a leftward direction towards the HindIII H fragment (Weinrich and Hruby, 1987; Rosel and Moss, 1985). Kinetic data from an S1 nuclease analysis suggested that, rather than being transcribed as a single long polycistronic message, these ORFs were independently transcribed (Weinrich and Hruby, 1987). In this communication, we further test this hypothesis by utilization of a transient expression system to identify promoter elements preceding each ORF. The identification of six VV late promoters significantly increases the number of vaccinia late promoters characterized to date. To further
define sequences important in the regulation of viral gene transcription, a series of deletions were made in two of the promoters (A1 and D13) and the 5' boundary of the minimal promoter regions were determined. Using the information gained from the deletion analysis, specific homology searches were conducted and several interesting homologies were observed.
MATERIALS AND METHODS

Plasmid Construction.
Recombinant plasmids were constructed, propagated, and analyzed essentially as previously described (Maniatis et al., 1982). Using the information previously published concerning the nucleotide sequence and RNA start sites used in the HindIII D/A portion of the genome (Weinrich and Hruby, 1986 and 1987), the regions immediately upstream of, and including, the 5' start sites were excised and cloned using the restriction enzyme cleavage sites indicated in Figure II.1. The isolated promoter fragments were ligated into recombinant plasmids at sites immediately upstream of the coding sequences of the bacterial chloramphenicol acetyl transferase gene (CAT) which was used as a reporter gene in these studies. Where possible, the promoter fragments were inserted in both the same and opposite orientation relative to the CAT gene. Final constructions were verified by both analytical restriction enzyme digestion and nucleic acid sequencing procedures. 5'-deletions of the D13 and Al ORF promoters were created by unidirectional Exo III deletion mutagenesis as previously described (Maniatis et al., 1982; Figure II.4). Deletion endpoints were determined by nucleotide sequencing.

Cells and Virus.
Growth, purification and plaque titration of vaccinia virus (strain WR) was carried out essentially as previously described (Hruby et al., 1979a). BSC-40 cells were grown in Earle's minimum essential medium (MEM,
supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine at 37°C, 5% CO₂, and 95% humidity. Ltk- cells were maintained in the same medium plus 25 ug of 5- bromodeoxyuridine per ml. Prior to being used for transient expression, Ltk- cells were passaged twice in medium lacking bromodeoxyuridine.

**Transient Expression.**

Transient expression assays were carried out essentially as previously described (Cochran et al., 1985; Gorman et al., 1982; Fuerst et al., 1986). Briefly, recombinant plasmids (5 ug) were co-precipitated with carrier salmon sperm DNA using the calcium phosphate method (Graham and Van Der Eb, 1973). Precipitated DNA was transfected into monolayers of Ltk- cells. Three to four hours later the transfected monolayers were glycerol shocked. After a one hour recovery period, the cells were superinfected with VV at a multiplicity of 30 plaque forming units/cell. The infected cells were incubated at 37°C for 24 h prior to being processed for CAT assays.

**CAT Assays.**

Cells were washed twice with ice-cold phosphate-buffered saline, pelleted and resuspended in 1 ml of CAT assay buffer [40mM Tris-Cl(pH 7.8), 1mM EDTA, 150mM NaCl] and incubated at 25°C for 5 min (Keller and Alwine, 1985; Fuerst et al., 1986). The cells were pelleted and resuspended in 0.1 ml of 0.25M Tris-Cl(pH 7.8). After subjecting the cells to three freeze-thaw cycles, the nuclei were pelleted at 12,000 rpm for 5 min in a TOMY 150A microcentrifuge and the supernatants removed and
stored at -20°C until subsequent CAT assays. CAT activity was measured by the ability of the extracts to transfer the acetyl group from acetyl CoA onto [14C] chloramphenicol (Hruby et al., 1979b). The acetylated chloramphenicol derivatives were resolved by thin-layer chromatography on silica plates, located by autoradiography, excised, and counted by liquid scintillation. Positive (CAT enzyme, Sigma) and negative (water) controls were run with each set of reactions. Extracts were diluted to ensure that the assay was within the linear range.

**Computer analyses.**

Nucleic acid sequences were analyzed for restriction enzyme cleavage sites and homologies using the microgenie (Beckman Instruments, Inc.) program on an IBM PC computer.

**Materials.**

Restriction endonucleases and other enzymes used for cloning manipulations were obtained from either Bethesda Research Laboratories or New England Biolabs Inc. Radioisotopes were from New England Nuclear. All tissue culture supplies were purchased from GIBCO.
RESULTS

**Cloning of VV late promoter elements.**

Initial transcriptional mapping studies of the vaccinia virus genome have not indicated any striking overall organizational motifs (Rosel and Moss, 1985; Belle Isle et al., 1981; Chipchase et al., 1980; Oda and Joklik, 1981; Paoletti and Grady, 1977). However, at the subgenomic level, detailed analyses have demonstrated that VV genes belonging to the same kinetic class are tightly packed as to suggest that the regulatory signals of each individual cistron may lie within the coding sequences of the neighboring upstream and downstream genes (Weinrich and Hruby, 1986). Although, 5'-termini of RNA's were detected for each putative gene by S1 nuclease mapping (Weinrich and Hruby, 1987), it was not obvious whether these ends represented transcriptional initiation from promoter elements upstream of each gene, or whether they were generated by posttranscriptional processing of a larger polycistronic transcript.

To distinguish between these possibilities, it was of interest to excise the 5'-proximal region upstream of each gene and to determine whether it could direct the expression of an abutted reporter gene, i.e., function as a cis-acting promoter element. Therefore, the indicated VV subgenomic fragments (Figure II.1) were cloned, making use of available restriction endonuclease sites. Depending on the fragment, the 5' boundaries of these putative promoters were between 114 and 283 base-pairs upstream of the late RNA start sites previously identified (Weinrich and Hruby, 1987). Based on earlier analyses of other VV late promoters, this should be more...
than enough DNA to encode a fully functional VV late promoter. On the 3' side, the cloned fragments contained from 1 to 33 bases downstream of the RNA start site and all but one included sequences corresponding to 5'-codons of the downstream ORF. The isolated VV DNA fragments were inserted into recombinant plasmids which contained the coding sequences of the bacterial chloramphenicol acetyltransferase gene (CAT, Figure II.2). The site of insertion was 5'-proximal to the CAT gene. With the exception of the putative A2/A3 promoter, all fragments were inserted in both the correct and incorrect orientation. Recent experiments in our laboratory have indicated that the bacterial lac promoter is recognized at least to some extent by the VV transcriptional apparatus (unpublished data). Thus it is of importance to note in the chimeric constructions assembled here (Figure II.2), that the lacZ gene contained in these plasmids is located downstream of the CAT gene and reading in the antisense direction relative to the CAT gene. This insures that any CAT activity directed by these plasmids is not the result of lac-directed transcripts.

**Transient expression analysis of VV promoters.**

A transient expression assay was used to assess the ability of the putative VV late promoter elements to be recognized by VV RNA polymerase and to direct the expression of functional CAT enzyme. Control experiments demonstrated that these plasmids were totally dependent on VV infection for activity. Cells were transfected with recombinant plasmids, infected with VV, and after 24 h lysates were assayed for CAT activity (Figure II.3). As positive and negative controls
Fig. II.1  Organization of the *Hind*III D/A region of the VV genome. At the top of the figure is the *Hind*III restriction map of the 185 Kb VV genome with the closed ends and inverted terminal repeats represented. The 5.1 Kb *Hind*III D/A region of interest has been expanded with the position and orientation of the open reading frames indicated as previously determined by Weinrich and Hruby 1986. The size of the ORFs and the distance between them are drawn to scale. ORF designations are according to the conventions proposed by Rosel et al. (Rosel et al., 1986). The lower portion of the figure indicates the genomic location, nomenclature, and restriction enzyme cleavage sites for each of the putative promoter fragments. The numbers below each potential promoter fragment indicate its size in base-pairs.
Figure II.1
Fig. II.2  Structure of recombinant plasmids containing chimeric VV late promoter:CAT gene cassettes. Potential VV late promoters from the H~{i}ndIII D/A region were inserted into plasmids containing the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene. Orientation of the promoter elements and CAT genes are indicated by the direction of the arrows. The restriction enzyme cleavage sites used for insertion of each fragment are listed (sites enclosed within parentheses were lost during cloning). The original parental plasmid, location of the lac promoter and its direction of transcription are also indicated.
Figure II.2
plasmids containing the CAT gene linked to the VV 7.5 Kd or no promoter element, respectively, were included in the experiments. The results indicate each of the six fragments cloned from the HindIII D/A region behaved as promoter elements, directing the expression of the CAT gene at levels ranging between 4- and 100-fold over the pUC:CAT control. In the inverted orientation, relative to the CAT gene with the exception of the A1 fragment, the promoter elements were essentially inactive. The reason for the weak (3-fold) activity of the negative orientation of the A1 fragment is unknown.

5' deletion analysis of promoter sequences.

To further delineate the nucleotide sequences that are important for the regulation of VV late gene transcription, the two most efficient promoter elements (A1 and D13) from the HindIII D/A cluster were chosen as substrates for 5' deletion mutagenesis. Using the approach outlined in Figure II.4, a family of unidirectional deletions were constructed which extended from the 5' boundary of the promoter fragment through the transcriptional start site. 5' deleted promoter:CAT constructs were assayed by transient expression.

The results of applying this approach to the A1 promoter element are displayed in Figure II.5A. High level promoter activity was maintained in the case of D+pPA1:CAT plasmids containing sequences from -114 to -24 bp upstream of the RNA start site. A deletion to +2, which
Fig. II.3. Transient expression of VV late promoter:CAT genes in VV-infected cells. Promoter elements from the HindIII D/A region were assayed for their ability to express an abutted CAT gene. The promoter fragments tested are indicated along with the ORF from which they were excised and their 5' and 3' limits relative to the major late start site of transcription (+1) previously determined (Weinrich and Hruby, 1987). As positive and negative controls, plasmids containing the CAT gene linked to the VV 7.5 Kd promoter, or no promoter, were included in these experiments. The recombinant plasmids were transfected into cells, the cells superinfected with WT VV, and after 24h, cytoplasmic extracts were prepared and assayed to determine the level of CAT activity present. The numbers represent nmoles of chloramphenicol acetylated per 5 X 10⁶ infected cells. The values given represent the average of at least three separate experiments in which the standard deviation of the measurements obtained was less than 18%. NT = not tested.
<table>
<thead>
<tr>
<th>OPEN READING FRAME</th>
<th>PROMOTER FRAGMENT</th>
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<td></td>
<td>5' LIMIT</td>
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<td>-398</td>
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<tr>
<td>D12</td>
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<td>A3</td>
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<tr>
<td>7.5K</td>
<td>-241</td>
<td>▼</td>
</tr>
<tr>
<td>pUC:CAT</td>
<td>NONE</td>
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Figure II.3
Exo III deletion mutagenesis of the ORF A1 (4A) and D13 (4B) promoter elements. The +pPA1:CAT and +pPD13:CAT plasmids were progressively deleted from the 5'- direction relative to the transcriptional start site using the procedures outlined. The resulting constructs were screened by restriction enzyme analysis and nucleotide sequencing protocols in order to obtain an appropriate library of deletions extending into or through the regions suspected to contain the regulatory elements of the A1 and D13 late promoters.
Figure II.4
removed the normal start site, completely eliminated CAT expression. Thus, characteristic of other VV late promoters analyzed thus far [11K (Bertholet et al., 1986; Hanggi et al., 1986) and 28K (Weir and Moss, 1987)], the A1 promoter apparently requires only a short upstream region for full transcriptional activity in transient expression assays. Deletion of sequences between -114 and -104 consistently resulted in an increase in CAT activity.

The results obtained from carrying out a similar analysis on the D13 promoter (Figure II.5B) were quite different. In this case, deletion of sequences between -235 and -170 caused a 40% loss in activity of the D13 promoter. Further deletions between -170 and -124 had little effect, whereas removal of the next 16 bp to -106 resulted in a substantial loss of promoter activity. None of these deletions removed the transcriptional start sites mapped by Weinrich and Hruby, 1987. The low level of D13 promoter activity was reduced to background levels by removal of the region between -88 and -41. Thus, the D13 promoter apparently contains at least two positive regulatory elements, one between -235 and -170, the other between -124 and -106.

Occurrence of inter-promoter nucleotide homologies.

The deletion mutagenesis data provided more precise information on the essential regions of the A1 and D13 promoters. Therefore the nucleotide sequences between -24 and +1 of the A1 promoter and -124 and -92 of the D13 promoter were compared by computer at high stringency to one another as well as with previously published vaccinia promoter sequences (Figure II.6).
Transient expression of the 5'-deleted ORF A1 and D13 promoter mutants. Recombinant plasmids containing the full VV promoter fragment, or the indicated 5'-deletions, abutted to the CAT gene were assayed for transcriptional activity by transient expression. Each experiment was repeated a minimum of 3 times. The average values are shown in bar graph form with the standard deviation of the various trials indicated by the thin line. CAT activity was calculated relative to the intact promoter fragment. Below each graph is displayed the nucleotide sequence of the promoter fragment along with the location of various deletions and the major late transcriptional start site (Weinrich and Hruby, 1987). A) ORF A1 promoter. B) ORF D13 promoter. The inset shows a log scale of activity levels of the indicated deletions. In all cases "C" = pUC:CAT control.
5A

![Bar graph showing % CAT activity for different deletions in ORF A1.](image)

5' DELETIONS
ORF A1

5B

![Bar graph showing % CAT activity for different deletions in ORF D13.](image)

5' DELETIONS
ORF D13

Figure II.5
We and others (Hanggi et al., 1986; Rosel et al., 1986) have noted the presence of the characteristic TAAAT(G) signal at or near the transcriptional start site of VV late genes. While this signal is present at the 5'-end of most of the HindIII D/A gene cluster, three other regions of significant homology were noted: 1) the sequence TAAACT(T)ACT is found 8 bp and 11 bp 5' to the upstream RNA start site of D13 and D11, respectively; 2) the sequence AAAA(A/T)ATAGTT is found 8 bp and 16 bp upstream from the first RNA start site for ORF A1 and the downstream RNA start site for ORF D11; and 3) the 13 nucleotide sequence, ATAAATACAATAAA is perfectly conserved between the late RNA start site of the 7.5 Kd gene promoter and the 5' RNA start site of ORF D13.
Fig. II.6. Inter-promoter sequence homologies present within kinetic sub-classes of VV late promoter elements. VV late ORFs A1, D11, D13, and the 7.5 Kd gene are diagrammed showing nucleotide sequences and location of the indicated regions upstream of the predicted coding regions. Both early and late RNA start sites (Weinrich and Hruby, 1987) are shown. Three regions of high homology (A,B, and C) detected by computer analysis are indicated.
Figure II.6
Based on the ability of cloned VV DNA fragments to catalyze CAT expression in a transient expression assay and on differential appearance of 5' ends by time course nuclease S1 analysis (Weinrich and Hruby, 1987), we conclude that regions upstream of each ORF in the VV HindIII D/A late gene cluster, contain a cis-acting promoter element. In two recent papers, data have been reported which suggest discontinuous synthesis of vaccinia late mRNA (Bertholet et al., 1987; Schwer et al., 1987). These authors have detected a 35-40 nucleotide leader RNA at the 5' end of late mRNA. The mechanism of addition is as yet unknown. The presumably extra-genomic location of transiently expressed plasmids prevents any cis genomic regulatory functions from operating. This indicates that a nascent RNA strand is synthesized from the promoter fragment, in the absence of readthrough. The episomal assay condition argues strongly in favor of promoter activity, and strongly against some other mechanism of transcript processing. The precise mechanisms of VV promoter function, i.e. leader primed transcription and possible trans splicing events occurring after RNA synthesis are not addressed by these experiments. Differing levels of CAT expression from individual promoter fragments would seem to support the hypothesis that the individual loci within this late gene cluster are differentially expressed and regulated. This latter conclusion must be qualified however since the six promoter fragments contain variable amounts of 3'- and 5'- flanking sequences which may or may not affect the measured level of expression.
of the abutted CAT gene. Of the promoter fragments, five produced only background CAT activity when present in the opposite orientation relative to CAT, whereas the ORF A1 promoter fragment demonstrated some measurable activity in both directions. It is not clear why this fragment is slightly active (3-fold) in the negative orientation. Arguments could be advanced for either an "enhancer"-like activity or a cryptic VV promoter. The latter possibility seems more likely for two reasons: 1) the former requires postulation of a promoter as well as an enhancer within the inverted fragment and 2) weak VV RNA start sites at locations not thought to contain VV promoters have previously been recognized (Weinrich and Hruby, 1987).

Nuclease S1 analyses of RNA generated from the chimeric genes during transient expression assays showed a CAT gene specific RNA with a 5' end corresponding to the in vivo 5' end previously mapped by Weinrich and Hruby, 1987 for ORF A1 (data not shown). Likewise, the ORF D13 promoter fragment protected a 5' end which corresponds to the upstream start site (-92) used in vivo. The cloning procedure used for the ORF D13 promoter fragment has eliminated the downstream site at (+1). Therefore regulatory signals described here are affecting expression of CAT from the upstream D13 start site exclusively. Experiments are currently in progress to determine the limits of the upstream signals which regulate the expression of the downstream (+1) D13 start site.

The 7.5 Kd promoter represents one of the most active VV promoters thus far recognized. Because of this, it has been used to direct the expression of foreign genes in many of the recombinant VV strains which are currently being evaluated as candidate vaccines (Mackett et al.,
1984; Werts et al., 1987). It is interesting to note on the basis of the data shown in Figure II.3, that the D13 promoter element is at least as efficient as the 7.5 Kd promoter element at expressing downstream CAT sequences (280-fold vs. 267-fold). Thus, the D13 promoter element may of be of potential use in future recombinant virus constructions.

The use of 5' deletion mutagenesis procedures revealed in the case of the ORF A1 promoter fragment that only the 5' proximal 24 base pairs is required for full CAT expression. The small size of this promoter is similar to that shown to be important for function of the 28K gene promoter (Weir and Moss, 1987). It was also of interest to note that deletion of the bases between -114 and -104 consistently increased the activity of the A1 promoter to a level of 124% of the intact fragment. This result could indicate that a negative regulatory element exists at this location. While further refinement of the transient expression system will be necessary before a detailed analysis of these sequences can be undertaken, there is evidence for negative regulatory ("silencer") signals being involved in the attenuation of late genes at early times during viral replication in other viral systems (Alwine and Picardi, 1986). A similar analysis of the ORF D13 promoter showed that in contrast to other VV promoters analyzed, the ORF D13 promoter contains at least two positively acting regulatory regions. The significance of this higher level of complexity is not known. Previous work indicated the presence of two transcriptional start sites for ORF D13 at +1 and -92 (Weinrich and Hruby, 1987). The -92 site was used transiently from 2-3 hpi and the +1 site was used from 3 to 12 hpi. The S1 analysis on CAT specific RNA mentioned earlier supports the notion that these two positive regulatory regions are
modulating levels of transcription from the upstream site at (-92). The presence of essential regulatory signals over 100 bp from the transcriptional start sites represents a significant departure from previously described VV late promoters. Furthermore, these results suggest that nucleotide sequences in this region of the VV genome may contain information for both transcriptional initiation and protein sequence, since the coding sequences for ORF A1 end at -17 relative to the D13 RNA start site.

Sequence comparisons between these regulatory elements and other VV late promoters revealed the existence of short regions of homology between several of the promoters. While the significance and possible function of these sequences remain to be established experimentally, it is of interest to note that in each case, these sequences are located in positions very close to established RNA start sites. More importantly, the kinetics of in vivo expression from the proximal RNA start are similar (Weinrich and Hruby, 1987), suggesting a role in regulation of the timing of gene expression. Thus a reasonable working hypothesis might be that the general A/T richness and the TAAATG motif found within VV late promoter elements may play a general role in identifying late genes as such, and other cis-acting information, such as that identified in Figure II.6, may serve to activate the expression of particular kinetic subclasses of vaccinia virus late genes.
CHAPTER III

DNA SEQUENCES THAT REGULATE THE EXPRESSION OF A VACCINIA VIRUS LATE GENE (L65) AND INTERACT WITH A DNA BINDING PROTEIN FROM INFECTED CELLS

Authors: Jeffrey N. Miner and Dennis E. Hruby
SUMMARY

To be efficiently expressed in vivo, the vaccinia virus (VV) late gene, L65, requires 5'-proximal cis-acting elements which bind a factor from infected cells. Deletion mutagenesis and VV helper-dependent transient expression procedures were used to demonstrate that two distinct late promoter elements direct transcription from two different start sites [proximal (+1) and distal (-92)]. The -128 to -112 region was essential for L65 distal promoter function, while sequences between -59 and +50 were sufficient for L65 proximal promoter function. The proximal DNA sequences bind a factor (BF-1), isolated and partially-purified from vaccinia-infected cells at late times post infection. This activity is not detectable in uninfected cells or in purified virions. This factor binds specifically to two different sites within the proximal promoter, one 5' and one 3' to the transcription start site, but does not bind to the distal promoter element.
INTRODUCTION

Within infected cells, the expression of vaccinia virus (VV) genes is regulated in a distinct temporal fashion (Moss, 1985; Wittek, 1982). During replication, VV proceeds through three general phases of gene expression: immediate-early, delayed-early, and late. Since the replication cycle of vaccinia virus occurs exclusively in the cytoplasm of infected cells, many of the regulatory functions affecting viral gene expression may be encoded by the virus (Villarreal et al., 1984). The lack of any apparent eukaryotic consensus signals within the promoter regions of VV genes tends to support this possibility (Rosel et al., 1986). Understanding the mechanisms responsible for the regulation of VV gene expression is of particular importance not only as a model system for a relatively complex genetic regulatory pathway, but also for facilitating the continued development of VV as a eukaryotic expression vector.

Experiments directed towards the identification and analysis of the cis and trans elements responsible for the regulated expression of VV genes in vivo are underway in a number of laboratories. The information accumulated to date indicates that VV early promoter elements are quite small, requiring only about 30 base-pairs (bp) of upstream sequence to achieve regulated expression of an abutted reporter gene (Cochran et al., 1985, Weir and Moss, 1987). Similarly, protein factors which bind to these early VV promoters have been identified in virion extracts, partially purified (Broyles et al., 1988, Wilson et al., 1988, Yuen et al., 1987), and shown to enhance transcription of cloned early genes in a fractionated cell-free transcription system prepared from VV virions (Broyles and
Moss, 1988, Broyles et al., 1988). However, in both cases the precise molecular identification of the cis and trans elements responsible for transcriptional enhancement has not yet been achieved.

Although relatively little is known regarding the signals which regulate the expression of VV early genes, even less is known concerning late gene expression, the study of which is complicated by two unusual transcriptional properties. First, VV late transcription is characterized by a lack of discrete 3' termination, generating long (>10 kilobase) transcripts traversing through other downstream late or early genes (Weir and Moss, 1984). Second, recent reports have indicated that VV late messages contain a 5' poly(A) head or leader sequence of variable size (Bertholet et al., 1987, Patel and Pickup, 1987, Schwer et al., 1987).

In previous work on late gene promoters, putative late promoter regions have been abutted to reporter genes and assayed by VV helper-dependent transient expression procedures or after recombination of the chimeric genes back into the viral genome (Bertholet et al., 1986, Cochran et al., 1985, Hanggi et al., 1986, Miner et al., 1988, Weir and Moss, 1987). Both of these approaches have given equivalent results (Cochran et al., 1985, Weir and Moss, 1987). Several studies have provided evidence that a TAAAT(G) motif, located at or near the transcription start site, is important for late gene expression (Bertholet et al., 1986, Hanggi et al., 1986). However, this motif alone is apparently insufficient to direct high level late gene transcription as a number of VV late genes have been shown to require additional short upstream elements for full promoter activity, including the L5 (28K) and A1 genes (Miner et al., 1988, Weir and Moss, 1987).
In contrast to previously mentioned VV late promoters, the regulatory sequences directing the expression of the L65 (VV D13) gene appear to be somewhat more complex. Utilizing two transcriptional start sites [distal (-92) and proximal (+1)] the L65 promoter governs the expression of a transcript encoding an abundant late VV protein of 65 kilodaltons (L65) to which rifampicin resistance has been mapped (Baldick and Moss, 1987, Tartaglia et al., 1986). The L65 gene is located within the Hind III D/A region of the VV genome (ORF D13) (Weinrich et al., 1985), and lies within a cluster of open reading frames which are noncoordinately expressed at late times post infection (Weinrich and Hruby, 1986, Weinrich and Hruby, 1987). The L65 promoter has been shown to contain two separate transcriptional elements, one between -128 and -112 (element I) and another between -235 and -175 (element II) (Miner et al., 1988).

In the present work we have used internally controlled S1 nuclease analysis of RNA from transiently expressed L65 promoter/chloramphenicol acetyltransferase (CAT) gene constructs to analyze the sequences required for transcription from the proximal site. Signals sufficient for expression from the proximal promoter lie between -59 and +50. To investigate whether viral-encoded regulatory factors may interact with elements in the L65 promoter, cytoplasmic extracts were made from uninfected and VV-infected HeLa cells and assayed for the presence of DNA binding proteins. DNA fragments corresponding to the L65 promoter region were isolated, labelled and used as probes in gel retention assays (Fried and Crothers, 1981) and were found to specifically bind a relatively heat-stable, proteinase K-sensitive factor [binding factor I
(BF-1)] from infected cell extracts. After partial purification, we were able to demonstrate by competition that this factor interacts specifically with regions of the proximal L65 promoter found to be essential for expression. To map the binding sites more closely, deleted versions of the promoter were used in both competition and direct binding studies, revealing the presence of two binding sites within the promoter.
MATERIALS AND METHODS

Plasmid construction

Recombinant plasmids were constructed, propagated, and analyzed essentially as described by Maniatis et al. (Maniatis et al., 1982). The pPD13:CAT plasmid contains 300 base pairs of upstream sequence from the L65 ORF (distal L65 promoter), fused to the bacterial chloramphenicol acetyl transferase gene (CAT) (Miner et al., 1988). pPD13L:CAT contains 440 base pairs of upstream sequence and 50 base pairs of downstream sequence from the distal and proximal L65 promoters fused to the CAT gene (see Fig III.1). This 490 bp Fok I/Pst I promoter fragment was trimmed with S1 nuclease and ligated to the Sma I site of pUC13:CAT generating pPD13L:CAT. Plasmid pPD13L:CAT served as the starting material for the deletion series. Digestion with Kpn I and XbaI, followed by limited exonuclease III/S1 nuclease treatment, allowed unidirectional 5'-deletion into the promoter fragment. Dideoxy DNA sequencing of the plasmids confirmed the extent of the deletions (Sanger et al., 1977).

Cells and virus.

Growth, purification and plaque titration of VV (strain WR) was carried out as previously described (Hruby et al., 1979). BSC-40 and L cells were maintained in Earle’s minimum essential medium (GIBCO Laboratories), supplemented with 10% heat inactivated fetal bovine serum, 50 ug/ml gentamycin sulphate and 2mM L-glutamine at 37C, 5% CO₂ and 95% humidity (Miner et al., 1988)
Transient expression

Transient expression assays were carried out as previously described (Cochran et al., 1985, Miner et al., 1988). Briefly, recombinant plasmids (5 ug internal control and 10 ug test plasmids) were co-precipitated with carrier salmon sperm DNA using the calcium phosphate method (Graham and Van Der Eb, 1973). Precipitated DNA was layered onto monolayers of L cells. Three hours later the monolayers were treated with glycerol at 37°C. After a one hour recovery period, the cells were superinfected with VV at a multiplicity of 30 PFU/cell. The infected cells were incubated at 37°C for 24 h prior to RNA extraction or CAT enzyme extract preparation.

CAT extracts and assays

Infected cells were harvested at 24 hours post infection (hpi), washed twice with ice cold phosphate-buffered saline, centrifuged and resuspended in 1 ml of CAT assay buffer [40mM Tris-Cl(pH 7.8), 1mM EDTA, 150mM NaCl] and incubated at 25°C for 5 min. The cells were centrifuged again and resuspended in 0.1 ml of 0.25M Tris-Cl (pH 7.8). After subjecting the cells to three freeze-thaw cycles, the nuclei were removed and the extract stored at -20°C. CAT assays were performed as previously described (Miner et al., 1988).
RNA preparation and S1 nuclease analysis

After transient expression, total cytoplasmic RNA was isolated from monolayers of transfected L cells. Cells were washed twice with phosphate-buffered saline, lysed with 2 ml guanidine isothiocyanate solution (4M guanidine isothiocyanate, 25mM sodium citrate (pH 7.0), 0.5% lauryl sarcosine, 0.1 M β-mercaptoethanol), sheared by 6 passages through a 23 gauge needle, layered onto a 3 ml CsCl cushion [5.7 M CsCl, 0.1M EDTA (pH 7.0)] and centrifuged for 12 hrs at 15C and 35,000 rpm in an SW50.1 rotor. RNA pellets were rinsed two times with ice-cold 70% ethanol, resuspended, and twice ethanol precipitated. DNA probes for the S1 nuclease analyses were isolated from agarose gels by electroelution of DNA fragments onto Whatman DE 81 paper after digestion of plasmid DNA with the appropriate restriction enzymes and agarose gel electrophoresis. The 5' ends were radiolabelled with [a 32P]ATP using polynucleotide kinase. Probes to be single end-labelled were asymmetrically cut with restriction enzymes and then re-isolated. Probes were denatured, annealed to 10 ug of infected cell cytoplasmic RNA and digested with S1 nuclease as described (Weinrich and Hruby, 1987). Reaction products were subjected to electrophoresis in 8M urea-8% polyacrylamide gels. Enzymes and radionucleotides were obtained from commercial suppliers (Bethesda Research Laboratories, Inc.; New England Biolabs, Inc.; Boehringer Mannheim Biochemicals; and New England Nuclear Corp.) and used according to the manufacturers instructions.
Cell extract and protein fractionation

Growth and infection of HeLa cells followed previously described protocols (Hruby et al., 1979). At either 3 or 8 hpi, two liters of infected cells were centrifuged (2000 rpm, 10 min), washed twice in buffer W (137mM NaCl, 10mM Na₂HPO₄, 2mM KH₂PO₄ 2.7mM KCl, 0.5mM MgCl₂) (Rosenfeld and Kelly, 1986) and then snap-frozen in liquid nitrogen and stored at -70°C. Cells prepared in this manner were stable for several months. To prepare cell extracts, twice the pellet volume of lysis buffer was added 0.5% NP-40, 20mM HEPES (pH 7.6), 2mM MgAc₂, 1mM DTT and the suspension mixed, incubated on ice for 5 min and remixed. Nuclei and cell debris were removed by centrifugation at 10,000 rpm for 2 min. One volume of buffer A [200 mM Tris·Cl (pH 8.0), 20mM DTT, 500mM KCl, and 0.2mM EDTA] was added to the supernatant fluid, which was then applied to a 1 ml DEAE-cellulose (DE-52, Whatman) column which had been equilibrated with buffer A [100mM Tris-Cl (pH 8.0), 10mM DTT, 250mM KCl, and 0.1mM EDTA]. Using buffer A as an eluant, 12 X 100 ul fractions were collected, and the protein concentration of each fraction determined using a BioRad protein assay kit. Peak fractions were pooled and dialyzed for 3 h against several changes of TGED buffer [50mM Tris-Cl (pH 8.0), 1mM EDTA, 1mM DTT, 0.1% Nonidet P-40, and 20% glycerol] containing 0.1M KCl. A one ml phosphocellulose column (BioRad Cellex-P), pre-equilibrated with TGED-0.1M KCl was used to fractionate the dialyzed extract. Extract was added to the column and then step-eluted with 1.0 ml aliquots each of TGED containing 0.1M KCl, 0.35M KCl, and 0.6M KCl, respectively followed by 4 ml TGED containing 1M KCl. Fractions (500 ul) were collected, assayed
for protein concentration and specific binding activity (data not shown). Alternatively, the phosphocellulose column was developed with a liner gradient from 0.1M to 1M KCl. Fractions which demonstrated the ability to bind specifically to L65 promoter sequences (BF-I) were pooled (usually fractions containing 0.8M KCl). These fractions were dialysed against buffer Z [25mM Hepes (pH 7.8), 12mM MgCl₂, 1mM DTT, 0.1% Nonidet P-40, and 20% glycerol] containing 10mM KCl (Kadonaga and Tjian, 1986) and were further purified using a salmon sperm DNA affinity column which consisted of sonicated salmon sperm DNA coupled to CNBr-activated Sepharose 4B (Pharmacia) (Briggs et al., 1986). Flow through fractions were collected and the column was step eluted with 4 x 1 ml aliquots of buffer Z-10mM KCl and then 2 x 1 ml aliquots each of buffer Z containing 0.1M KCl, 0.35M KCl, 0.6M KCl, and 1.0M KCl respectively. After adding glycerol to a concentration of 30%, protein concentrations were established and fractions assayed for BF-I activity. BF-I containing fractions were pooled, concentrated by dialysis against dry Aquacide III (Calbiochem) at 4C, and stored at -20C.

**Gel retention assays**

Binding reactions contained the protein fraction being tested, 1ug poly I-C, 4 ul buffer B [10mM Hepes (pH 8.0), 10% glycerol, 0.5mM EDTA, 0.5mM dithiothreitol (DTT), and 15mM KCl], and the indicated amount of unlabelled competitor fragment in a total volume of 20 ul. Heat stability of BF-I was tested by heating the extracts prior to addition to the reaction. Sensitivity to SDS was tested by addition of SDS to a final concentration of 0.01%. The reactions were incubated on ice for 10 min prior to addition of
1 ul radiolabelled DNA probe fragment (20 femtomoles, ~10,000 cpm). Reactions were incubated an additional 5' on ice and subsequently transferred to room temperature for 30 min (Samuels et al., 1982). DNA-protein complexes were electrophoretically separated on low ionic strength, prewarmed 7.5% polyacrylamide gels [1.7 mM Tris-Cl (pH 8.0), 0.37 mM sodium acetate, 1 mM EDTA] at a constant current of 20 mA with recirculated buffer. After approximately 3 h, gels were dried and radioactivity was detected by exposure to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen at -70°C.
RESULTS

Transcription from the L65 promoter

Previous analysis of the L65 promoter has shown that transcriptional initiation from the distal site is governed by two upstream signals, one between -123 and -106 and the other between -235 and -170 (Miner et al., 1988). The experiments described below seek to delineate the sequences required for proximal start site function and determine whether these sequences specifically bind trans-acting factors from infected cells.

To facilitate these experiments, plasmids were assembled which contained various portions of the region upstream from the L65 open reading frame abutted to the CAT gene. The first plasmid (pPD13:CAT) was described previously (Miner et al., 1988) and consists of the L65 upstream region from +1 to -300 adjacent to the CAT gene within a pUC13 vector (figure III.1). The second plasmid (pPD13L:CAT) contained additional 3' and 5' sequences which spanned from +50 to -440 (figure III.1). The probe fragments used to detect transcripts from each of these constructions by S1 nuclease analysis are shown below each construction (figure III.1).

S1 nuclease protection assays using single end-labelled probe D13 and cytoplasmic RNA generated by VV helper-dependent transient expression of plasmid pPD13:CAT produced two bands, one which corresponds to full length protection of the probe at 450 nucleotides and another at 258 nucleotides, mapping to the distal transcriptional start site.
Fig. III.1  Plasmids and probes derived from the *Hind*III D/A region of the VV genome. The location of the L65 (orf D13) gene, promoter and direction of transcription are shown. The inverted triangles indicate the previously mapped start sites at +1 (proximal) and -92 (distal) on the L65 promoter (Miner et al., 1988). Two VV L65 promoter/CAT constructs (pPD13L:CAT and pPD13L:CAT) used in these experiments are drawn to scale. The black bars indicate the probes utilized in various S1 nuclease protection experiments. The star indicates the position of the radiolabelled nucleotide. Probe D13 was isolated from pPD13:CAT as a PvuII fragment, labelled, digested with SstI and purified, generating a single end-labelled fragment of 450 base pairs. Probe D13L was isolated from pPD13L:CAT by precisely the same protocol generating a single end-labelled fragment of 607 base pairs. The 680 bp D13Q probe was isolated from pPD13L:CAT by digestion with HpaII, which cuts within the CAT gene just 5' to the PvuII site, end-labelled, digested with EcoRI and re-isolated.
Figure III.1
of the L65 promoter (figure III.2A). A protected fragment of 166 nucleotides would have been expected if transcription from the proximal start site had occurred. Likewise, using probe D13X, and the RNA transcribed from pPD13:CAT-235, a 258 nucleotide band which corresponds to transcription from the distal site is protected is present in figure III.2B, lane 3 (pPD13:CAT-235 is a plasmid with a small 70 bp 5' deletion (Miner et al., 1988). CAT gene expression driven by the -235 deletion mutant is identical to the full length construction as measured by CAT assay. An additional band is also visible at 390 nucleotides which corresponds to transcripts reading through the 5' end of the L65 promoter and protecting the D13 probe up to the -235 position (figure III.2B, lane 3; see below). No 166 nucleotide band was detected which would correspond to transcription from the proximal start site. Therefore, in both the full length pPD13:CAT construction (figure III.2A) and the -235 deletion mutant (figure III.2B), transcription from the proximal start site was virtually undetectable. The lack of transcripts mapping to the proximal start site suggested that either the proximal transcriptional start site had been destroyed by truncation at +1 or that additional sequences 3' or 5' to +1 were required for full proximal promoter function. Thus the plasmid pPD13L:CAT, which contains 50 additional base pairs of 3' sequence in addition to 440 base pairs of 5' sequence was assembled and used in similar transient expression assays. S1 nuclease analysis with RNA derived from this construct and probe D13L shows transcriptional activity from both sites (Figure III.2B, lane 4). The three protected fragments are 607, 309 and 217 nucleotides in size, corresponding to full length probe protection, distal, and proximal start sites, respectively. This result
Fig. III.2. S1 analysis of RNA derived from transient expression of pPD13:CAT and pPD13L:CAT. S1 nuclease analysis was performed as described in the experimental procedures. A) Lane 1 is probe D13 digested with S1 in the presence of calf liver tRNA only. Lane 2 is a reaction with probe D13 and RNA isolated from VV infected cells transiently expressing the pPD13:CAT construct. B) As a control, lane 1 is probe D13X hybridized to 10 ug calf liver tRNA and digested with S1 under the same conditions as lanes 3 and 4, lane 2 is identical except the probe is D13L. Lanes 3 and 4 are identical to lanes 1 and 2 except that RNA was used which had been isolated at 24 hpi from cells transiently expressing plasmids -235:D13:CAT and D13L:CAT respectively.
Figure III.2
indicates the importance of the sequences between +1 and +50 in pPD13L:CAT. Deletion of the upstream sequences to -128 did not affect the levels of transcription from the proximal start site which strongly suggests that it is the additional sequences 3' to +1 that are required for proximal start site function (figure III.4, lane -128). A TAAAT motif implicated in the regulation of other late genes is located between +2 and -3 and is restored by the additional sequences.

Specific sequences involved in L65 transcriptional regulation

To further dissect the L65 promoter element, a series of 5' deletions of pPD13L:CAT were prepared which spanned both the upstream elements responsible for the transcriptional activity of the distal start site and the region immediately upstream of the proximal site (figure III.3A). These deletion mutants were used in concert with transient expression assays. Relative expression of these mutants was measured by CAT enzyme assays (figure III.3B) and quantitative S1 analysis (figure III.4).

Figure III.3B shows that deletion from -440 to -128 had no reproducible effect on CAT activity from pPD13L:CAT. Further deletion of 16 bp to -112, resulted in a 2-fold drop in CAT gene expression to 60%. This deleted region corresponds to the distal promoter element I previously discussed (Miner et al., 1988). Deletion of sequences between -59 and -35 resulted in an additional 3-fold decrease in CAT enzyme produced, to 20% that of the full length promoter. Removal of the next 26 base pairs to -9 decreased activity 5-fold below the -35 deletion signal to 4%, effectively eliminating all CAT expression above background levels.
Fig. III.3. Effect of 5' deletions into the full length L65 promoter (pPD13L:CAT) on CAT gene expression. A) A map showing the positions of each deletion tested relative to the CAT reading frame and S1 mapped start sites. B) The CAT activity generated from the full length construct (-440) is compared to CAT activity from various 5' deletions. All values were normalized to CAT activity generated by the full length promoter (-440) and the standard deviations were calculated after normalization of each of three independent trials. Column C is the CAT activity produced by the identical vector without VV sequences (pUC:CAT).
Figure III.3
**Fig. III.4.** S1 nuclease analysis of RNA derived from transient expression of 5' deletion constructs using the D13L probe. A) Each lane is designated by the deletion construct used in transient expression (-440 to -9). The large arrows mark bands of interest: FL 680 - full length probe protection and probe rehybridization; D 368 - distal site; P 274 - proximal site; IC 222 - internal control signal. The smaller arrows indicate bands generated by readthrough into the deleted promoter constructs. Size markers are shown at the right of the figure. The tRNA lane is the identical reaction except that tRNA is substituted for VV RNA. B) Quantitation of transcription signals from the L65 promoter/CAT shown in figure III.4A provided data regarding transcription from both the distal and proximal start sites. These data, after normalization to the internal control are shown in graph form. The Y axis is relative counts per minute and the particular deletion mutant analyzed is shown along the X axis. In addition, the readthrough data are included.
Figure III.4

pPD13L:CAT Deletions

-440 -128 -112 -59 -35 -9

distal
proximal
readthrough

Relative counts/minute

FL 680
D 368
P 274
IC 222

-440 -128 -112 -59 -35 -9
When taken together with the results of the experiments discussed earlier, this indicates that the elements which are responsible for expression of CAT from pPD13L:CAT appear to be located between -128 and -9.

To examine the effects of the deletions at the level of RNA synthesis, internally controlled S1 nuclease analysis was used on the RNA generated from transient expression of the deletion series. Cells were transfected with 10 μg of a given deletion plasmid plus 5 μg of pPA1:CAT [pPA1:CAT is a plasmid previously described (Miner et al., 1988) which contains the promoter for orf A1 abutted to the CAT gene]. The orf A1 promoter is a strong late promoter with a single start site. The transfected cells were infected with vaccinia virus, and then cytoplasmic RNA was harvested at 24 hpi. Probes which detect transcription from pPD13L:CAT also protect a fragment of 222 nucleotides due to transcription from pPA1:CAT allowing internal control of transfection efficiency and RNA extraction procedures (see figure III.4). Titration of pPA1:CAT and pPD13L:CAT plasmids shows that at the concentrations used, no competitive effects are detected which affect RNA synthesis or expression from either plasmid during transient expression (data not shown). Figure III.4A shows the results of this S1 analysis using the D13Q probe (figure III.1); protected fragments at P 274 nucleotides and D 368 nucleotides are signals from the proximal and distal start sites of parental pPD13L:CAT, respectively (see figures III.1 and III.3). The internal control migrates at 222 nucleotides and is designated by IC 222. Bands marked by small arrows correspond to protection of the probe fragment which maps to the 5' end of the particular deletion used,
suggesting transcriptional readthrough from the circular vector into each of these deletion mutants from the 5' side (figure III.2B; lane 3 and figure III.4A). Densitometric scanning of appropriate exposures and normalization to the internal control enabled quantitation of relative transcriptional signal strengths (summarized in figure III.4B). Figure III.4 shows that in agreement with the CAT expression data (figure III.3) as well as previous work (Miner et al., 1988), this assay detects a positive regulatory region acting on transcription from the distal start site which resides between -128 and -112, and two other positive regulatory regions acting on the proximal start site between -59 and -9. Sequences between -128 and -440 have only minor effects on the distal site in this context, in contrast to earlier work showing a 2-fold decrease due to these sequences in the pPD13:CAT deletion mutants (Miner et al., 1988). Deletion of the distal elements had little effect on the transcriptional activity of the proximal site. Deletion of the region between -128 and -112 reduces the signal from the distal start site by 65-fold; the proximal site is relatively unaffected. A 2.5-fold reduction in transcription from the proximal start site is seen when sequences from -59 to -35 are removed and a further 25-fold decrease occurs upon deletion to -9. This region between -59 and -9 does not contain any previously described regulatory signals. The TAAAT motif implicated in the regulation of several late genes (Rosel et al., 1986) is found at the proximal start site. This TAAAT motif is also found within the -128 to -112 region governing activity of the distal promoter (Miner et al., 1988).
Fig. III.5. Schematic representation of various probes and competitors used in gel retention assays. The L65 promoter and part of the L65 ORF are shown. Each probe is named by its end points on the L65 promoter. Each of these probes contains flanking puc sequences to correct for differences in size.

$+50/-59 = 180 \text{ bp}$; $+1/-88 = 120 \text{ bp}$; $-181/-300 = 120 \text{ bp}$; $-99/-181 = 100 \text{ bp}$; $+50/+9 = 150 \text{ bp}$; $+1/-13 = 110 \text{ bp}$. 
Figure III.5
VV promoter binding factor

It is reasonable to predict that both the distal and proximal L65 promoter elements function in vivo through interaction with trans-activating factors that stimulate or repress transcription. Using a gel retention assay, previous workers have isolated from VV virions, putative transcription factors which interact with VV early promoters (Yuen et al., 1987; Broyles and Moss, 1988; Broyles et al., 1988). We have used a similar strategy to search for factors from VV-infected cells that bind specifically to the proximal L65 promoter region. The probes and competitors used in these experiments are shown in figure III.5. DNA-protein complexes were resolved from free DNA by native gel electrophoresis (Fried and Crothers, 1981). The crude cytoplasmic extract had to be subjected to both DEAE-cellulose and phosphocellulose ion-exchange chromatography steps before individual DNA-protein complexes could be resolved (see experimental procedures). The protein eluted from the phosphocellulose column at different KCl concentrations contained a number of DNA-binding activities which, when incubated with a labelled probe containing the proximal promoter region from +50 to -59 (figure III.5), generated a number of DNA-protein complexes that migrated slower than free probe in native gels. However, only the protein in the eluate from the 1M KCl wash generated a DNA-protein complex which could be competed by prior incubation with a 10- to 20-fold molar excess of unlabelled +50/-59 L65 promoter fragment, but not by preincubation with the same molar ratio of a similarly sized unlabelled pUC fragment (figure III.6A and data not shown). This activity or
Fig. III.6. Gel retention assays establishing the specificity of BF-I binding to the L65 proximal promoter region. All reactions contain radioactively labelled +50/-59 probe incubated with BF-I containing fractions from the salmon sperm column under the conditions described in the Materials and Methods. A) Lane 1 is no competitor. Lanes 2-13 are identical except that unlabelled competitor DNA is included in the reaction. Lanes 2 and 3: +50/-59 (proximal promoter) 10 and 20-fold molar excess; lanes 4 and 5: +1/-88 (5' portion of proximal promoter) 10- and 20-fold molar excess; lanes 6 and 7: -99/-181 [region upstream from distal promoter (element II)] 10- and 20-fold molar excess; lanes 8 and 9: -99/-181 [distal promoter (element I)] 10- and 20-fold molar excess; lanes 10 and 11: +50/+9 (3' portion of proximal promoter) 10- and 20-fold molar excess; lanes 12 and 13: +1/-13 (proximal start site only) 10- and 20-fold molar excess; lane 14 is probe without protein. B) Lane 1 is no protein, lane 2 is no competitor and lanes 3-5 are 1, 10 and 100 fold molar excess of the +50/+9 competitor. Lanes 6-8 are 1-, 10- and 100- fold molar excess of the -181/-300 competitor.
Figure III.6
activities were designated binding factor I (BF-I). The specificity of BF-I binding for the proximal promoter was confirmed by competition with 10 to 20-fold molar excess of a fragment containing -99/-181 or a 10 to 20-fold molar excess of a fragment containing -181/-300 from the distal promoter (figure III.6A). At the concentrations tested, neither of these elements affected formation of the complex. Competitor (+50/+9) and (-99/-181) to probe (+50/-59) molar ratios of 1, 10 and 100 were used to further demonstrate the specificity of the interaction (figure III.6B). The fragment containing the 3' portion of the L65 proximal promoter competed efficiently at low molar ratios while the competitor from the distal promoter competed only above molar ratios of 100. BF-I activity appears to be resistant to mild heating because incubation at 65C for ten min did not affect complex formation, whereas incubation of the extracts at 90C for ten min eliminated complex formation. In addition, the BF-I complex was sensitive to either 0.01% SDS or proteinase K in the reactions (data not shown). As a nonspecific competitor, double stranded polyribonucleotide poly (I-C) was used. Poly d(I-C) or poly d(A-T), generally produced less distinct retarded bands. Using promoter fragments derived from the pPD13L:CAT and pPD13:CAT deletion families, the binding sites can be positioned on the promoter fragment either by testing for the ability of a given deletion to compete for complex formation (figure III.6) or by testing for binding of BF-I to each deletion mutant (figure III.7). Competition with fragments from either 5' or 3' regions relative to the proximal start site demonstrate the existence of two separable binding sites (figure III.5,6). A fragment containing sequences between +1 and -88 (+1/-88) competes equally as well as a fragment containing sequences between +50...
Fig. III.7. Specific binding of BF-I to two sites near the L65 promoter and cross-competition between them. A) Standard binding reaction including probe +1/-88. Lane 1 and 2 are no BF-I protein and no competitor respectively. Lanes 3-6 are the results of binding reactions which contained 1-, 10-, and 100-fold molar excess of +1/-88 unlabelled competitor fragment. Lanes 7-9 show competition with a 1-, 10- and 100-fold molar excess of the unlabelled +50/+9 fragment. B) Binding reactions which contain labelled probe +50/+9. Lanes 1 and 2 are controls, - and + BF-I protein. Lanes 3-6 show binding reactions which contain 1-, 10- and 100- fold molar excess of the +50/+9 fragment. Lanes 7-9 are binding reactions which contain 1-, 10-, and 100-fold molar excess of +1/-88 unlabelled competitor fragment. C) Binding reaction with probe +1/-13. Lane 1 and 2 are - and + BF-I protein respectively.
Figure III.7
and +9 (+50/+9), although neither fragment has the competing activity of the entire +50 to -59 (+50/-59) fragment (figure III.6A). Fragments containing sequences between +1 and -13 (+1/-13) do not compete for binding. These results were confirmed by using these same fragments as labelled probes (figure III.7A,B,C lanes 1 and 2). Fragments containing either the +1/-88 or +50/+9 regions form complexes similar to -59/+50 (figure III.7A,B lanes 1 and 2); whereas a fragment with the +1/-13 region does not (figure III.7C lane 1,2). All of the fragments in the binding and competition studies contain 88 base pairs of flanking sequences of the pUC vector from which they were derived, however these flanking sequences are not involved in the binding because the fragment +1/-13 which contains these same pUC sequences does not form a complex itself or competitively inhibit +50/-59 complex formation.

Because the data suggest the presence of two BF-I binding sites within the L65 proximal promoter, and because fractions containing BF-I were not highly purified, it was of interest to determine whether one binding site could compete effectively for BF-I binding to the other site. The results of this so called cross-competition experiment are shown in figure III.7A,B. In both cases, each binding site was capable of competing for BF-I bound to the other site which suggests that the same factor is interacting with each fragment.

Increasing the amount of BF-I protein in a reaction with the +50/-59 probe generates what appears to be a multimeric form of the probe-BF-I complex (figure III.8A). This result would be expected if the +50/-59 fragment contains two binding sites which are both bound by BF-I at a high protein to probe ratio. Competition experiments show that both
complexes are competed by fragments from the proximal promoter region. However, complex II is affected at a lower concentration of competitor when compared to complex I, consistent with the model that the same protein or proteins generates this complex (figure III.6A and data not shown). It follows that a fragment containing only a single binding site (+1/-88) should only generate a single bound complex, as shown in figure III.8B
Fig. III.8. The effect of increasing protein concentration on retarded species. BF-I is added to binding reactions in concentrations of 0ng, 50ng, 100ng, 200ng, and 400ng with both +50/-59 and +1/-88 probes. Complex I corresponds to the usual BF-I complex and complex II is the additional species formed on high protein concentrations with the probe containing two binding sites. In the 400ng lane, the +1/-88 probe is retained at the origin of the gel (not shown) similar to that seen in the 400ng lane using probe +50/-59.
Figure III.8
DISCUSSION

To investigate cis-acting signals affecting this promoter, S1 analysis of RNA generated from transiently expressed promoter/CAT plasmids was used to monitor the effects of various promoter deletion mutations on transcription efficiency. The pPD13:CAT plasmid initially used in these studies showed undetectable levels of transcription from the proximal site. In contrast, high levels of genomic L65 specific RNA from the proximal site were generated during infection. To analyze sequences required for transcription from the proximal site, additional VV sequences, both 5' and 3' to those already present in pPD13:CAT, were included in another plasmid (pPD13L:CAT). S1 nuclease analysis of RNA from this plasmid showed that the additional sequences activated the proximal site without affecting the distal site. Deletion of the additional 5' sequences -440 to -128 had no effect on the level of transcription from the proximal site, strongly suggesting that the additional 50 bp of 3' sequence contained additional information, required for transcription from the proximal site. These sequences contain part of a TAAAT motif found at the proximal start site, and it is possible that restoration of this signal was necessary for the transcriptional activity of the proximal promoter.

To dissect functional regions within the proximal promoter, deletions that removed the distal start site and the region up to and including the proximal start site were made in pPD13L:CAT. These plasmids were used in transient expression assays and the level of CAT enzyme produced from each construct was measured. Cotransfection of a control plasmid in these experiments allowed quantitative S1 analysis on
the RNA generated by these plasmids. Results obtained from the S1 analysis and CAT assays generally agreed, although the CAT assays presumably measure expression from both sites whereas the S1 analysis differentiates between the two. Deletion of the region from -440 to -128 has been previously shown to cause a two-fold decrease in CAT gene expression from pPD13:CAT. We were unable to fully confirm this result using the longer pPD13L:CAT construct. In this case, CAT activity is not affected significantly when these sequences are deleted and S1 analysis shows only a moderate 1.4-fold decrease in transcription from the proximal start site. The distal start site was also relatively unaffected by this deletion. Removal of the next 16 bp to -112 effectively eliminates the signal from the distal start site, causing a 60-fold reduction in its intensity. These results confirm and refine previous work showing the presence of a distal promoter regulatory element between -128 and -106. Taken together, these deletions map the 5' end of a distal promoter regulatory element to a 12 bp segment between -128 and -112, the presence of which causes a 60-fold increase in the level of transcription from a site located 30 bp downstream. The start site affected by this element maps to a TAAAG at - 92 using S1 analysis of both genomic RNA (Weinrich and Hruby, 1987), and RNA from transient expression of pPD13:CAT and pPD13L:CAT (figure III.4). The TAAAG start site is downstream from a TAAAT motif (at -113 and -117) which has been shown to be important for the expression in several other late genes (Bertholet et al., 1986; Hanggi et al., 1986; Rosel et al., 1986). In these previously characterized promoters, the TAAAT signal is located at or within a few base pairs of the apparent start site. In the context of the L65 distal promoter, the TAAAT motif may
be functioning in another role since it lies within an element shown to be crucial to a transcription start 30 bp downstream. It is also possible that in this context, the TAAAT motif is nonessential and that other signals within this short segment constitute the distal promoter.

The sequences responsible for the transcriptional activity of the proximal promoter are located further downstream from the distal promoter, between -59 and -9. Sequences between -59 and -35 are responsible for a 2.5-fold effect on transcription from the proximal start site. When the remaining sequences between -35 and -9 are deleted, an additional 20-fold decrease in activity occurs essentially eliminating the signal, thus mapping the 5' end of the element to this region. Sequence similarity searches of this segment have revealed none of the previously characterized vaccinia virus regulatory elements.

In addition to the S1 nuclease signals generated by the two start sites and the internal control, a signal is present in each lane in figure III.4 and lane 3 in figure III.2B which corresponds to a readthrough transcript from each of the deleted plasmids. The 680 bp probe is complementary to the deletions up to the deletion endpoint, any RNA generated which reads through the junction is detected as a higher molecular weight species which migrates according to the extent of the deletion. The presence of readthrough transcripts is explained by the fact that transcripts from the L65 promoter do not terminate, reading around the circular plasmid and back into the promoter region. The S1 mapped RNA is in fact bonafide mature VV late RNA since both the distal and proximal start site derived transcripts contain a 5' poly-A leader sequence (J. Miner unpublished results).
The trans-activation of these promoters during transient expression in VV infected cells implies that diffusable factors are involved in recognition of, and transcription from, these sequences. A factor (Binding Factor I) has been detected in infected cell extracts at late times post-infection that specifically binds to the proximal promoter region shown to be involved in regulation of the proximal site. This activity was not detectable in uninfected cells, virion extracts or infected cells at early times post-infection (data not shown). A specific complex is formed in gel retention assays which is competitive only with fragments containing parts of the proximal promoter. Fragments containing the distal promoter (-181/-300) do not compete for complex formation, nor do fragments containing 120 bp of sequence upstream from the distal promoter (-99/-181) or fragments from pUC19 (figure III.6A,B). This suggests that BF-I binding is specific for the proximal promoter region. BF-I is sensitive to proteinase K digestion, heat and SDS which strongly argues that BF-I is a protein.

Using appropriate fragments from the deletion constructs described above, we have mapped what appear to be two BF-I binding sites between -13 and -59 and between +50 and +9 on the proximal promoter. Non-overlapping fragments from the proximal promoter (+50/+9 and +1/-88) will form specific complexes with BF-I while fragments corresponding to other regions cannot (Figure III.7A, B, C). That both sites can be filled at once in vitro is implied by results shown in figure III.8. Using a probe with two putative binding sites (+50/-59) another more slowly migrating complex is generated at high BF-I to probe ratios. Filling of these two sites does not appear to be highly cooperative. These two sites presumably
bind identical BF-I molecules, this presumption is based on the fact that
cross-competition experiments show that fragments containing one
binding site can compete for binding of BF-I to the other and vice-versa
(figure III.7A,B). Though, it should be noted that in several experiments,
the +50/+9 fragment competed less well than the +1/-88 fragment
implying a qualitative difference in BF-I affinity for the two sites (Figures
III.6B, and III.7A,B). Sequences 3' to the proximal start site bind to BF-I,
but it is not yet clear which portion of the 3' element is responsible for
activating the proximal promoter in pPD13L:CAT. The TAAAT motif that
is located between +2 and -3 is likely to be important for proximal
promoter function which leaves open the importance of the 3' BF-I
binding site especially in light of its reduced affinity for this site. The
same proximal DNA sequences which are responsible for transcriptional
activation of the proximal site, also bind avidly to BF-I. The association of
BF-I with transcriptionally important DNA sequences has several
interesting interpretations. First, it is possible that BF-I functions to
modify the structure of the DNA which lies between the two proteins,
allowing an entry point for VV RNA polymerase. Second, it is possible
that BF-I interacts directly with VV RNA polymerase to provide a surface
that positions the polymerase at the start site. The fact that BF-I binds
only to the proximal promoter and not to the distal promoter may in part
explain the differential regulation of these two start sites (Weinrich and
Hruby, 1987).

In addition, we have preliminary data indicating that this factor
also binds to the VV thymidine kinase (TK) early promoter (L. Wilson and
J. Miner unpublished results). It may be relevant that TK RNA synthesis
is negatively regulated at 2-3 hpi; the same time that transcription from
the L65 promoter is beginning (Hruby and Ball, 1981). The sequence
AATTA T/A A is conserved between the 5' and 3' portions of the L65
proximal promoter and is found in the inverted orientation at -43 within
the TK promoter. The assessment of the importance of this sequence will
await DNase I footprinting of BF-1 on the L65 and TK promoters and
demonstration that BF-I occupancy of these sites correlates with
transcriptional activation in vitro and in vivo. Thus far footprinting of
DNA binding proteins on VV DNA has proved to be problematic. This
may be in part due to the extreme A/T richness of the VV promoter
regions and/or relatively low affinity of the binding factors. In either case
it is likely that it will be necessary to preparatively purify large quantities
of biologically active BF-1 before such studies can be undertaken. Perhaps
of more immediate importance will be to make use of recently described
VV cell-free transcription systems (Puckett and Moss, 1983; Schwer et al.,
1987) which are responsive to exogenously added VV late genes to
determine whether BF-1 has a positive or negative effect on transcription
from the L65 proximal promoter element.
CHAPTER IV

RIFAMPICIN PREVENTS VIROSONME LOCALIZATION OF L65 AN ESSENTIAL VACCINIA VIRUS POLYPEPTIDE

Jeffrey N. Miner and Dennis E. Hruby
SUMMARY

In contrast to its irreversible effect on the *E. coli* RNA polymerase β-subunit, the antibiotic rifampicin reversibly inhibits vaccinia virus morphogenesis at a step during the formation of immature viral particles. The protein affected by the presence of rifampicin is L65, a major late vaccinia polypeptide to which mutations that confer rifampicin resistance have been mapped. We now provide evidence using a monospecific anti-L65 serum in concert with immunofluorescence and sucrose gradient analysis, that the mechanism of action of rifampicin on vaccinia virus replication involves the inhibition of localization of L65 to the viral factories (virosomes) thereby blocking further development. Studies on the expression and distribution of L65 during the infection cycle reveal that L65 is a stable, nonglycosylated late protein associated with virions. These results are discussed in relationship to the possible in vivo functions of the L65 protein.
INTRODUCTION

The sequence of steps between initial infection and the development of mature vaccinia virus particles is quite complex (Moss, 1974; Dales and Pogo, 1981). Replication and packaging of the 180 kilobase (Kb) DNA genome occurs within large cytoplasmic inclusion bodies or virosomes (Dahl and Kates, 1970). Rifampicin, an antibiotic which irreversibly binds to and inhibits the activity of the β-subunit of E. coli RNA polymerase, also inhibits a discrete step in the replication cycle of vaccinia virus (Boothroyd et al., 1983; Moss et al., 1971a; Orchinnokov et al., 1983). The molecular mechanism by which rifampicin inhibits vaccinia replication is not known. However, there are several lines of evidence which suggest that rifampicin affects the growth of vaccinia virus by a different mechanism than it affects the growth of procaryotic organisms (Moss et al., 1971). First, the presence of rifampicin during vaccinia infection has little or no effect on viral DNA, RNA or protein syntheses (Moss et al., 1969; Subak-Sharpe et al., 1970; Ben-Ishai et al., 1969; Pennington, 1973), and second, the effect of rifampicin on vaccinia infection is reversible (Nagayama et al., 1970). Electron micrographs of cells infected with wild type vaccinia virus in the presence of rifampicin have shown that membranes which surround the maturing viral particles lack a dense layer of spicules normally present at this stage during infection (Nagayama et al., 1970; Pennington et al., 1970 Moss, 1969). Biochemical analyses demonstrated that rifampicin inhibited the normal transition of the viral DNA from a DNase-sensitive state to a DNase-insensitive state, suggesting that the DNA failed to be packaged into mature virions (Moss et al., 1969). In
addition, proteolytic processing of the major virion core precursors fails to occur in the presence of rifampicin (Katz and Moss, 1970; Rosenblum, 1973). The reversibility of the rifampicin inhibition is evidenced by the fact that within 10 min after removal of the drug, viral morphogenesis resumes. In experiments using protein synthesis inhibitors, it was suggested that de novo macromolecular synthesis was not required for the reversal of the rifampicin block, or for the initial stages of viral morphogenesis occurring after rifampicin reversal (Moss et al., 1971b). However, several other labs have demonstrated a requirement for at least some protein synthesis during the later stages of morphogenesis (Nagayama et al., 1970; Pennington et al., 1970).

Viral mutants have been isolated which are resistant to levels of rifampicin (100 ug/ml) which completely inhibit the growth of wild type virus. These rifampicin resistant mutants (RifR) can rescue wild-type vaccinia virus during co-infections by providing a diffusible factor needed in stoichiometric amounts, presumably the product of the RifR locus (Moss et al., 1971a). Two vaccinia virus rifampicin resistant mutants have been studied in detail. Nucleotide sequencing procedures have shown that these two mutants contain different point mutations located at opposite ends of the coding region of the L65 gene (Tartaglia and Paoletti, 1985; Tartaglia et al., 1986; Baldick and Moss, 1987; Weinrich and Hruby, 1986; Niles et al., 1986). The L65 gene is the same as that designated as D13 using the nomenclature system of Rosel and Moss (Rosel et al., 1986). Transcriptional and translational analysis of the RifR/L65 locus indicates that the gene is expressed by a strong late promoter after the initiation of viral DNA replication (Tartaglia et al., 1986; Weinrich and Hruby, 1986;
Weinrich and Hruby, 1987; Miner et al., 1988). Since no immunological reagents or functional assays were available to measure the expression of the L65-encoded gene product, it has not thus far been possible to examine the synthesis or activity of this protein in infected cells.

Because the action of rifampicin on vaccinia virus replication appears to be unique, and because our understanding of vaccinia morphogenesis is quite limited, we undertook a functional analysis of L65, the gene product believed to be involved in rifampicin sensitivity. Using monospecific polyclonal antisera, the expression and distribution of L65 in cells was measured during infection. In addition both immunofluorescence and biochemical criteria were used to demonstrate that rifampicin reversibly inhibits the association of L65 with viral particles in the vaccinia viroosome.
MATERIALS AND METHODS

Expression vector/fusion protein purification

An 800 base pair HpaII fragment of the L65 open reading frame (amino acids 40-307) was excised from the plasmid pEcoRI C which contains the EcoRI C fragment of the HindIII D fragment cloned into pUC13 (Weinrich and Hruby, 1986). This DNA molecule was treated with the Klenow fragment of DNA polymerase I and cloned into pATH III (trpE fusion expression vector), which had been cut with BamHI and filled in with Klenow, resulting in an in-frame fusion which has at its amino-terminus, the E. coli trpE gene and at its carboxyl-terminus, the cloned portion of the L65 gene, representing about half of the fusion protein (T.J. Koerner and A. Tzagoloff, personal communication; Gilmore et al., 1988).

The resulting plasmid (ptrp:L65) was propagated in E. coli, strain JM83. The trpE:L65 fusion protein was induced by growing the cells in M9 media with 20 ug/ml tryptophan overnight, then diluting the culture 1:10 in media without tryptophan. The diluted cultures were grown at 30C for 1 h with vigorous aeration, at which time 5 ug/ml of indoleacrylic acid was added and the culture was left at 30C for 2 h. The insoluble fraction of the cells was isolated by resuspending the cell pellet in 0.1 ml TEN [50 mM Tris, (pH 7.5), 0.5 mM EDTA 0.3M NaCl] buffer containing 2 mg/ml lysozyme at 12C for 20 min. The cells were subjected to 3 cycles of freezing thawing and lysed by the addition of NP40 to a final concentration of 0.2% v/v. The extract was then treated with DNase I (6 ug/ml) for 1 h on ice after adjusting the NaCl and MgCl2 concentrations to 1M and 8mM,
respectively. The samples were centrifuged for 10 min at 10,000 rpm, the pellet washed 2X in TEN buffer + 1% NP40, resuspended in cracking buffer [0.01 M sodium phosphate (pH 7.2), 1% β-mercaptoethanol, 1% sodium dodecyl sulphate, (SDS) 6M urea] and incubated at 37C for 1 h. Samples could be stored frozen at -20C for several months.

**Production of Antisera**

The induced trpE/L65 fusion protein was isolated by preparative SDS polyacrylamide gel electrophoresis, the protein band was excised, emulsified in Freunds complete adjuvant and injected intradermally into 2 Kg female New Zealand white rabbits. Approximately 200 ug of fusion protein was used per rabbit per injection. At 7 and 14 days after the initial injection, the rabbits were injected with another 200 ug of emulsified (Freunds incomplete) fusion protein intradermally and intramuscularly, respectively. Rabbits were periodically test bled and the sera checked for the presence of L65-immunoreactive antisera by immunoprecipitation of radiolabeled L65 proteins prepared by metabolically-labeling VV infected cells at 6 hpi (h.p.i.) with $^{35}$S methionine.

**Immunoprecipitation**

Confluent monolayers of BSC-40 cells were pulse labeled at 0 or 6 hpi with $[^{35}\text{S}]$methionine as described (Weinrich et al., 1985) lysed with 0.5% SDS and 20 ug/ml PMSF at 60C, sheared by 3 passages through a 24 gauge needle and heated at 80C for 10 min. 200 ul of the extract was mixed with 3 ul rabbit anti-L65 serum in 800 ul RIPA + 0.1% SDS [0.1% sodium deoxycholate (w/v), 1% triton X-100 (v/v), 0.1% SDS (w/v), 150 mM NaCl, 50
mM Tris-Cl (pH 7.4)] and incubated for 2 h on ice. Protein-A sepharose beads were hydrated overnight in RIPA buffer, washed in the same buffer twice, added to the reaction [200 ul, 10% (v/v) RIPA] and the mixture was shaken overnight at 4C. After being pelleted, the beads were washed twice with RIPA + 0.2% SDS, resuspended in SDS:PAGE loading buffer and boiled for 3 min. The samples were centrifuged at 15,000 rpm for 20 s, the supernatant was removed, loaded onto an SDS:polyacrylamide gel system and electrophoresis, fluorography and autoradiography procedures were carried out (Weinrich et al., 1985).

Cells and Virus

Growth, purification and plaque titration of VV (strain WR) was carried out as previously described (Hruby et al., 1979). BSC-40 cells were maintained in Earle's minimum essential medium (GIBCO Laboratories), supplemented with 10% heat inactivated fetal bovine serum and 2mM L- glutamine, 10 ug of gentamycin per ml, at 37C, 5% CO$_2$ and 95% humidity (Miner et al., 1988).

Immunofluoresence

Cells were grown and infected in multi-well dishes on 18 mm circular coverslips. At the indicated times post infection, the coverslips were washed once in phosphate buffered saline (PBS), fixed in 100% methanol at -20C for 8 min, dried and mounted on microscope slides. The coverslips were treated with the indicated dilution of L65 antisera in PBS for 20 min, washed 2 X 10 min in PBS and stained in the dark with goat anti-rabbit FITC conjugate (Sigma) at a dilution of 1:25 in PBS for 20 min.
After washing, the coverslips were stained with 0.01% Evans blue for 2 min, washed with PBS again and air-dried briefly. Cells were prepared for microscopy using DABCO mounting medium (Sigma) and fluorescence was visualized on a Zeiss standard microscope with an ultraviolet light source and 450-490 nm filter (FT 510-LP520).

Sucrose Gradient Analysis

Plates (100 mm) of confluent BSC-40 cells were infected as described, continuously labeled with 5 uCi/ml [3H] thymidine (New England Nuclear) from 1 hpi, (Miner et al., 1988), washed at 6 hpi in PBS, and resuspended in 0.7 ml hypotonic buffer [10 mM Tris-Cl (pH 8.0), 5 mM EDTA, 10 mM KCl]. They were allowed to swell for 10 min on ice and were broken by 12 strokes of a tight fitting dounce homogenizer. Cell nuclei were pelleted in a Beckman TJ-6 centrifuge at 2500 RPM for 2 min at 4C. The supernatant was removed, made 0.5% NP40 and loaded onto a 15 ml 38-50% sucrose gradient with an 84% sucrose cushion in 50 mM KCl, 5mM EDTA, 50 mM Tris-Cl (pH 8.0), 0.5% NP40. After centrifugation in a Beckman LM-8 ultracentrifuge using a SW41 TI rotor at 20,000 RPM at 4C for 20 h, fractions were collected from the bottom and either assayed for [3H] thymidine as described (Dahl and Kates, 1970), or subjected to gel electrophoresis and western blotting procedures.

Western Blotting

Proteins were separated by SDS:polyacrylamide gel electrophoresis, and transferred to nitrocellulose using Towbin transfer buffer (TTB) [5mM Tris-Cl base (pH 8.3), 192mM glycine, 20% methanol (v/v)] for 30
min at 25V (E-C Apparatus, Inc.). The nitrocellulose filter was washed at 23C for 10 min in Tris-buffered saline (TBS) [20mM Tris-Cl base, 500mM NaCl (pH 7.5)] and then incubated in blocking solution (3% gelatin in TBS) for 4 h at 23C. After washing 2 x 5 min in TTBS (0.05% Tween-20 in TBS), the filter was incubated overnight with primary antibody (anti-L65) diluted in antibody buffer (1% gelatin-TTBS). The filter was washed 3 x 10 min in TTBS prior to addition of the secondary antibody (alkaline phosphatase conjugated goat anti-rabbit, BioRad immunoblot assay kit; Blake et al., 1984) which was diluted in antibody solution and incubated for 1 h. The filter was washed 3 x 10 min in TTBS and 1 x 10 min in TBS and developed with 0.15 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt) 0.2 mg/ml NBT (p-nitro blue tetrazolium chloride) in carbonate buffer [0.1M NaHCO₃ 1.0 mM MgCl₂, (pH 9.8)] for 30 min.
RESULTS

Production and characterization of anti-L65 sera

Using the pATH III trpE expression vector, we have constructed a gene fusion between the trpE gene and the amino terminus of the L65 gene. Induction with indoleacrylic acid of bacteria harboring the plasmid encoding this fusion gene resulted in the presence of a strong 65 kd protein band in coomassie stained SDS:protein gels of trpE/L65 E. coli lysates. This band was not present in extracts of cells containing either the parental pATH III vector or clones containing the L65 N-terminus coding region in the opposite orientation relative to the trpE gene (data not shown). Rabbits injected with emulsified gel slices containing this band generated antisera which was capable of immunoprecipitating a 68 Kd protein from infected cells at late times post infection, but not from extracts of uninfected or early-infected cells or cells infected in the presence of hydroxyurea (Data not shown. Figure IV.1). This correlates well with several pieces of data: 1) The detection of L65 RNA only at late times post infection (Weinrich and Hruby, 1985; Tartaglia et al., 1986); 2) Hybrid selection and translation of RNA using DNA fragments spanning the L65 locus generates a 68 Kd protein (Weinrich et al., 1985); and, 3) The predicted L65 open reading frame is large enough to encode a 62 Kd protein; however, the protein appears to migrate at 68 Kd in SDS:PAGE gels (Weinrich et al., 1986). The minor bands which are
Fig. IV.1. Immunoprecipitation of infected all extracts with anti-L65 sera. $^{35}$S labeled proteins from mock infected cells (MI), or virus-infected cells (labeled at 2 (E) or 6 (L) hpi) were immunoprecipitated with either preimmune (PI) or anti-L65 antisera (I). The positions of molecular weight markers is shown on the left. L65 is indicated on the right.
visible above the L65 band in Figure IV. 1 are most likely not cross-reactive proteins, but rather proteins which remain associated with L65 during immunoprecipitation. This conclusion is based on an analysis of infected cell lysates by western blotting which demonstrates the existence of only a single L65 specific polypeptide migrating at 68 Kd (Figs. 3 and 5).

In vivo expression of L65

Examination of autoradiographs of 35S-labeled total infected cell extracts led to the suggestion that L65 comigrates with precursor p4b, a protein which is processed during infection and incorporated into mature virions as a major core component (Weinrich and Hruby, 1985). L65 however, apparently remained unprocessed. To firmly establish the identity, stability and expression kinetics of L65, a time course pulse-chase procedure using vaccinia virus-infected cells was carried out and labeled-extracts from these experiments were immunoprecipitated with anti-L65 sera. The results, which are shown in Figure IV.2, demonstrate that L65 is expressed at times after 4 hpi (Figure IV. 2) and was not detectable at earlier times post infection, except for a low level of L65 at 3 hpi (data not shown). In addition, monitoring a specific population of L65 molecules over time by chasing with an excess of unlabeled methionine demonstrated no effect on the migration of immunoprecipitated L65 suggesting that the protein is not processed and has a half-life of approximately 4 h (Figure IV. 2). In contrast, examination of these same
Fig. IV.2. Immunoprecipitation of pulse labeled VV infected cell extracts. Infected cells were pulse-labeled with $[^{35}\text{S}]$methionine for 1 h prior to the indicated times post-infection in media containing 0.1X the normal amount of methionine (pulse samples). At 8 hpi the radioactive media was replaced with media containing 100X the normal amount of methionine. Hours post chase are indicated (chase samples). Radioactive molecular weight markers are shown in lane M. L65 is indicated on right. Densitometric scanning of appropriate exposures allowed quantitation of the relative signal strengths of L65 during the chase period.
Figure IV.2
extracts by SDS:PAGE and autoradiography without immunoprecipitation clearly shows processing of the precursor p4b to its major 4b product (data not shown).

The abundance of L65 in the cytoplasm of infected cells suggested that it may be a structural component of virions. Analysis of $^{14}$C- and $^{35}$S-labeled purified virions by both SDS:PAGE or immunoprecipitation failed to demonstrate the presence of virion-associated L65 (data not shown). This was probably due to difficulty in disrupting the viral particles, because using the more sensitive western blot protocol, a signal was detected from purified virions (Figure IV. 3).

**L65 is not post-translationally modified by glycosylation**

The possibility that L65 was post-translationally modified by glycosylation was investigated for several reasons: 1) The presence of 10 consensus N-linked glycosylation sites (ASN-X-ser/thr) within the predicted amino acid sequence of the L65 protein (Weinrich and Hruby, 1986). 2) the observation that the L65 protein migrates slightly slower (68K) than its predicted molecular weight of 62K (Figure IV. 1). 3) a mutation which confers rifampicin resistance maps to one of the putative glycosylation signals in L65, destroying the consensus site (Tartaglia et al., 1986). However, by four separate criteria we have been unable to detect glycosylation of L65 (Figure IV. 4 and data not shown).
Fig. IV.3. Western blot analysis of purified vaccinia virions with anti-L65 sera. Density gradient purified vaccinia virus (0.23 mg) was denatured, subjected to SDS-PAGE and western blotting procedures using anti-L65 sera as described in the Materials and Methods (Lane V). No immunoreactivity was detected, using pre-immune sera (data not shown). Prestained molecular weight (BRL) markers were run on the same gel (lane M).
Fig. IV.4. Tunicamycin effects on L65. Radiolabelled protein extracts were made from wild-type VV and vaccinia recombinant VV3S infected cells which had been incubated in either the presence or absence of (1 ug/ml) tunicamycin. The VV3S recombinant contains and expresses the Sindbis virus glycoprotein genes (E1 and E2) and functioned as an internal control for the activity of the tunicamycin (Rice et al., 1985). Immunoprecipitation of wildtype or VV3S extracts with either anti-Sindbis virus glycoprotein antisera (anti-Sindbis) or with anti-L65 antisera (anti-L65) is shown. Molecular weight markers are indicated at the left of each panel.
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Figure IV.4
Treatment of cells with tunicamycin during vaccinia infection failed to alter the migration of immunoprecipitated L65 in SDS:PAGE, under conditions where the migration of an internal control, Sindbis virus glycoproteins E1 and E2, was affected (Figure IV. 4). This was also true for treatment of L65 with N-glycanase and hydrofluoric acid, reagents which are reported to remove N-linked or all sugar residues, respectively (Mort and Lamport, 1977; Papkoff et al., 1987), from proteins (data not shown). Labeling infected cells with tritiated N-acetyl-glucosamine and immunoprecipitation of L65 from these extracts also failed to label L65 under conditions where several viral glycoproteins were detectable (data not shown).

**L65 association with VV virosomes**

The function of L65 during the VV infection cycle is unknown. As one step toward answering that question, we examined the distribution of L65 during infection by sucrose density gradient fractionation. Fractionation of VV-infected cell cytoplasmic components under nondenaturing conditions through 38-50% sucrose density gradients separates virosomes (viral factories) from developing and mature viral particles (Dahl and Kates, 1970). These assays were conducted using tritiated thymidine and DNase to monitor the position of newly synthesized viral DNA and its DNase sensitivity. The viral factories sedimented through the 50% sucrose but not through an 84% cushion while viral particles in various stages of maturation migrate in the upper portion of the gradient (Dahl and Kates, 1970; Figure IV.5). Analysis of the distribution of L65 in these gradients by western blotting revealed that
L65 was likely associated with viral particles or membranes in the upper fractions of the gradient (Figure IV.5). The results of three separate trials were identical; L65 is present primarily in fractions 10 and 11 with some residual in fraction 12. These same fractions appear to contain viral particles in various stages of morphogenesis based on DNase insensitivity and the presence of newly replicated DNA (Dahl and Kates, 1970; Figure IV.5). When the same procedures were carried out on extracts of cells infected with VV in the presence of rifampicin, a change in the distribution of L65 was observed (Figure IV.5). In the presence of rifampicin, L65 was found primarily at the top of gradient, in fraction 12 and occasionally in fraction 11 while most viral DNA was found on the 84% cushion in fraction 2 viral factories and uncoated membrane bound DNA. However, a small amount of partially DNase sensitive DNA migrates in fractions 9 and 10 (immature viral particles). A DNase insensitive peak corresponding to mature virus was not seen in the presence of rifampicin (data not shown). Finding L65 in the top fraction in the presence of rifampicin suggested that L65 was no longer associated with a denser material under these conditions.

**Cytolocalization of L65 by immunofluorescence**

To examine the effect of rifampicin by another approach, we used immunofluorescence to determine the location of L65 within infected cells. Several experiments were done to establish the specificity of the fluorescence and to define the optimal conditions for detecting L65. BSC-40 cells were fixed onto coverslips in methanol and probed with either preimmune sera or anti-L65 sera, washed, and then reacted with goat anti-rabbit IgG FITC conjugated antibody.
Sucrose gradient/western blot analysis of L65 distribution. Western blots were done on $^3$H-thymidine labeled VV-infected cytoplasmic extracts which had been fractionated on a sucrose gradient (38-50%, 84% cushion). Fractions were collected from the bottom beginning with number 1. The top panel is a sucrose gradient of infected cell extracts in the absence rifampicin and the bottom panel is in the presence rifampicin. Molecular weight markers are shown at the right, lane M. Cells were continuously labeled with $^3$H thymidine from 1 hpi. The distribution of viral DNA was monitored by assaying each fraction for TCA precipitable radioactivity. Aliquots were tested for DNase sensitivity by incubation with 50 uM pancreatic DNase at 37C for 30 min in the presence of 0.01 M MgCl$_2$. Purified vaccinia virus migrates in fractions 10 and 11 and is indicated by the arrow.
Figure IV.5
Using either uninfected cells or preimmune sera resulted in similar low background immunofluorescence at 4 hpi (Figure IV.6E). Within infected cells, in the presence of anti-L65 antiserum, strongly fluorescing granular cytoplasmic structures were visible (Figure IV.6A). They appeared to be similar to viral factories or virus inclusion bodies described previously (Carins, 1960; Becker and Joklik, 1964; Dales, 1963). To determine whether these fluorescing structures also contained viral DNA, cells were stained with Hoechst 33342 (Bisbenzimide) and processed for immunofluorescence. Hoechst 33342 stains double stranded nucleic acid and fluoresces blue at ultraviolet wavelengths. This stain can be used to detect DNA-containing virosomes during infection (Esteban, 1977). As evidenced by the photo at UV wavelengths in Figure IV.6B, blue cytoplasmic DNA structures are formed during the infection (nuclei are visible as large round structures). Figure IV.6C is the same field of cells viewed with the FITC filter, showing that the same structures which contain the viral DNA, also contain L65 and fluoresce greenish-yellow.

Cells infected in the presence of rifampicin show a marked change in the distribution of L65 (Figure IV.6D). Immunofluorescence within these cells is not localized to the virosome, but appears as dispersed pinpoints of fluorescence throughout the cytoplasm. The red color is due to the presence of Evans blue stain which increases the visibility of the small foci. Staining with Hoechst 33342 demonstrates that the virosomes remain in the presence of the drug (Esteban, 1977 and data not shown).
Fig. IV.6. Immunofluorescence of VV infected cells (6 hpi) was carried out using: A) anti-L65 and Evans blue stain. B) anti-L65 and Hoechst 33342 stain, viewed at UV wavelengths (in the absence of the Hoechst stain no fluorescence was seen at UV wavelengths in the presence of anti-L65). C) Same frame as B, but viewed under 450-490 nm wavelength light. D) Same as A, except infection was carried out in the presence of rifampicin. E) preimmune sera. No L65 fluorescence was detectable prior to 4 hpi.
Several workers have shown the reversibility of the rifampicin inhibition by replacement with media without rifampicin (Moss et al., 1971a and Nagayama et al., 1970). The effect of removing the rifampicin appears to be a resumption of viral morphogenesis. Two changes can be rapidly observed: 1) the formation of spicules on trilaminar precursor membranes, and 2) the cleavage of the core precursor polypeptides (Moss et al., 1981b). These effects were seen to occur within 10 min after removal of the rifampicin. Figure IV.7 documents the effects of such treatments on the virosomal localization of L65. Within 2 min after removal of the rifampicin, L65 was localized to the virosomes. This rapid virosome localization of L65 does not require protein synthesis because incubation of the infected cells with both rifampicin and cycloheximide followed by the removal of the rifampicin allowed virosome localization of L65 (Figure IV.7 Plate 5). The complex between L65 and the virosoome is stable as incubation of cells with rifampicin had no effect on the association of L65 previously associated with the virosome (Figure IV.7 Plate 6). Another inhibitor of vaccinia virus morphogenesis, α-amanitin, prevents cleavage of the viral core precursors (Villarreal and Hruby, 1986; and data not shown). However this drug does not affect the localization of L65 to the virosomes (Figure IV.7 Plate 7). Similarly, the presence of nicotinamide, an inhibitor of ADP-ribosylation and consequently cleavage of the viral core precursors, has no effect on the distribution of L65 (Child et al., 1988; data not shown).
Tabulation of immunofluorescence data. Infected cells were treated as indicated and subjected to immunofluorescence with anti-L65. The horizontal bars indicate both the time and conditions of incubation. Plate 1 is a positive control. All plates were coded and assayed by 2 independent observers with identical results. Plates 2 and 3 were treated with rifampicin and then washed with rifampicin-free media and left for 0', 5' and 15' respectively. Plate 5 is identical to plate 4 except cycloheximide was added 15' before the removal of rifampicin. Plate 6 is identical with plate 4 except that rifampicin was replaced for 1 hour after the 15' washout. Plate 7 is incubated in media + α-amanitin (6ug/ml).
Figure IV.7
DISCUSSION

As a step toward understanding vaccinia virus morphogenesis we have undertaken an analysis of the expression and possible functions of a major late VV polypeptide L65 which appears to be involved in a crucial step during the maturation of viral particles. Using specific polyclonal antisera directed against the N-terminal portion of the L65 protein, L65 is detected in cells only after vaccinia infection and after viral DNA replication (Figure IV.1). L65 is clearly a late gene, by both RNA transcription criteria (Weinrich and Hruby, 1987; Lee-Chen and Niles, 1988a; Lee-Chen and Niles, 1988b) and now by the expression of protein (Figures 1 and 2). The timing of L65 RNA transcription generally coincides with the expression of protein. However, the L65 promoter has two transcription start sites, the distal site of which is most active at 2 hpi (Weinrich and Hruby, 1987) does not apparently give rise to detectable levels of L65 protein. By 3 hpi, however, the proximal site is active and L65 is detectable. It is unclear whether transcripts from the distal site, which contain a 90 nucleotide leader sequence are translationally inactive or the stability of the L65 protein at 2 hpi is reduced. The 90 nucleotide leader itself doesn't appear to inhibit translation because when it is attached to the chloramphenicol acetyl transferase (CAT) coding sequences, CAT enzyme is efficiently expressed in VV-infected cells (Miner et al., 1988).

Confirming previous work, it is clear that L65 remains stable to at least 16 hpi, with a half life of about 4 h(Figure IV.2). In contrast, P4b is cleaved into a smaller processed polypeptide (4b) (Weinrich et al., 1985 and J. Miner, unpublished results). This confirms the identity of the L65 band
and reinforces the specificity of the anti-L65 antisera. With anti-L65 antisera available, it was then possible to ask specific questions about the structure of the L65 protein which in turn may provide information regarding function. A large number of VV polypeptides are subject to post-translational modifications (Child et al., 1988; Sarov and Joklik, 1972; Hiller and Weber, 1985) which may have an effect on function. For the reasons mentioned previously, L65 was tested for the presence of sugar residues. By every criteria tried we were unable to detect glycosylation of L65. The analysis of L65 for other post-translational modifications is currently in progress.

The large amount of L65 synthesized in infected cells suggested that perhaps it was a structural protein. An examination of purified virions by western blotting analysis demonstrated that L65 was present in the virion, however, only a trace amount of the L65 made is actually packaged in the virion. L65 may function as a virion constituent, however, it is also possible that L65's primary function may be during morphogenesis and the amount present in the virion particles is due to the nonspecific packaging of proteins within mature virions (Franke and Hruby, 1987).

Because mutations within the L65 gene have been implicated in rifampicin resistance and because rifampicin disturbs a discrete step during morphogenesis, we analyzed infected cells for the distribution of L65 by both biochemical and immunological means. These approaches provided evidence that L65 is probably associated with virions present within virosomes and that rifampicin appears to inhibit this association (Figs. IV 5 and IV 6). L65 comigrates in sucrose gradients with immature and mature viral particles, however in the presence of
rifampicin, L65 is found in a less dense form at the top of the gradient. Immunofluorescence demonstrates the association of L65 with the virosome. The L65 immunofluorescence signal from the virosome is somewhat granular in nature suggesting some type substructure containing high levels of L65 (Figure IV.6). It is unlikely that additional resolution can be obtained by light microscopy, therefore anti-L65 sera is currently being used in immuno-electron microscopy to identify the specific structures with which L65 is associating.

The presence of rifampicin during viral infection prevents normal morphogenesis, halting the process at a step just prior to formation of rigid immature viral membrane crescents and prior to cleavage of the core precursors. This drug also prevents the association of L65 with the virosome during infection (Figure IV.5), because in spite of the fact that the virosomes form under these conditions (Esteban, 1977 and J. Miner, unpublished results), no L65 specific fluorescence is localized to these structures. Instead, small granules of fluorescing material are visible throughout the cytoplasm. The significance of these putative substructures is not known.

We were able to demonstrate rapid reversibility of the rifampicin inhibition. Within 2 min after removal of the rifampicin, the L65 was localized to the virosome (Figure IV.7 plate 3,4). This reversibility did not require protein synthesis and once bound, L65 was not affected by rifampicin (Figure IV.7 plate 5,6). The cleavage of the core precursors is tightly-linked in a temporal sense to the reversal-reaction after removal of rifampicin. To test whether other inhibitors which prevent processing of the core precursors also prevent L65 virosome localization, cells were
incubated with either α-amanitin or nicotinamide. These treatments had no effect on the distribution of L65 (Figure IV.7 Plate 7; data not shown). The lack of effect implies that the targets of α-amanitin and nicotinamide inhibition function between the point where L65 is required and processing of the core precursors during the morphogenic pathway.

Since both temperature-sensitive (R. Condit, pers. comm.) and rifampicin resistant mutants have been mapped to L65, this protein is likely essential to the virus. It is possible that by preventing the virosomal association of this essential polypeptide, rifampicin blocks further morphogenesis. This model predicts that the regions of L65 which confer resistance to rifampicin, are involved in the localization of L65 to the virosome. Experiments to address the possibility of a virosomal localization signal (VLS) in L65, and perhaps other VV late proteins, are underway.
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