AN ABSTRACT OF THE THESIS OF

Jay T. Evans for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on February 17, 1998. Title: Protein-Protein Interactions Involved in Baculovirus DNA Replication.

Abstract approved: ________________________________ George F. Rohrmann

The yeast two-hybrid system was used to examine interactions between the nine proteins involved in baculovirus DNA replication. From the six proteins required for DNA replication, four protein-protein interactions were identified, including an interaction between LEF-1 and LEF-2, LEF-3 and itself, LEF-3 and P143 (Helicase), and an interaction between IE-1 and itself.

The replication factors LEF-1 and LEF-2 interacted in both yeast two-hybrid assays and glutathione S-transferase fusion affinity assays. Using the yeast two-hybrid system, we mapped the interaction domain of LEF-2 to amino acids between positions 20 and 60. Deletion analysis of LEF-1 failed to reveal an interaction domain, suggesting that either multiple interaction domains exists or the deletions disrupted secondary structures required for the interaction. All of the deletions which were unable to interact also failed to support significant levels of transient DNA replication, suggesting that this interaction plays a significant role in DNA replication.
The baculovirus single-stranded DNA binding protein, LEF-3, interacts with itself in yeast two-hybrid assays and glutathione S-transferase fusion affinity assays. Deletions of LEF-3, which were unable to interact with full length LEF-3, also failed to support transient DNA replication, suggesting that this interaction is required for the proper function of LEF-3. LEF-3 was purified to apparent homogeneity and analyzed by analytical ultracentrifugation, native PAGE and MALDI mass spectrometry, identifying the oligomeric structure of LEF-3 as a homotrimer.

In addition to interacting with itself, LEF-3 also interacts with P143 in yeast two-hybrid assays, immunoprecipitation experiments, and co-purification from a single-stranded DNA agarose column. The yeast two-hybrid system was used to map the LEF-3 interaction domain to the N-terminal 165 amino acids of LEF-3. Deletion analysis of P143 failed to reveal a delimited interaction domain. C-terminal deletions of LEF-3 containing amino acids 1 to 165 were unable to interact with full length LEF-3, indicating that the interaction of LEF-3 with itself (trimerization) is not required for the interaction between LEF-3 with P143.
Protein-Protein Interactions Involved in Baculovirus DNA Replication

by

Jay T. Evans

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APPROVED:

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Major Professor, representing Molecular and Cellular Biology

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Director of Molecular and Cellular Biology Program

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Jay T. Evans, Author
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CONTRIBUTION OF AUTHORS

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Greg Rosenblatt, currently a medical student at George Washington University School of Medicine and Health Sciences, provided technical assistance to many of the experiments described in Chapter 5, including cloning of constructs, immunoprecipitations, westerns, and the preparation of nuclear extracts.

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Protein-Protein Interactions Involved in Baculovirus DNA Replication

CHAPTER 1: Introduction
The Baculoviridae are a large family of viral pathogens which are infectious predominantly for holometabolous insects (Blissard and Rohrmann, 1990). Over 400 viruses have been described that are pathogenic for the larval stages of at least 600 insect species, primarily of the order Lepidoptera (moths and butterflies), but some strains infect species of Hymenoptera (sawflies), Diptera (mosquitoes), and Trichoptera (caddis flies) (Adams and McClintock, 1991; Granados and Federici, 1986; Martignoni and Iwai, 1986). In addition, several baculoviruses have been identified which infect crustaceans of the order Decapoda (shrimp) (Couch, 1974a; Couch, 1974b; Summers, 1977). Each virus isolate is usually restricted to a small host range of one or a few related species. However, several viruses, such as Autographa californica multinucleocapsid nuclear polyhedrosis virus (AcMNPV), have been identified which infect greater than 25 different insect species.

The earliest historical accounts of baculovirus infections are found in ancient Chinese literature describing a “jaundice disease” of the silkworm, Bombyx mori (Benz, 1986). The identification of baculoviruses as the infectious agent responsible for “jaundice disease” was first demonstrated by Bergold in 1947 (Bergold, 1947). Due to the limited host range, non-pathogenicity for mammals, and long-term persistence of baculoviruses in the environment, early baculovirologists quickly realized the potential of baculoviruses as possible biological control agents. Several baculoviruses were tested in field trials during the 1950’s and 60’s and in 1975, the first baculovirus pesticide, Elcar®, was registered in the United States. While many of the early viral pesticides showed some promise as insect control agents, they also suffered from a variety
of problems which limited their widespread use. Baculoviruses have a relatively slow time-to-death when compared to chemical alternatives and infection often stimulates feeding of the larval host. The limited host range of the viruses is a benefit for environmental concerns when testing baculoviruses as biological insecticides; however, it makes developing baculovirus insecticides for a wide variety of insect pests more difficult. In addition, the development of baculovirus insecticides has been limited due to high production costs, variable potency, and availability of characterized baculoviruses.

The advent of recombinant-DNA technologies in the 1970’s and 1980’s has allowed researchers to engineer recombinant baculoviruses which overcome some of the limitations described above. Recombinant baculoviruses have been constructed that express insect hormones or insect specific toxins resulting in a reduced time-to-death (Stewart et al, 1991; Tomalski and Miller, 1991). In addition, research is underway to identify genes involved in host range functions and to produce recombinant baculoviruses with alterations in these genes which will allow the killing of a greater variety of insect pests.
CHAPTER 2

Baculovirus Taxonomy, Life Cycle,
Genome Organization and DNA Replication
Taxonomy

The baculoviridae are a large family of occluded viruses characterized by rod-shaped virions which contain double-stranded, supercoiled DNA genomes ranging in size from 88 to over 170 kb pairs. They are divided into two genera that are distinguished by the structure of their occlusion bodies. The nucleopolyhedroviruses [nuclear polyhedrosis viruses (NPV's)] have large crystalline proteinaceous structures called polyhedra that are 1-15 mm in diameter and contain multiple virions (Rohrmann, 1994). The NPV's are further divided into either single or multiple nucleopolyhedroviruses (SNPV or MNPV), depending on the number of virion nucleocapsids per envelope. The granuloviruses [granulosis viruses (GV's)] have a single virion embedded within a smaller occlusion body or granule (0.25-0.5 mm in diameter) (Crook, 1994). NPV's are better characterized than the GV's, and therefore will be the focus of the remainder of this chapter.

Life Cycle (Fig. 2.1)

Baculovirus occlusion bodies (polyhedra) can be found in the environment both on plant tissues and in soil where they can remain infectious for decades. Upon ingestion by an insect host, the proteinaceous coat of the occlusion body is dissolved in the alkaline pH of the insect midgut. Breakdown of the occlusion body releases the enveloped "occlusion-derived virus" (ODV) into the lumen of the insect midgut followed by infection of insect midgut columnar epithelial cells (Fig. 2.1 and 2.2). The ODV travel to the nucleus
where they are uncoated, followed by early transcription and DNA replication in permissive insect hosts. Early genes are transcribed by the RNA polymerase II of the host cell (Fuchs et al, 1983; Hoopes and Rohrmann, 1991; Huh and Weaver, 1990) and include genes involved in DNA replication, inhibition of apoptosis, host range factors, and early transcription. Soon after the onset of viral DNA replication, a unique viral encoded α-amanatin- and tagetitoxin-resistant RNA polymerase begins transcription of late viral genes (Glocker et al, 1993; Grula et al, 1981; Yang et al, 1991) (Fig. 2.3). Late transcription initiates within a five base pair late promoter element with the sequence A/G/T TAAG (Blissard and Rohrmann, 1990). DNA replication and late transcription leads to the formation of ODV and a second morphologically distinct viral particle (Fig. 2.2). Budded virus (BV) is released from infected cells and is responsible for cell-to-cell transmission of the virus throughout the insect (Volkman and Summers, 1977). At very late stages of the infection cycle the 'very late' genes polyhedrin and p10 are highly overexpressed (Fig. 2.3). ODV particles are embedded within a crystalline protein matrix made predominantly of the polyhedrin protein, followed by lysis of the infected cell. Eventually the insect dies and decays, releasing occluded virions (polyhedra) into the environment where they can be ingested by other insects.

Baculoviruses have received widespread attention due to their ability to express foreign proteins under control of the polyhedrin promoter. The ability to passage budded virus in cell culture has allowed scientists to replace the highly overexpressed polyhedrin gene with other eukaryotic genes of interest, creating a recombinant baculovirus which highly overexpresses the foreign gene (Miller,
Figure 2.1. Nuclear polyhedrovirus infection cycle. Polyhedra are ingested by the host insect and solubilized in the midgut of the insect. ODV are released into the midgut lumen and enter the midgut epithelium cells by fusion with microvilli. The nucleocapsids are transported to the nucleus, followed by uncoating, early transcription, DNA replication, late transcription and very late transcription (Fig. 2.3). Progeny virions are assembled in the virogenic stroma. At late times in the infection cycle, budded virus (BV) is released from the infected cell by budding through the cytoplasmic membrane containing the virus encoded envelope glycoprotein gp64. BV is responsible for cell-to-cell transmission of the virus throughout the insect, eventually leading to a systemic infection. At very late times in the infection cycle, ODV are occluded within a crystalline protein matrix made predominantly of the polyhedrin protein. Infected cells eventually lyse, killing the insect host and releasing polyhedra into the environment.

Figure 2.2. Diagram of two morphologically distinct baculovirus phenotypes which occur during the infection cycle (Fig. 2.1). Unique and shared viral components and lipid composition (%) are indicated for both ODV and BV. The ODV diagrammed is representative of a MNPV. References for the structural proteins and lipid composition represented in this figure can be found in The Baculoviruses, 1997 (Miller, 1997).
Figure 2.1 Baculovirus Life Cycle (Beek, 1980)
Budded Virus (BV)  

**BV specific Components**
- gp64 Envelope Fusion Protein (gp64 EFP)
- Virion Envelope Lipid Composition (%)
  - LPC 5.9
  - SPH 13.2
  - PC 10.7
  - PI 12.3
  - PS 50.2
  - PE 7.6

**Common Virion Components**
- Virus DNA
- Basic DNA Binding Protein (p6.9)
- Major Capsid Proteins (vp39; p80; p24, p91)
- Capsid End Structure (ORF 1629 [pp78/83])

Occulusion Derived Virus (ODV)

**Common Virion Components**
- Virus DNA
- Basic DNA Binding Protein (p6.9)
- Major Capsid Proteins (vp39; p80; p24, p91)
- Capsid End Structure (ORF 1629 [pp78/83])

**ODV specific Components**
- Envelope proteins (ODV E18/35; E25; EC27*; E35; E56; E66)
- gp41 (tegument)
- p74
- Virion Envelope Lipid Composition (%)
  - LPC 0.8
  - SPH 1.5
  - PC 39.0
  - PI 8.2
  - PS 20.5
  - PE 30.1

* also present in ODV capsid

Figure 2.2: Baculovirus Phenotypes (Blissard and Rohrmann, 1990)
Infection begins

**Classes of mRNA**

- **Early**
  - Host cell RNA pol II, transcription factors, viral transactivators, viral enhancers
  - Genes transcribed: transcriptional activators, enzymes associated with DNA replication, etc.

- **Late**
  - \(\alpha\) - amanitin resistant RNA polymerase
  - Genes transcribed: structural proteins, etc.

- **Very Late**
  - Polyhedrin p10

**DNA Replication**

- Shut off of some genes transcribed by RNA polymerase II
- Budded Virus Production
- Occluded Virus Production

Figure 2.3: Baculovirus Transcriptional Cascade (Rohrmann, 1992)
1988; Pennock et al, 1984; Smith et al, 1983). The ability to express large quantities of a recombinant protein (20-50% of total protein) in a eukaryotic cell line has made recombinant baculovirus expression systems one of the most popular eukaryotic expression systems for use in both basic and applied research. Proteins expressed in insect cells can be post-translationally modified by phosphorylation, glycosylation, and proteolysis. In addition, proteins can be targeted for secretion, to the membrane or other cellular compartments and multiple proteins can be simultaneously expressed to form oligomeric complexes [for reviews, see (Luckow and Summers, 1988; Maeda, 1989; Miller, 1988)].

**Genome Organization**

The baculovirus genome consists of circular, supercoiled, double-stranded DNA ranging in size from 88 to over 170 kb, depending on the viral strain. Five baculoviruses have been completely sequenced including the NPV's *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV), *Orgyia pseudotsugata* MNPV (OpMNPV), *Bombyx mori* NPV (BmNPV), and *Lymantria dispar* MNPV (LdMNPV) (Ahrens et al, 1997; Ayres et al, 1994; Genebank 133180; unpublished data) and the GV XcGV (personal communications, S. Maeda). While the NPV's have many genes in common both in sequence homology and location, they vary greatly in size and G + C content (Table 2.1). One characteristic unique to many NPV's is the presence of homologous regions (*hrs*) interspersed throughout their genomes.
Table 2.1: NPV Genome Size and Content

<table>
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<tr>
<th>Virus</th>
<th>Genome Size&lt;sup&gt;1&lt;/sup&gt; (base pairs)</th>
<th>ORF's&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Hr's&lt;sup&gt;3&lt;/sup&gt;</th>
<th>% G + C&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
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<tr>
<td>AcMNPV</td>
<td>133,894</td>
<td>155</td>
<td>8</td>
<td>41</td>
</tr>
<tr>
<td>BmNPV</td>
<td>128,413</td>
<td>135</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>OpMNPV</td>
<td>131,990</td>
<td>152</td>
<td>5</td>
<td>55</td>
</tr>
</tbody>
</table>

<sup>1</sup> Genome sizes in base pairs  
<sup>2</sup> Open reading frames of greater than 50 aa which showed minimal overlap (Ahrens et al, 1997; Ayres et al, 1994)  
<sup>3</sup> Number of homologous regions in genome  
<sup>4</sup> % G + C content of entire genome
OpMNPV has 5 *hrs* while AcMNPV has 8 *hrs* located throughout the genome (Fig. 2.4) (Ahrens *et al.*, 1997; Ayres *et al.*, 1994). The *hrs* consist of imperfect palindromes embedded within a larger direct repeat. *Hrs* will be discussed in greater detail below.

AcMNPV and OpMNPV sequence analysis identified 155 and 152 possible open reading frames (ORF’s) of 50 amino acids or larger that showed minimal overlap with other ORF’s (Table 2.1). Of the 155 ORF’s in AcMNPV 127 have homologs in OpMNPV. All of the 18 genes involved in late transcription and DNA replication in AcMNPV are present in both genomes; except p35 from AcMNPV, which has a functional homolog, *iap*-3, in OpMNPV (Ahrens *et al.*, 1997). OpMNPV has several genes which are not found in AcMNPV. These include homologs of genes involved in nucleotide metabolism (dUTPase and ribonucleotide reductase homologs) and two additional *iap* homologs not found in AcMNPV. Homologies between ORF’s vary considerably from the highly conserved polyhedrin protein with 89% amino acid sequence identity between AcMNPV and OpMNPV, to other proteins ranging as low as 24% (Ahrens *et al.*, 1997).

The genome organization between AcMNPV and OpMNPV is very similar with many contiguous ORF’s in the same linear order and orientation. There are two large inversions in OpMNPV (ORF’s 1-10 and 24-38) (relative to AcMNPV) that are located near *hrs*, and are likely the result of homologous recombination events between *hrs*. In addition, there are several insertions or deletions that have occurred, leading the presence of unique ORF’s and/or repeated sequences not found in the other viruses.
Figure 2.4. Location of homologous regions (hrs), non-hr origins (HdIII-K) of DNA replication, and genes involved in DNA replication on the AcMNPV genome. The number within the circles (representing hr positions) indicate the number of repeated sequences within each hr (Fig. 2.6a). Arrows indicate the position and orientation of genes involved in AcMNPV DNA replication. The polyhedrin gene is shown as a point of reference. Data for this figure are from Ayres et al (1994).
Figure 2.4: Map of AcMNPV (Kool et al, 1995)
Baculovirus DNA Replication

INTRODUCTION

Baculovirus DNA replication is an intermediate step in the ordered cascade of events leading to the production of BV and ODV (Fig. 2.1 and 2.3). This cascade begins with the expression of early genes by the host RNA polymerase II, followed by DNA replication, late and very late transcription. Late genes are transcribed by a unique viral encoded RNA polymerase (Fig. 2.3). Late transcription is dependent upon viral DNA replication and is prevented when DNA replication is inhibited (Friesen and Miller, 1986; Rice and Miller, 1986/87).

The most well characterized baculovirus, AcMNPV, has been used to identify a number of cis-acting and trans-acting factors involved in the regulation of transcription and DNA replication in an infected cell. Early investigations transfecting purified viral DNA into uninfected insect cells demonstrated that viral DNA is all that is required to initiate the infection cycle and to produce mature virions (Burand et al., 1980; Carstens et al., 1980; Potter and Miller, 1980), and that viral protein synthesis was required for viral DNA replication (Erlandson et al., 1985; Gordon and Carstens, 1984; Kelly, 1982; Kelly and Lescott, 1976).

BACULOVIRUS ORIGINS OF DNA REPLICATION

Two different methods have been used to identify putative viral origins of DNA replication. The first method used the serial
passage of baculoviruses to produce defective interfering particles in which large genomic deletions have taken place and origin-containing sequences have been amplified. The second relies on the ability of cloned baculovirus sequences to replicate after transfection into infected insect cells.

Defective interfering particles

The identification of possible origins of DNA replication in AcMNPV was assisted by the production of defective interfering (DI) particles (defective genomes). The undiluted serial passage of AcMNPV results in the production of DI particles which contain major genomic deletions and tandemly repeated sequences that are believed to represent viral origins of DNA replication (Kool et al, 1993a; Lee and Krell, 1992; Lee and Krell, 1994). DI particles have been used to identify both hr and non-hr origins of DNA replication. Kool et al (1993a) reported the amplification of two different regions of the AcMNPV genome after 40 serial passages [map units (m.u.) 50.1 to 53.2 and m.u. 87.2 to 88.9]. Both of these regions were found to contain hr sequences, suggesting that hrs may function as viral origins. In a separate study, Lee and Krell (Lee and Krell, 1992; Lee and Krell, 1994) identified a non-hr origin (m.u. 85.0 to 87.2) after 81 serial passages with AcMNPV. Transient DNA replication assays have been used to confirm that both hr and non-hr sequences can function as origins (Kool et al, 1994b), suggesting that AcMNPV may have multiple hr and non-hr origins of DNA replication. The ability of these origins to function in the context of the entire AcMNPV genome is still unclear.
Homologous regions

A unique feature of many NPV's are the presence of homologous repeats (hrs) interspersed throughout their genomes. Each hr consists of one or more imperfect palindromes which are contained within a larger direct repeat (Fig. 2.5a, b). These hrs can be found throughout the genome and some appear to be conserved in both location and sequence between different baculoviruses (Ahrens et al, 1997). It has been hypothesized that these sequences may form a cruciform structure centered around the imperfect palindrome; however, the role of these structures in hr function is still unknown (Rasmussen et al, 1996).

Hrs have been identified in several baculoviruses, including AcMNPV, OpMNPV, BmNPV, LdMNPV, Choristoneura fumiferana MNPV (CfMNPV), and Anticarsia gemmatalis MNPV (AgMNPV) (Ahrens et al, 1997; Ayres et al, 1994; Garcia-Maruniak et al, 1996; Xie et al, 1995). The hrs of AcMNPV consist of one to eight copies of a 30 bp imperfect palindrome flanked by 22 bp and 20 bp direct repeats and separated by 0 to 131 bp of DNA (Ayres et al, 1994; Guarino and Summers, 1986a) (Fig. 2.4 and 2.5a). In OpMNPV, the hrs contain 2 to 10 repeats of a 30 bp imperfect palindrome flanked by 21 bp and 15 bp direct repeats (Fig. 2.5b). The palindrome consensus sequences for AcMNPV and OpMNPV are 57% identical. While both AcMNPV and OpMNPV hrs contain multiple repeats, the OpMNPV repeats are not separated by intervening DNA (Ahrens et al, 1997; Ayres et al, 1994).

Hrs have been shown to act as enhancers of RNA polymerase II mediated transcription (Guarino et al, 1986; Guarino and Summers, 1986b; Theilmann and Stewart, 1992) and as origins of DNA
replication in transient DNA replication assays (Ahrens et al, 1995b; Ahrens and Rohrmann, 1995b; Kool et al, 1995; Pearson et al, 1992). The ability of hrs to function as cis-acting transcriptional activators is the result of an interaction between specific hr sequences and the viral transcriptional activator IE-1, which binds to these sites as a dimer (Choi and Guarino, 1995; Guarino and Dong, 1991; Guarino and Dong, 1994; Leisy et al, 1995; Rasmussen et al, 1996; Rodems and Friesen, 1995). Hrs were identified as possible origins of DNA replication using DI particles and by their ability to function in infection-dependent transient DNA replication assays (Ahrens and Rohrmann, 1995b; Kool et al, 1993a; Kool et al, 1993b; Lee and Krell, 1994; Leisy and Rohrmann, 1993; Pearson et al, 1993; Pearson et al, 1992; Pearson and Rohrmann, 1995; Xie et al, 1995). In this assay, plasmid DNA containing fragments from the viral genome are prepared in dam+ Escherichia coli and transfected into infected insect cells. If the plasmid replicates it will become hemi-methylated or un-methylated and resistant to digestion with Dpnl, whereas the original bacterial produced plasmid DNA is sensitive to digestion with Dpnl. The replicated (Dpnl resistant) plasmids can be detected by Southern blot analysis using labeled plasmid DNA as a probe.
Figure 2.5. Sequences and organization of homologous regions from AcMNPV and OpMNPV. (a) AcMNPV hr structure and sequence. Shown on top is a schematic representation of a single AcMNPV hr repeat, found in 1-8 copies within each of the 8 AcMNPV hrs (Fig. 2.4). A consensus sequence for the 20 and 22 bp direct repeats, from 20 different hr repeats, is shown on the bottom. The 30 bp palindrome is shown above the direct repeat consensus and represents 32 of the 35 palindromes found in AcMNPV. The frequency of variable nucleotides in the palindrome are shown as subscripted numbers below each nucleotide. The highly conserved 12 bp core and EcoRI site are boxed and underlined. (b) OpMNPV hr structure and sequence. Shown on top is a schematic representation of a single hr repeat, found in 2-10 copies within each of the 5 OpMNPV hrs, (Ahrens et al, 1997). A consensus sequence for the 30 bp imperfect palindrome is shown below the schematic representation. The subscripted numbers represent identical matches from the 27 hr repeats found in OpMNPV. A consensus sequence for the 21 and 15 bp direct repeats is shown below the palindrome.
Figure 2.5: Homologous Repeats of AcMNPV and OpMNPV
Non-\textit{hr} origins of DNA replication

Non-\textit{hr} origins have been identified using both DI particles and infection-dependent transient DNA replication assays. In AcMNPV, the HindIII-K (84.9 to 87.3 m.u.) region of the genome was identified as a putative origin from its enrichment in AcMNPV DI particles (Lee and Krell, 1994) (Fig. 2.4). The ability of this region to function as an origin was confirmed in infection-dependent transient DNA replication assays (Kool \textit{et al}, 1994b; Lee and Krell, 1994). This region contains multiple palindromic and repetitive sequences which are not found in \textit{hr} sequences and are unique to this element. In addition to the HindIII-K region of AcMNPV, promoters from several early baculovirus genes have been shown to function as origins in infection-dependent transient DNA replication assays (Wu and Carstens, 1996). One non-\textit{hr} origin has been identified in the HindIII-N region of the OpMNPV genome (7.0 to 11.3 m.u.) (Pearson \textit{et al}, 1993). This region of the genome contains multiple palindromes, direct and inverted repeats, and transcription factor binding sites; however, the correlation between these areas and origin activity has not been determined.

THE STRUCTURE OF REPLICATED DNA

Replicated plasmids from AcMNPV infected insect cells appear to form high molecular weight structures, indicating that replication of origin containing plasmids does not result in exact replicas of the input plasmid. In addition, linearized plasmids containing origins are unable to replicate in these assays (Kool \textit{et al}, 1993b). Partial digestion of these high molecular weight
structures with an enzyme that cuts the plasmid in a single location resulted in the production of a ladder with multiples of unit length, indicating that the DNA was present as a concatemer of the original plasmid (Leisy and Rohrmann, 1993; Xie et al, 1995). The amplification of origins identified by DI particle analysis is also consistent with these results (Lee and Krell, 1992; Lee and Krell, 1994). These data are suggestive of a rolling circle type of DNA replication. The identification of similar intermediates during viral replication and the enzymes required for the resolution of these structures has not been reported.

GENES INVOLVED IN BACULOVIRUS DNA REPLICATION

A transient DNA replication assay, similar to the one described above, was used to identify viral proteins which are required or stimulatory for the replication of an origin (hr) containing plasmid. A similar assay was used to identify proteins from herpes simplex virus 1 (HSV-1), which are involved in DNA replication (Challberg, 1986). In this assay, a set of overlapping cosmids and an origin (hr) containing plasmid were cotransfected into insect cells and a DpnI assay (described above) was used to identify a minimal set of cosmids required for replication of the origin containing plasmid. Subclones of these cosmids were then used to identify a subset of proteins which are involved in origin dependent DNA replication. Six genes from AcMNPV were found to be essential for DNA replication and encode the following proteins: DNA polymerase, P143 (putative helicase), LEF-3 (SSB), LEF-1 (putative primase), LEF-2, and IE-1. In addition, three genes were identified which are stimulatory for DNA
replication and encode two transcriptional activators: IE-2 and PE-38 and an inhibitor of apoptosis, P35 (Kool et al, 1994a; Lu and Miller, 1995b). A similar set of proteins was identified in OpMNPV (Ahrens and Rohrmann, 1995a; Ahrens et al, 1995a; Ahrens and Rohrmann, 1995b; Ahrens and Rohrmann, 1996). The proteins involved in AcMNPV DNA replication are described below, in Table 2.2, and their locations are shown in Figure 2.4.

DNA Polymerase

Early investigations using fractionated extracts from infected insect cells identified a new DNA polymerase activity which was separated from the cellular DNA polymerases (Kelly, 1981; Kelly, 1982; Miller et al, 1981; Wang and Kelly, 1983). Further investigation identified a 3'-5' exonuclease activity which copurified with the BmNPV DNA polymerase activity (Mikhailov et al, 1986b). A 3'-5' exonuclease activity has been found associated with other eukaryotic DNA polymerases and is responsible for proofreading the newly synthesized DNA by hydrolyzing mismatched nucleotides from the primer terminus (Kornberg and Baker, 1992).

DNA polymerase homologs have been identified from six different baculoviruses and encode proteins of about 115 kDa (Ahrens and Rohrmann, 1996; Bjornson et al, 1992; Chaeychomsri et al, 1995; Cowan et al, 1994; Liu and Carstens, 1995; Tomalski et al, 1988). These DNA polymerases contain multiple conserved motifs found in other DNA polymerases with 3'-5' exonuclease activity. In AcMNPV and OpMNPV, the DNA polymerase homologs were also identified using DpnI based transient DNA replication assays. Several reports have indicated that the baculovirus DNA
Table 2.2: AcMNPV and OpMNPV Replication Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>MW (kDa)(^1)</th>
<th>Function(s)</th>
<th>Motifs(^2)</th>
<th>Essential(E)</th>
<th>Stimulatory(S)</th>
<th>Amino acid identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA pol</td>
<td>114.3/112.6</td>
<td></td>
<td>DNA Pol</td>
<td>E</td>
<td></td>
<td>63.1%</td>
</tr>
<tr>
<td>helicase</td>
<td>143.2/140.5</td>
<td></td>
<td>Helicase</td>
<td>E</td>
<td></td>
<td>59.1%</td>
</tr>
<tr>
<td>ie-1</td>
<td>66.9/ 64.3</td>
<td>transcriptional activator</td>
<td>SSB</td>
<td>E</td>
<td></td>
<td>48.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>binds hr sequences</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lef-1</td>
<td>30.8/ 27.9</td>
<td>NTPase</td>
<td>E</td>
<td></td>
<td></td>
<td>58.1%</td>
</tr>
<tr>
<td>lef-2</td>
<td>23.9/ 22.7</td>
<td></td>
<td></td>
<td>E</td>
<td></td>
<td>54.7%</td>
</tr>
<tr>
<td>lef-3</td>
<td>44.6/ 42.6</td>
<td>SSB</td>
<td>SSB</td>
<td>E</td>
<td></td>
<td>41.5%</td>
</tr>
<tr>
<td>p35</td>
<td>34.8/ -</td>
<td>inhibits apoptosis</td>
<td></td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>transcriptional activator</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Op-iap(^3)</td>
<td>33.3 / 30.1</td>
<td>inhibits apoptosis</td>
<td>S</td>
<td></td>
<td></td>
<td>28.4%</td>
</tr>
<tr>
<td>ie-2</td>
<td>47.0/ 45.7</td>
<td>transcriptional activator</td>
<td>S</td>
<td></td>
<td></td>
<td>38.8%</td>
</tr>
<tr>
<td>pe-38/p34</td>
<td>37.4/ 34.7</td>
<td>transcriptional activator</td>
<td>S</td>
<td></td>
<td></td>
<td>37.1%</td>
</tr>
<tr>
<td>lef-7</td>
<td>26.6/ -</td>
<td>SSB</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Molecular weights of predicted AcMNPV and OpMNPV proteins are from (Ayres et al., 1994, Ahrens et al, 1997).

\(^2\) Listed motifs are confined to motifs commonly found in components of replication systems (Kornberg and Baker, 1992).

\(^3\) An AcMNPV homolog (Ac-iap1) has been reported but is nonfunctional in preventing apoptosis (Crook, 1993).
polymerase was required for DNA replication in a transient DNA replication assay (Ahrens and Rohrmann, 1995b; Kool et al, 1994a; Kool, Voeten, and Vlak, 1994; Pearson et al, 1993); however, one report identified the DNA polymerase as stimulatory in this assay (Lu and Miller, 1995b). The ability of a cellular DNA polymerase to function in this assay (at levels far less than the viral DNA polymerase), suggests a conservation in eukaryotic replicative polymerases and larger replication complexes. This conservation is further supported by substitution studies where AcMNPV and OpMNPV DNA polymerases are able to substitute for each other in transient DNA replication assays (Ahrens and Rohrmann, 1996). In addition, an ascovirus DNA polymerase was shown to substitute for the AcMNPV DNA polymerase in a similar assay (Pellock et al, 1996). This strong conservation in both sequence homology and function suggests that further research into baculovirus DNA replication may be helpful in understanding not only baculovirus DNA replication but also other eukaryotic replication systems.

Several classes of DNA polymerases are stimulated by an accessory protein called proliferating cell nuclear antigen (PCNA). PCNA forms a homotrimeric ring structure around double-stranded DNA and may function as a sliding clamp to assist in the loading and processivity of DNA polymerases (Krishna et al, 1994). In addition to its obvious role in DNA replication and repair, PCNA may also play a role in cell cycle regulation, due to its association with a variety of cyclins and cyclin dependent kinases (Xiong et al, 1993; Zhang et al, 1993). AcMNPV contains a PCNA homolog with 42% amino acid identity with rat PCNA (O'Reilly et al, 1989); however, it is neither essential or stimulatory when used in a transient DNA replication
assay (Kool et al, 1994a). A PCNA homolog was also identified in OpMNPV and has 31% amino acid identity with AcMNPV PCNA and no homolog was found in the genome of BmNPV or LdMNPV (Ahrens et al, 1997; unpublished data). Deletion of the PCNA homolog from the AcMNPV genome resulted in a mutant virus with a slightly delayed pattern of DNA replication and late gene expression when compared with wild type (wt) virus (Crawford and Miller, 1988). Further investigations are required to identify the possible roles of PCNA in viral DNA replication, repair, and cell cycle control.

P143 (Helicase)

Sequencing of an AcMNPV temperature sensitive mutant defective in DNA synthesis revealed a gene (p143) containing significant sequence homology with known helicases (Lu and Carstens, 1991). The requirement of this gene for origin specific replication was confirmed in transient DNA replication assays. Helicases are large class of DNA and/or RNA modifying enzymes which use the energy of NTP hydrolysis to disrupt hydrogen bonds resulting in the separation of DNA, RNA or DNA/RNA duplexes. The putative baculovirus helicase (p143) encodes a 143 kDa protein with homology to other known helicases, including DNA/RNA unwinding and NTP binding motifs which are common to many helicases (Hodgeman, 1988). A recent report by Laufs et al (Laufs et al, 1997) describes the purification of P143 from infected insect cells and demonstrates a nonspecific double-stranded DNA binding activity associated with the purified protein. In addition, P143 interacts with LEF-3, a viral single-stranded DNA binding protein
(SSB), which is also required in transient DNA replication assays (Chapter 5).

In addition to their role in baculovirus DNA replication, the helicases of AcMNPV and BmNPV have also been found to play a role in host range functions (Croizier et al., 1994; Kamita and Maeda, 1996; Maeda et al., 1993). Both AcMNPV and BmNPV are able to infect S. frugiperda (Sf-21) and B. mori (Bm) cells; however, DNA replication only occurs in the permissive cell line. AcMNPV recombinants in which a 79 bp section of the p143 gene was replaced with the corresponding BmNPV sequence (resulting in four altered codons) were able to replicate in both Sf-21 and Bm cell lines (Croizier et al., 1994; Maeda et al., 1993). In addition, the recombinant virus was capable of replicating in B. mori larvae, which are not permissive for wt AcMNPV replication (Croizier et al., 1994). Further investigations are necessary to determine the functional significance P143 in both baculovirus DNA replication and host range.

LEF-1 and LEF-2

AcMNPV lef-1 and lef-2 were first identified as being essential in late and very late transcription assays (Passarelli and Miller, 1993a; Passarelli and Miller, 1993b) and were also found to be required for DNA replication in transient DNA replication assays (Ahrens and Rohrmann, 1995b; Kool et al., 1994a; Lu and Miller, 1995b).

LEF-1 homologs have been identified in several baculoviruses, including AcMNPV, OpMNPV, BmNPV, LdMNPV, CfMNPV, CfDEF (defective strain of CfMNPV) and Buzura suppressaria NPV
Alignment of LEF-1 sequences from five of these viruses (AcMNPV, OpMNPV, BmNPV, CfMNPV and CfDEF) identified four conserved domains with 69% to 100% amino acid identity (Barrett et al, 1996), including domains common to DNA primases from several organisms. A putative primase domain in LEF-1 was mutated by a single conserved amino acid change (WVVDAD to WVVQAD) which eliminated the ability of this clone to replace wt LEF-1 in a transient DNA replication assay, suggesting that LEF-1 may function as a DNA primase (Chapter 3) (Evans et al, 1997).

In addition to a possible role in DNA replication, LEF-2 may have an independent role in late gene expression. Merrington et al (Merrington et al, 1996) identified an AcMNPV mutant in which the lef-2 gene had been modified causing an alteration in late gene expression without affecting DNA replication.

Protein-protein interaction studies involving LEF-1 and LEF-2 identified an interaction between these two proteins using the yeast two-hybrid system and affinity assays employing a LEF-2 glutathione S-transferase fusion and radiolabeled LEF-1 produced using a coupled in vitro transcription and translation reaction (Chapter 3). In addition, deletions which disrupted this interaction also disrupted DNA replication in transient DNA replication assays, suggesting that this interaction is required for the proper function of these two proteins (Chapter 3) (Evans et al, 1997).

LEF-3 (SSB)

LEF-3 was identified in both AcMNPV and OpMNPV as being required for DNA replication in transient DNA replication assays (Ahrens et al, 1995a; Kool et al, 1994a; Lu and Miller, 1995b). Fractionation of extracts from infected insect cells by single-
stranded DNA agarose chromatography identified a protein of 44 kDa which eluted from the column in 800 mM to 1000 mM NaCl. Immunological analysis using anti-LEF-3 polyclonal antiserum identified this protein as the product of the AcMNPV lef-3 gene. Further biochemical analysis of the purified protein demonstrated a non-specific single-stranded DNA binding activity associated with the purified protein (Hang et al, 1995).

In a separate study, LEF-3 was shown to interact with itself and with P143 (helicase) in yeast two-hybrid assays, co-immunoprecipitations, co-purification, and affinity assays using glutathione S-transferase fusions and in vitro radiolabeled proteins (Chapters 4 and 5). Purification of LEF-3 by single-stranded DNA agarose and Superose 12 gel filtration chromatography resulted in a highly purified LEF-3 which was analyzed by native PAGE, MALDI mass spectrometry, and analytical ultracentrifugation, identifying the oligomeric structure as a homotrimer (Chapter 4) (Evans and Rohrmann, 1997).

IE-1

Immediate early gene 1 (ie-1) activates or negatively regulates transcription from a variety of early gene promoter constructs after cotransfection into uninfected insect cells (Blissard and Rohrmann, 1991; Carson et al, 1991b; Guarino and Summers, 1986a; Kovacs et al, 1991; Lu and Carstens, 1993; Rodems and Friesen, 1993). Negative regulation of specific promoters by IE-1 is due to the binding of IE-1 to promoters containing a specific IE-1 binding motif near their mRNA start sites (Leisy et al, 1997). Promoters without IE-1 binding motifs near
their mRNA start sites are activated by IE-1 and can be further stimulated by the presence of a cis-acting hr sequence. Extracts from insect cells transfected with an ie-1 containing plasmid causes the retardation of hr containing fragments in gel shift assays, indicating that IE-1 is specifically binds to DNA fragments which contain hr sequences (Choi and Guarino, 1995; Leisy et al, 1995; Rodems and Friesen, 1995). Further analysis using a combination of full length and deletions of ie-1 in gel shift assays revealed that IE-1 binds hr sequences as a dimer (Rodems and Friesen, 1995). Deletion analysis of AcMNPV IE-1 has identified distinct DNA binding and transcriptional activation domains (Kovacs et al, 1992; Rodems et al, 1997; Slack and Blissard, 1997). In addition to its role as an early gene transcriptional activator, IE-1 is also required in transient DNA replication assays (Ahrens and Rohrmann, 1995a; Kool et al, 1994a; Lu and Miller, 1995b). The requirement for IE-1 in transient DNA replication assays may be due to its activation of other early genes directly involved in DNA replication. Alternatively, IE-1 may function directly in origin specific DNA replication by binding to origins (hrs) as an early step in the assembly of a DNA replication complex.

The ie-1 gene is the only baculovirus gene for which unspliced and spliced forms have been identified (Chisholm and Henner, 1988; Pearson and Rohrmann, 1997; Theilmann and Stewart, 1991). In AcMNPV the unspliced form of IE-1 produces a predicted protein of 582 amino acids (67 kDa) and the spliced form has an additional 54 amino acids on the N-terminus of IE-1 (Chisholm and Henner, 1988). Both the spliced and unspliced forms of AcMNPV IE-1 are active in transcription and replication assays. In LdMNPV, the unspliced form
of IE-1 (566 aa) appears to be inactive in both transcription and transient DNA replication assays, whereas the spliced form of IE-1, with an additional 44 aa (610 aa), is active in both assays (Pearson and Rohrmann, 1997). Further functional analysis of the spliced and unspliced forms of IE-1 are needed to determine their role in early transcription and DNA replication.

**IE-2/PE38**

In addition to the six genes which are required in transient DNA replication assays, three genes were found to be stimulatory in these assays. Two of these genes, *ie-2* (previously called *ie-n*) (Carson *et al*, 1988) and *pe-38* (also called *ie-3*) (Krappa and Knebel-Mörsdorf, 1991), encode two early gene transcriptional activators. IE-2 has been shown to activate the transcription of *pe-38* and *ie-1*, whereas PE-38 stimulates expression of the baculovirus helicase homolog, *p143* (Lu and Carstens, 1993; Yoo and Guarino, 1994). IE-2 is not stimulatory for DNA replication in *Tn-368* cells (cell line from *Trichoplusia ni*), suggesting that IE-2 may have species-specific effects on early viral transcription and DNA replication (Lu and Miller, 1995a). Stimulation of DNA replication by IE-2 and PE-38 is most likely due to the transcriptional enhancement of viral proteins directly involved in DNA replication.

IE-2 has also been reported to cause cell cycle arrest in cells transfected with an *ie-2* containing plasmid (Prikhod'ko and Miller, 1998). Mutations within its RING finger motif disrupted the ability of IE-2 to arrest cell cycle progression, but did not affect its ability to transactivate the *ie-1* promoter. In addition, deletions were created which eliminated transcriptional activation, but were still
able to block cell cycle progression, suggesting that IE-2 has multiple independent functions in cell cycle arrest and transcriptional activation (Prikhod'ko and Miller, 1998).

P35

The AcMNPV p35 gene has been shown to greatly stimulate replication in one study (Kool et al, 1994a), whereas an independent study using different assay condition found P35 essential in a transient DNA replication assay (Lu and Miller, 1995b). P35 functions as an inhibitor of apoptosis in S. frugiperda cells (Lu and Miller, 1995b), suggesting the role of P35 in transient DNA replication assays is to suppress apoptosis induced by the transfection of replication genes. This is supported by reports that the transfection of ie-1 into uninfected S. frugiperda cells induces apoptosis which can be blocked by cotransfection with p35 (Prikhod'ko and Miller, 1996). In addition, cells infected with a p35 mutant of AcMNPV can be treated with aphidicolin to block the induction of apoptosis, suggesting that DNA replication or expression of late gene product(s) is responsible for the onset of apoptosis in infected cells (Clem and Miller, 1994).

P35 inhibits apoptosis in a wide variety of organisms including Caenorhabditis elegans, drosophila, and mammalian B lymphocytes, fibroblasts, breast carcinoma cells, and neurons (Beidler et al, 1995; Hay et al, 1994; Rabizadeh et al, 1993; Sugimoto et al, 1994). P35 blocks apoptosis by inhibiting several members of the ICE family of proteases (Bertin et al, 1996; Bump et al, 1995; Xue and Horvitz, 1995). The ICE family of proteases are a highly conserved family of proteases which play a central role in the
cascade of events which leads to apoptotic cell death (Martin and Green, 1995). P35 is cleaved into two fragments by ICE proteases and forms a stable complex with the protease resulting in an inactive complex (Bump et al, 1995).

OpMNPV and LdMNPV lack homologs of p35. Instead, they contain a functional homolog of P35, IAP (inhibitor of apoptosis), which has no sequence similarities with P35 (Birnbaum et al, 1994; Crook et al, 1993). OpMNPV and CpMNPV iap genes have been shown to functionally replace p35 in transient DNA replication assays using AcMNPV replication genes (Lu and Miller, 1995b). The cellular target for IAP inhibition of apoptosis is unknown; however, it appears to be upstream of P35 action. Baculovirus IAP’s contain a conserved zinc-finger like motif and two BIR (baculovirus iap repeat) motifs which are highly conserved in several drosophilas and human proteins (Duckett et al, 1996; Hay et al, 1995; Listen et al, 1996; Rothe et al, 1995; Uren et al, 1996).

LEF-7

The AcMNPV lef-7 gene encodes a protein estimated to be 26.7 kDa (226 aa) and contains two single-stranded DNA binding motifs (Lu and Miller, 1995b). It was originally identified by its ability to stimulate expression of a chloramphenicol acetyltransferase (cat) reporter under control of either the polyhedrin or p39-capsid promoters (Morris et al, 1994). It was subsequently shown to stimulate DNA replication in a transient DNA replication assay (Lu and Miller, 1995b). An independent study by Kool et al (Kool et al, 1994a) failed to identify lef-7 as being involved in DNA replication. (Ahrens et al, 1997). Similar to ie-2, p35, and hcf-1 (see
below), _lef-7_ appears to have cell-line-specific effects, stimulating plasmid replication in _Sf-21_ but not in _TN-368_ cells, suggesting a possible role as a host range factor (Lu and Miller, 1995a).

**HCF-1**

Host cell-specific factor 1 (_hcf-1_) from AcMNPV encodes a cystine-rich 34 kDa protein which has been shown to affect cell-line-specific transient DNA replication (Lu and Miller, 1995a). HCF-1 is required for transient DNA replication in _T. ni_ cells but appears to have no affect in _Sf-21_ cells. In addition, deletion of _hcf-1_ from the AcMNPV genome compromises DNA replication in _T. ni_ cells (Lu and Miller, 1995a; Lu and Miller, 1996). A homolog of _hcf-1_ has not been identified in BmNPV, OpMNPV, or LdMNPV (Ayres _et al_, 1994; Genebank 133180; unpublished data).
CHAPTER 3

Characterization of the Interaction Between the Baculovirus Replication Factors, LEF-1 and LEF-2

JAY T. EVANS, DOUG J. LEISY AND GEORGE F. ROHRMANN

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Abstract

The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) has six genes required and three genes stimulatory for transient DNA replication. We demonstrate that the products of two of these genes, LEF-1 and LEF-2, interact in both yeast two-hybrid assays and glutathione S-transferase fusion affinity assays. Using yeast two-hybrid assays, we mapped the interaction domain of LEF-2 to amino acids between positions 20 to 60. Extensive deletion analyses of LEF-1 failed to reveal a delimited interaction domain, suggesting there may be essential secondary structural elements that are disrupted by these deletions. All clones expressing LEF-1 and LEF-2 that were unable to interact also failed to support significant levels of transient DNA replication, suggesting that this interaction is required for DNA replication. Sequence analysis of LEF-1 revealed a primase-like motif, WVVDAD. When this motif was mutated to WVVQAD, LEF-1 was unable to substitute for wt LEF-1 in a transient DNA replication assay.

Introduction

Baculoviruses are a diverse family of invertebrate viruses with large covalently closed, circular, double-stranded DNA genomes (Blissard and Rohrmann, 1990). The most well-characterized baculovirus, *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV), has a genome of 134 kb which encodes approximately 150 genes (Ayres *et al.*, 1994). Although baculoviruses have received widespread attention in recent years
due to their ability to hyperexpress heterologous genes under the control of very late promoter elements (Bishop, 1992; Miller, 1988), little is known about their replication cycle. Recently, methods for the analysis of defective genomes and the development of transient replication assays have been used to identify putative origins of DNA replication (Kool et al, 1993a; Kool et al, 1993b; Lee and Krell, 1994; Leisy and Rohrmann, 1993; Pearson et al, 1992).

Subsequently, a modification of the transient replication assay system was used to identify a subset of six essential and three stimulatory genes involved in baculovirus DNA replication (Kool et al, 1994a; Lu and Miller, 1995b). The six genes required for DNA replication encode the following proteins: DNA polymerase and helicase, whose functions are implied by DNA sequence homology (Lu and Carstens, 1991; Tomalski et al, 1988); LEF-3, a single-stranded DNA binding protein (Hang et al, 1995); IE-1, a transcriptional activator (Guarino and Summers, 1987) which also binds putative replication origins (Choi and Guarino, 1995; Guarino and Dong, 1991; Kovacs et al, 1992; Rodems and Friesen, 1995); and LEF-1 and LEF-2 to which functions have not yet been assigned. The three stimulatory gene products include two additional transcriptional activators, IE-2 (Carson et al, 1991a) and PE-38 (Krappa and Knebel-Mörsdorf, 1991) and P35, which blocks apoptosis (Clem et al, 1991; Herschberger et al, 1992) and therefore may not function directly in DNA replication.

In order to examine the functional interrelationships of baculovirus replication factors, we have characterized interactions occurring between the nine proteins involved in replication and investigated the functional significance of these interactions. In
In this report, we describe the interaction between AcMNPV LEF-1 and LEF-2 using yeast two-hybrid analyses and glutathione S-transferase (GST) fusion affinity experiments.

Materials and Methods

INSECT CELLS

_Spodoptera frugiperda_ (Sf-9) cells (Vaughn _et al_, 1977) were cultured in TNM-FH medium [Graces Medium (Gibco BRL), lactalbumin (3.3 mg/ml), TC Yeastolate (3.3 mg/ml) (Difco), penicillin G (50 U/ml), streptomycin (50 µg/ml) (BioWhittaker Inc.), and fungisone (375 ng/ml) (Gibco-BRL)], (Hink, 1970), supplemented with 10% fetal bovine serum (FBS). Cell culture maintenance was carried out according to published procedures (Summers and Smith, 1987).

BACTERIAL AND YEAST CELLS

All bacterial plasmids were maintained in _Escherichia coli_ DH5α. _Saccharomyces cerevisiae_ Y166 (MATα; _ura3-52; leu2-3,-112; his3Δ200; ade2-101; trp1-901; gal4Δ; gal80Δ; RNR::GAL-URA3; LYS2::GAL-HIS3; GAL->lacZ) was used for the yeast two-hybrid assays and was the gift of Steve Elledge (Baylor College of Medicine, Houston, TX).
PLASMID CONSTRUCTS

All baculovirus constructs were originally derived from the AcMNPV E2 strain (Smith and Summers, 1978).

Yeast two-hybrid clones

*lef-1* was originally cloned as a *NruI*-EcoRI fragment [map units (m.u.) 7.5 to 8.7] (Kool *et al*, 1994a; Kool and Vlak, 1993) into pUC19. *lef-2* was cloned as a *MluI* fragment (m.u. 1.9 to 2.6) into pUC19. *lef-1* and *lef-2* were both subcloned into pBluescript (pKS+) (Stratagene, Inc.) and *Ncol* sites were generated at the ATG start codon of each gene by site-directed mutagenesis (Sambrook *et al*, 1989) to form pKSLEF1(Ncol) and pKSLEF2(Ncol), respectively. The primers used are shown in Table 3.1. Mutagenesis changed the second amino acid (aa) of LEF-1 from leucine to valine and left the LEF-2 aa sequence unchanged. *Ncol*-BamHI inserts from these constructs were subcloned into the yeast vectors, pAS1 and pACTII (gifts from Steve Elledge) (Durfee *et al*, 1993), creating fusions with the GAL4 DNA binding and GAL4 activation domains, respectively.

C-terminal deletion constructs of LEF-1 (aa 1-241, aa 1-222, aa 1-215 and aa 1-197) were made by exonuclease III digestion (Henikoff, 1987). pASLEF1 aa 1-129 was created by digesting pASLEF1 with EcoRI and Dral (Dral cuts at codon 129) followed by subcloning the fragment into EcoRI-Smal digested pAS1. C-terminal deletion aa 1-175 and N-terminal deletion aa 175-266 were created by digesting pKSLEF1(Ncol) with Ndel, which cuts at codon 175 of *lef-1*, filling in with T4 DNA polymerase, and then
digesting with Ncol and BamHI. The resulting fragments were subcloned into pAS1 cut with Ncol-Smal (for N-terminal deletion clone pASLEF1 aa 1-175) or Smal-BamHI (for C-terminal deletion clone pASLEF1 aa 175-266). The remaining N-terminal deletions were constructed by introducing Ncol sites at codon positions 243, 223, 192, 134, 98, and 46 (Table 3.1) followed by subcloning of Ncol-BamHI fragments into pAS1. Site-directed mutagenesis was used to change the putative primase domain WVVDAD, located at aa 73-78, to WVVQAD creating pKSLEF1(Ncol)(WVVQAD) (Table 3.1). The mutant was subcloned into pAS1 with Ncol-BamHI. All mutations were confirmed by DNA sequence analysis.

LEF-2 deletions were constructed as follows: aa 1-96, aa 1-60, and aa 60-210 were removed from pKSLEF2(Ncol) with Ncol-SalI, Ncol-Hpal, and Hpal-BamHI respectively, and cloned into pAS1. The two remaining LEF-2 C-terminal deletion clones were created using site-directed mutagenesis to insert Ncol sites at the indicated positions (codon 20, and codon 40) followed by subcloning into pAS1 with Ncol-BamHI (Table 3.1).

GST fusions and transcription/translation (TnT) constructs

Glutathione S-transferase (GST) fusion proteins were constructed using pGEX CS-1 (a gift from Bill Dougherty) modified from pGEX (Pharmacia) such that it contained an Ncol site downstream of the GST gene that allowed direct cloning of our constructs. lef-1, lef-2, and lef-3 were subcloned by ligating Ncol-BamHI inserts into pGEX CS-1 cut with the same enzymes, resulting in pGEXLEF1, pGEXLEF2, and pGEXLEF3, respectively. The in vitro transcription and translation plasmid, pKSLEF1(TnT), was
constructed by digesting pKSLEF1 with Ncol-HindIII and re-ligating the vector, eliminating the viral promoter region and bringing the bacterial T3 promoter (from pKS\(^{-}\)) within 40 bp of the translational start site for \textit{lef}-1.

Replication assay clones

\textit{lef}-1, \textit{lef}-2, and \textit{lef}-3 were cloned into pKS\(^{-}\) as described above. DNA polymerase, \textit{p143} (helicase), \textit{ie}-1, \textit{p35}, \textit{pe}-38 and \textit{ie}-2 were subcloned from pUC19 into pKS\(^{-}\) (Kool \textit{et al}, 1994a). The \textit{lef}-1 construct with the mutated putative primase domain (WVVQAD) was cloned as described above. The reporter plasmid (pAcHdL) contained the putative replication origin, \textit{hr}2, in the HindIII-L fragment (m.u. 18.4-20.5) cloned into pKS\(^{-}\). pKSLEF1 aa 1-241, and aa 1-222 were cloned downstream of the \textit{lef}-1 promoter by digesting pASLEF1 (aa 1-241 or aa 1-222) with \textit{SalI}-\textit{BamHI} (\textit{SalI} is at codon 11 of \textit{lef}-1 and \textit{BamHI} is in the pAS1 multiple cloning site) and ligating the fragment into pKSLEF1 cut with the same enzymes. pKSLEF1 aa 134-266, aa 98-266 and aa 46-266 were created by cutting pKSLEF1(NcoI) (\textit{NcoI} site at ATG and codon 134, codon 98 or codon 46) with \textit{NcoI}, religating, and screening for clones that lacked the \textit{NcoI} fragment. pKSLEF2 aa 1-96 was created by cutting pKSLEF2 with \textit{SalI}-\textit{BamHI}, blunt ending with T4 DNA polymerase and re-ligating with T4 DNA ligase. pKSLEF2 aa 1-60 was constructed similarly with \textit{Hpal}-\textit{Xbal}. pKSLEF2 aa 40-210 and aa 60-210 were created by digesting pASLEF2 aa 20-210 or aa 40-210 with \textit{NcoI}-\textit{BamHI} and ligating the fragment behind the \textit{lef}-1 promoter using pKSLEF1(Nco-1) digested with \textit{NcoI}-\textit{BamHI}. 
Table 3.1: Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Clone*</th>
<th>sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pKSLEF-1(Ncol @ codon 1)</td>
<td>GTTCAAAGGGCACCATGGTAGTGCTGTGCAATTAT</td>
</tr>
<tr>
<td>2. pKSLEF-1(WVVDAD)</td>
<td>GAATGGGTCGTACAGGCGGATTACAAAA</td>
</tr>
<tr>
<td>3. pKSLEF-1(Ncol @ codon 46)</td>
<td>ATTTTGATACCTCCATGCGCAAAATTGTATA</td>
</tr>
<tr>
<td>4. pKSLEF-1(Ncol @ codon 98)</td>
<td>TTTACATTGTGCGCCATGGCTCAAATGTTCG</td>
</tr>
<tr>
<td>5. pKSLEF-1(Ncol @ codon 134)</td>
<td>AGTATTGGCCGCGCCATGGACAGGGATATT</td>
</tr>
<tr>
<td>6. pKSLEF-1(Ncol @ codon 192)</td>
<td>TTCTCGTTGTATAACCATGGAATTGCTAGACA</td>
</tr>
<tr>
<td>7. pKSLEF-1(Ncol @ codon 223)</td>
<td>ACTGGTGGATGTGCCATGGTGACAAGCA</td>
</tr>
<tr>
<td>8. pKSLEF-1(Ncol @ codon 243)</td>
<td>AGAAGCCGCGCAACCATGGCGAATGCA</td>
</tr>
<tr>
<td>9. pKSLEF-2(Ncol @ codon 1)</td>
<td>AGCGTCATGTTTAGCCATGGAAGCTACATATTTAATT</td>
</tr>
<tr>
<td>10. pKSLEF-2(Ncol @ codon 20)</td>
<td>ACCCTAACCTCCATCCGATTTCTACAAATG</td>
</tr>
<tr>
<td>11. pKSLEF-2(Ncol @ codon 40)</td>
<td>TCGAACAACAGCACCATGGCGACCAAA</td>
</tr>
<tr>
<td>12. pKSLEF-3(Ncol @ codon 1)</td>
<td>TCGAACAACAGCACCATGGCGACCAAA</td>
</tr>
</tbody>
</table>

*Clones 2-8 were created using clone 1. Clones 10 and 11 were created using clone 9.
SITE DIRECTED MUTAGENESIS

Site directed mutagenesis (Kunkel et al., 1987; Sambrook et al., 1989) employed the primers shown in Table 3.1 to generate the clones used in this study.

YEAST TWO-HYBRID ASSAYS

Transfections

All DNA used for transfections was prepared by alkaline lysis plasmid purification followed by phenol extraction (Sambrook et al., 1989). Yeast transformations were performed by spinning down 1 ml of overnight yeast culture (Y166 or single recombinant clones) and resuspending the cell pellet in 500 μl transformation buffer (40% PEG 3350, 100 mM LiCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 11 μg sonicated calf thymus DNA). Plasmid DNA (1-2 μg) was added to cells plus transfection buffer and left at 23°C for 6-8 h. Cells were then washed three times with H₂O and plated on appropriate media.

Yeast two-hybrid liquid assays

β-galactosidase assays (Himmelfarb et al., 1990) were performed as follows. Single colonies were inoculated into 2 ml of appropriate media and incubated overnight at 30°C with shaking. Overnight culture (1.7 ml) was centrifuged at 13,000 rpm for 1 min and the pellet was resuspended in 100 μl of Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄·H₂O, 0.01 M KCl, 0.001 M MgSO₄·7H₂O, 0.04 M β-mercaptoethanol). An equal volume of 0.5 mm glass beads (Biospec Products) were added followed by vortexing 3-4 times for 1
An additional 100 μl of Z buffer was added and the extract was spun in a microcentrifuge for 5 min at 10,000 X g. Supernatant (50 μl) was added to 950 μl of Z buffer and 200 μl of o-nitrophenol b-D-galactopyranoside (ONPG) [4 mg/ml ONPG, 0.1 M NaPO₄ (pH 7.0)], incubated at 30°C for 1 h, and 400 μl of 1 M NaCO₃ was added to stop the reaction. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Inc). The specific activity of the extract was calculated using the following formula:

\[
\frac{\text{OD}_{420} \times 1.6}{0.0045 \times \text{protein (mg/ml)} \times \text{extract volume (ml)} \times \text{time (min)}}
\]

\(\text{OD}_{420}\) is the optical density of the product, o-nitrophenol, at 420 nm. The factor 1.6 corrects for the reaction volume. Extract volume is the volume assayed in ml. The factor 0.0045 is the optical density of a 1 nmol/ml solution of o-nitrophenol. Specific activity is expressed as nmol/minute/mg protein.

The expression of fusion proteins was confirmed by Western blot analysis using an antibody to the HA epitope (Babco). The HA epitope is located between the GAL4 activation or DNA binding domains and the fusion protein in each construct.

GST FUSION AFFINITY ASSAYS

GST fusion constructs were grown in 100 ml of 2XYT (Sambrook et al, 1989) to OD595 0.5-0.6 and induced with 0.1 mM IPTG for 2 h at 37°C. Cells were pelleted, resuspended in 5 ml of PBS (120 mM NaCl, 2.7 mM KPO₄, pH 7.4), and sonicated three times for 1 min. Triton-X-100 was added to a final concentration of 1%
and the mixture was incubated at 23°C for 30 min. The bacterial cell extract was spun in a Sorvall centrifuge at 12,000 X g for 10 min at 4°C and the supernatant was frozen in 1 ml aliquots. The expression of GST fusion proteins was confirmed by SDS polyacrylamide gel electrophoresis (PAGE) analysis.

*In vitro* transcription-translation reactions were performed with a rabbit reticulocyte lysate coupled transcription and translation (TnT) system (Promega) according to manufacturer’s instructions. TnT reactions were labeled with \[^{35}\text{S}\]\text{methionine} (New England Nuclear). For interactions, 25 µl of glutathione Sepharose beads (Pharmacia Biotech) were incubated with 500 µl of bacterial extract, containing the appropriate fusion protein, for 30 min at 23°C. The beads were washed three times with 1 ml of PBS. TnT reaction products (5 µl) plus 50 µl of PBS was added to the beads, incubated for 1 h at 23°C and washed five times with 1 ml PBS. SDS PAGE sample buffer (45 µl) (Sambrook *et al*, 1989) was added to beads, boiled for 5 min, resolved by 12% SDS PAGE, and analyzed by autoradiography.

REPLICATION ASSAY

Transient replication assays were performed as described previously (Kool *et al*, 1994a, Kool *et al*, 1994c). Briefly, Sf-9 cells were plated into 6-well plates at a density of 1.25 X 10⁶ cells/well in TNM-FH plus 10% fetal bovine serum (FBS) and allowed to attach overnight at 30°C. DNA constructs (0.5 µg of each) to be transfected (including reporter construct pAcHdL) were mixed with 400 µl of transfection buffer (25 mM HEPES-KOH, 140 mM NaCl, 125
mM CaCl₂·2H₂O, pH 7.1). All DNAs were prepared using Qiagen midi columns (Qiagen, Inc.). The medium was replaced with 400 µl of Grace's medium +10% FBS, and the 400 µl of DNA plus transfection buffer was added. Transfection was allowed to proceed for 4 h at 27°C and the transfection buffer was then replaced with 1 ml TNM-FH (10% FBS). After 72 h at 27°C, total DNA was isolated from the cells (Summers and Smith, 1987) and resuspended in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA (10 µg) from each sample (concentration determined by OD260) were digested overnight with HindIII-DpnI, electrophoresed on a 0.8% agarose gel, transferred to membrane filters (GeneScreen Plus, New England Nuclear), and probed with ³²P-labeled pKS⁻.

Results

INTERACTION OF FULL-LENGTH LEF-1 AND LEF-2

A yeast two-hybrid system based on the modular nature of the GAL4 DNA binding and activation domains (Chien et al, 1991; Fields and Song, 1989; Guarente, 1993; Keegan et al, 1986; Ma and Ptashne, 1987) was used to examine interactions between the nine baculovirus gene products implicated in previous studies (Kool et al, 1994a; Lu and Miller, 1995b) as being involved in DNA replication. In this system, the GAL4 DNA binding and activation domains have been separated onto two different plasmids (pAS1 and pACTII, respectively) which have different auxotrophic markers for selection of single or double recombinants in yeast (Durfee et al, 1993). These plasmids can be used to make fusions with proteins of
interest and the GAL4 activation domain or GAL4 DNA binding
domain. When cotransfected, if the fusion partners with the DNA
binding domain and activation domain interact, the GAL4 activation
domain is brought into proximity with the lacZ and ura promoters,
causing transactivation of these genes. Interaction can be tested by
both β-galactosidase assays (colormetric colony lift assay or liquid
assay) and growth on plates lacking uracil.

All nine of the replication genes were cloned into pAS1 and
pACTII (Durfee et al, 1993). An initial screen of these clones
revealed several interactions, including one involving LEF-1 and LEF-
2 (Fig. 3.1, row 1 and Fig. 3.2, row 1). No β-galactosidase activity
was detected when double recombinants were made containing the
DNA binding domain or activation domain fusions and the control
reciprocal vectors encoding no fusion protein (Fig 3.1, row 1 and Fig.
3.2, row 1). This indicated that the full-length fusion proteins had
no intrinsic capacity to transcriptionally activate the
β-galactosidase promoter. In addition, no oligomerization was
detected between LEF-1 and itself or LEF-2 and itself when pACTII
and pAS1 fusions of LEF-1 or LEF-2 were cotransfected into yeast
(data not shown).
Figure 3.1. Characterization of the interaction domain of LEF-1. On the left are diagrams showing the portions of LEF-1 present in the mutants analyzed. Clone #1 is the full-length LEF-1 and Clone #2 is a full-length construct with a mutation in a putative primase domain. In the middle columns, the levels of \( \textit{lacZ} \) expression are shown from yeast that contained both the LEF-1 deletions diagrammed (in pAS1) and full-length \( \textit{lef-2} \) in pACTII or pACTII with no insert (negative control). \( \textit{lacZ} \) expression was calculated from four liquid assays from at least two independent transformations (± standard deviation). Specific activity is expressed as nmoles/minute/mg protein. Growth on plates lacking uracil is shown in the right columns. (+, -, ± indicate growth, no growth, or slow growth, respectively on plates lacking uracil after four days at 30°C). Protein expression was confirmed by Western blot analysis using a monoclonal antibody (Babco) to the HA epitope.

Figure 3.2. Characterization of the interaction domain of LEF-2. On the left are diagrams showing the portions of LEF-2 present in the mutants analyzed. Clone #1 contains the full-length \( \textit{lef-2} \) sequence. In the middle column are shown the levels of \( \textit{lacZ} \) expression from yeast that contained both the LEF-2 deletion clones diagrammed (in pAS1) and full-length \( \textit{lef-1} \) in pACTII or pACTII alone with no fusion protein (negative control). For details see Figure 3.1 legend.
<table>
<thead>
<tr>
<th>LEF-1 DNA Binding Domain Fusions</th>
<th>LacZ Expression</th>
<th>Growth on URA- plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pACTII-lef-2</td>
<td>pACTII-lef-2</td>
</tr>
<tr>
<td>1</td>
<td>aa 1 WVVDAD</td>
<td>aa 266</td>
</tr>
<tr>
<td>2</td>
<td>aa 1 WVVDAD</td>
<td>aa 266</td>
</tr>
<tr>
<td>3</td>
<td>aa 1</td>
<td>aa 241</td>
</tr>
<tr>
<td>4</td>
<td>aa 1</td>
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<td>5</td>
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<td>6</td>
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</tr>
<tr>
<td>15</td>
<td>aa 46</td>
<td>aa 266</td>
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Figure 3.1
<table>
<thead>
<tr>
<th>LEF-2 DNA Binding Domain Fusions</th>
<th>LacZ Expression</th>
<th>Growth on URA- plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pACTII-lef-1</td>
<td>pACTII</td>
</tr>
<tr>
<td>1  aa 1_________________________aa 210</td>
<td>20.3±14.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2  aa 1_______________________aa 96</td>
<td>152.7±106</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3  aa 1________aa 60</td>
<td>8.0±1.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4  aa 20________________________aa 210</td>
<td>7.2±2.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5  aa 40________________________aa 210</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6  aa 60________________________aa 210</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Figure 3.2
To confirm the interaction observed in the yeast two-hybrid system, we used GST fusion affinity experiments (Fig. 3.3). GST fusion constructs were made with *lef-*1, *lef-*2, and *lef-*3 (called pGEXLEF1, pGEXLEF2, and pGEXLEF3, respectively), expressed in *E. coli*, and whole cell extracts were prepared. The expression of a fusion protein of the expected size was confirmed by incubating the whole cell extract with glutathione Sepharose beads, washing with PBS to remove unbound proteins, and sizing the retained proteins by SDS PAGE. All three constructs showed protein fusion products of the correct size (data not shown). Each GST fusion protein was bound to glutathione Sepharose beads and incubated with $^{35}$S]methionine labeled LEF-1 generated by *in vitro* transcription and translation reactions. After extensive washing, the bound proteins were eluted, separated by SDS PAGE and analyzed by autoradiography (Fig. 3.3). The input, *in vitro* transcribed and translated, LEF-1 is shown in lane 1. A major band of 31 kDa is present which is consistent with initiation at the first ATG. A second minor band of smaller molecular weight is also evident and corresponds to a pair of in-frame downstream ATG's at codon positions 13 and 14. When the radiolabeled LEF-1 was incubated with GST fusions of LEF-1, LEF-2 and LEF-3 bound to glutathione Sepharose beads, the following was observed (Fig. 3.3). LEF-1 did not interact with GST-LEF-1 (lane 2), it did interact with GST-LEF-2 (lane 3) and did not interact with GST-LEF-3 (lane 4). The interaction of LEF-2 (lane 3) appeared to be with the larger (full-length) species of LEF-1, whereas the smaller protein that is likely to be missing 13-14 aa from the N-terminus did not interact with GST-LEF-2.
Figure 3.3. SDS PAGE (12%) and autoradiography of the interaction of glutathione S-transferase fusions with *in vitro* transcribed and translated LEF-1. Lane 1 shows the input *in vitro* transcribed and translated ([³⁵S]methionine labeled) LEF-1. The subsequent lanes show the radiolabeled LEF-1 which was retained by glutathione Sepharose beads previously incubated with glutathione S-transferase fusions of LEF-1 (lane 2), LEF-2 (lane 3), or LEF-3 (lane 4). Molecular weight markers (Gibco BRL), in kDa, are indicated to the left of the gel.
Figure 3.3
INTERACTION OF LEF-1 DELETION CLONES WITH FULL-LENGTH LEF-2

To map the amino acid sequence involved in LEF-1 interaction with LEF-2, a set of *lef-1* deletion clones were constructed and cloned into the yeast two-hybrid vector, pAS1. These constructs were then transfected into yeast containing pACTLEF2, and the double recombinants were tested for *lacZ* expression. The full-length LEF-1 (Fig. 3.1, row 1) and one C-terminal deletion clone (aa 1-241) (row 3) interacted with full-length LEF-2. One N-terminal deletion clone (aa 175-266) (row 12) gave some activity in the β-galactosidase liquid assay and grew slightly on plates lacking uracil. Similarly, two other N-terminal deletion clones (rows 9 and 10) showed some limited growth on plates lacking. Two of these clones also showed some activity when cotransfected with pACTII alone indicating that these constructs had intrinsic transactivation capabilities in this system (Fig. 3.1, rows 9 and 12). In a separate test, all of the LEF-1 deletion constructs were cloned into pACTII as GAL4 activation domain fusions and cotransfected with pASLEF2. The full-length LEF-1 and aa 1-241 resulted in β-galactosidase activity, whereas those that showed slight positive reactions when cloned as DNA binding domain fusions, (aa 243-266, aa 223-266, aa 175-266) gave no activity (data not shown). All deletion clones expressed fusion proteins of the expected sizes as demonstrated by SDS-PAGE followed by Western blot analysis using an HA monoclonal antibody (data not shown).
INTERACTION OF LEF-2 DELETION CLONES WITH FULL-LENGTH LEF-1

In order to map the interaction domain of LEF-2, a set of LEF-2 deletion clones were constructed in pAS1 (GAL4 DNA binding domain). Deletion clones aa 1-96, aa 1-60, and aa 20-210 gave β-galactosidase activity when cotransfected with pACTLEF1 (GAL4 activation domain fusion) (Fig. 3.2, rows 2-4). These double recombinants were also able to grow on plates lacking uracil (Fig. 3.2). Deletion clones aa 40-210 and aa 60-210 resulted in no β-galactosidase activity or growth on plates lacking uracil when cotransfected with pACTLEF1 (rows 5, 6). Double recombinants with pASLEF2 clones and pACTII (no insert) were negative indicating that the observed activities were due to interactions with pACTLEF1 and not the result of transactivation by these deletion clones (Fig. 3.2). These results indicate that a domain located between aa 20-60 of LEF-2 is essential for interaction with LEF-1.

REPLICATION ASSAYS WITH LEF-1 AND LEF-2 DELETION CLONES

LEF-1 and LEF-2 are required for transient replication of an origin containing plasmid (Kool et al, 1994a; Lu and Miller, 1995b). To determine if the interaction of LEF-1 and LEF-2 was a prerequisite for their ability to function in baculovirus DNA replication, selected clones were tested in a transient replication assay. Inserts from these clones were placed behind their native promoters (see Materials and Methods) and used in a transient DNA replication assay (Kool et al, 1994a; Kool et al, 1994c). As shown in Figure 3.4, both LEF-1 (lane 2) and LEF-2 (lane 8) are required for
DNA replication. The lef-1 clone encoding amino acids 1-241 which showed interaction with LEF-2 in the yeast two-hybrid system (Fig. 3.1, row 3) demonstrated the ability to support high levels of replication. The clone encoding amino acids 1-222 which demonstrated no interaction in the yeast two-hybrid assay allowed trace amounts of replication (Fig. 3.4, lane 4). The remaining LEF-1 deletions that showed no interaction in the yeast two-hybrid assay, were unable to support replication (Fig. 3.4, lanes 5-7).

Deletion clones of LEF-2 showed that, although clones aa 1-96 and aa 1-60 are capable of interacting with LEF-1 (Fig. 3.2, rows 2, 3), they are unable to support replication (Fig. 3.4, lanes 9, 10). However, the clone containing aa 20-210 that interacted with LEF-1 (Fig. 3.2, row 4) also supported replication (Fig. 3.4, lane 11) and clone aa 40-210 which did not interact with LEF-1 (Fig. 3.2, row 5) failed to support replication (Fig. 3.4, lane 12).

These data showed that in every case, clones which failed to interact also failed to show high levels of replication. The very weak replication signal observed with LEF-1 aa 1-222 may be due to a weak interaction between LEF-1 aa 1-222 and LEF-2 which is not detectable in the yeast two-hybrid system. The interaction domain of LEF-2 is located near the N-terminus as deletion of the first 40 amino acids (Fig. 3.2, row 5) eliminated interaction. Deletion of this region also abolished replication (Fig. 3.4, lane 12). However, clones that contained the interaction domain but had major deletions of the C-terminal region (Fig. 3.4, lanes 9, 10) did not support replication. These data indicate that regions of the LEF-2 ORF in addition to the oligomerization domain are required for DNA replication.
Figure 3.4. Characterization of the ability of LEF-1 and LEF-2 mutants to support transient DNA replication. Lane 1 shows a transfection with the nine previously characterized replication genes [lef-1, lef-2, lef-3 (SSB), DNA polymerase, p143 (Helicase), ie-1, ie-2, pe-38 and p35] (Kool et al, 1994a; Lu and Miller, 1995b). Lanes 2 and 8 show replication when LEF-1 or LEF-2, respectively, is not present. Lanes 3-7 and 9-12 show the replacement of LEF-1 or LEF-2 with the indicated deletion clones.
Figure 3.4

pAcHdL (5.2 Kb)
PRIMASE DOMAIN MUTATION IN LEF-1

Characterization of DNA replication in a variety of other viruses has led to the identification of a similar set of genes essential for DNA replication (Kornberg and Baker, 1992). One of the common components not yet identified as essential for baculovirus DNA replication is a primase. Therefore, we examined the LEF-1 and LEF-2 sequences to determine if they had features of this enzyme. A moderately conserved primase motif comprised of invariant DXD residues separated by a single hydrophobic amino acid and preceded by three hydrophobic amino acids has been described in primases from a number of herpesviridae, bacteriophage T7, yeast, and mice (Klinedinst and Challberg, 1994). Similar motifs (WVI/DAD) were also found in AcMNPV and the Orgyia pseudotsugata (OpMNPV) LEF-1 sequence. A conserved aspartate to glutamine (IILDLD to IILQLD) change of amino acid 628 in HSV-1 UL52 completely eliminated the primase activity associated with the HSV helicase-primase UL5-UL8-UL52 complex (Klinedinst and Challberg, 1994). We made a similar change in AcMNPV LEF-1 whereby amino acid 76 was converted from aspartate to glutamine (WVVDAD to WVVQAD). This mutation eliminated the ability of LEF-1 to function in the transient replication assay (Fig. 3.5, lanes 7, 8), without disrupting the interaction between LEF-1 and LEF-2 (Fig. 3.1, row 2). The loss of ability to support replication was not caused by the insertion of the Ncol site at the start of the ORF, as the parent Ncol-containing clone supported replication at the wild type level (Fig. 3.5, compare lanes 5, 6 to lanes 1, 2).
Figure 3.5. Characterization of the ability of a LEF-1 mutation in the putative primase domain to support transient DNA replication. The figure shows results from two independent replication assays. Lanes 1 and 2; transfection with all replication genes (lef-1, lef-2, lef-3 [SSB], DNA polymerase, p143 (Helicase), ie-1, ie-2, p38 and p35). Lanes 3 and 4; all replication genes minus LEF-1. Lanes 5 and 6; all replication genes minus LEF-1, plus LEF-1 with an NcoI site at the ATG start codon [pKSLEF1(NcoI)]. Lanes 7 and 8; all replication genes minus LEF-1, plus LEF-1 with a mutation in the putative primase domain.
Figure 3.5
Discussion

We have demonstrated using both yeast two-hybrid assays and GST-fusion affinity experiments that the products of the AcMNPV replication genes, \textit{lef-1} and \textit{lef-2} form oligomers. We were able to map the interaction domain of LEF-2 to the region between amino acids 20 and 60. However in LEF-1, the only deletion that was able to interact with LEF-2 was a C-terminal deletion of 25 amino acids. These data suggest that the interaction domain in LEF-1 contains components of both the N- and C-terminal regions or that the deletions we examined disrupted secondary structures required for this interaction. By testing the mutants in a DNA replication assay, we found that clones which failed to interact, also failed to support significant levels of DNA replication, suggesting that interaction of these two proteins is essential for DNA replication.

We identified a motif (WVVDAD) in the LEF-1 amino acid sequence that resembles a primase motif that is essential for DNA replication in other systems. When the first aspartate in this motif was changed to a glutamine, the resulting mutant was unable to substitute for LEF-1 in transient replication assays. This mutant was still able to interact with full-length LEF-2 in yeast two-hybrid assays. The interaction appeared weaker than seen with wild type LEF-1; however, it is difficult to correlate the results from yeast two-hybrid liquid assays with binding strength (Estojak \textit{et al}, 1995).

In other well characterized replication systems such as HSV-1, SV40, T4, T7, ColE1 and \textit{E.coli} there is a core set of proteins involved in replication. These include a DNA polymerase, helicase,
primase, SSB, origin binding protein, and one or more accessory or processivity factors (Kornberg and Baker, 1992). In AcMNPV, genes with homology to DNA polymerase, helicase and PCNA are present but only DNA polymerase and helicase homologs are essential for DNA replication (Kool et al, 1994a). In addition, LEF-3 has the properties of a single-stranded DNA binding protein (Hang et al, 1995) and IE-1 has been shown to bind to putative origins of DNA replication (Choi and Guarino, 1995; Guarino and Dong, 1991; Kovacs et al, 1992; Rodems and Friesen, 1995). The presence of a primase motif in LEF-1 suggests that LEF-1 may be a primase and LEF-2 a primase accessory factor. Primases are often associated with one or more other proteins. For example, HSV-1 primase (UL52) forms a complex with two other proteins, UL8 and UL52 (helicase), to form a helicase-primase complex (Klinedinst and Challberg, 1994). Future investigations undertaken to define the role of LEF-1 and LEF-2 in baculovirus DNA replication should consider these proteins together as it is likely that if studied in isolation, they will not form a functional complex. Further protein purification and biochemical characterization of LEF-1 is currently underway to determine the role that LEF-1 and LEF-2 play in baculovirus DNA replication.

Acknowledgments

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CHAPTER 4

The Baculovirus Single-Stranded DNA Binding Protein, LEF-3, Forms a Homotrimer in Solution

JAY T. EVANS AND GEORGE F. ROHRMANN

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Abstract

LEF-3 is one of six proteins from *Autographa californica* multinucleocapsid nuclear polyhedrosis virus required for transient DNA replication and has the properties of a single-stranded DNA binding protein (SSB). In this report we demonstrate that LEF-3 interacts with itself in both yeast two-hybrid assays and glutathione S-transferase fusion affinity assays. LEF-3 deletion clones which were unable to interact with full length LEF-3 also failed to support transient DNA replication, suggesting that this interaction is required for the proper function of LEF-3. LEF-3 was purified to homogeneity and characterized by analytical ultracentrifugation and native polyacrylamide gel electrophoresis. These studies revealed that LEF-3 was present as a 132 kDa complex, indicating that it's native conformation is that of a homotrimer. This result was confirmed by cross-linking with glutaraldehyde followed by matrix-assisted laser desorption/ionization mass spectrometry.

Introduction

*Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) has a genome of 134 kb that encodes approximately 150 genes (Ayres *et al*, 1994). Transient replication assays have been used to identify six essential and three stimulatory genes involved in baculovirus DNA replication (Kool *et al*, 1994a; Lu and Miller, 1995b). The six genes required for DNA replication encode the following proteins: DNA polymerase and
helicase, whose functions are implied by DNA sequence homology (Lu and Carstens, 1991; Tomalski et al, 1988); LEF-3, a single-stranded DNA binding protein (Hang et al, 1995); IE-1, a transcriptional activator (Guarino and Summers, 1987) which also binds to putative replication origins (Choi and Guarino, 1995; Guarino and Dong, 1991; Kovacs et al, 1992; Leisy et al, 1995; Rodems and Friesen, 1995); and two proteins, LEF-1 and LEF-2, neither of which have been assigned specific functions, but which have been shown to interact with one another (Evans et al, 1997). The three gene products which are stimulatory for DNA replication include two additional transcriptional activators, IE-2 (Carson et al, 1991a) and PE-38 (Krappa and Knebel-Mörsdorf, 1991), and P35, which blocks apoptosis (Clem et al, 1991; Herschberger et al, 1992) and therefore may not function directly in DNA replication.

Characterization of the interactions between the products of these nine genes using the yeast two-hybrid system revealed several interactions. We recently described the interaction of LEF-1 and LEF-2 and demonstrated that mutants which failed to interact also failed to support transient DNA replication (Evans et al, 1997). In this report, we describe the interaction between LEF-3 and itself and provide evidence indicating that LEF-3 forms a homotrimer in solution.

Materials and Methods

INSECT CELLS

Spodoptera frugiperda (Sf-9) cell (Vaughn et al, 1977) monolayers were cultured in TNM-FH medium (Hink, 1970),
supplemented with 10% fetal bovine serum (FBS). Suspension cultures were maintained in serum free SF900 II SFM medium (GIBCO-BRL) supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) (BioWhittaker Inc.), and fungisone (375 ng/ml) (Gibco-BRL). Cell culture maintenance was carried out according to published procedures (Summers and Smith, 1987).

BACTERIAL AND YEAST CELLS

All bacterial plasmids were maintained in *Escherichia coli* DH5α. *Saccharomyces cerevisiae* Y166 (MATa; ura3-52; leu2-3,-112; his3Δ200; ade2-101; trp1-901; gal4Δ; gal80Δ; RNR::GAL-URA3; LYS2::GAL-HIS3; GAL-lacZ) was used for the yeast two-hybrid assays.

PLASMID CONSTRUCTS

All baculovirus constructs were originally derived from the AcMNPV E2 strain (Smith and Summers, 1978). *lef-1* was originally cloned as an *NruI-EcoRI* fragment [map units (m.u.) 7.5 to 8.7] (Kool *et al.*, 1994a; Kool and Vlak, 1993) into pUC19. *lef-2* was cloned as a *MluI* fragment (m.u. 1.9 to 2.6), and *lef-3* was cloned as an *EcoRI-Apal* fragment (m.u. 42.8 to 44.5) into pUC19. *lef-1*, *lef-2*, and *lef-3* were subcloned into pBluescript (pKS-) (Stratagene, Inc.). *NcoI* sites were generated at the ATG start codon of each gene by site-directed mutagenesis (Sambrook *et al.*, 1989) to form pKSLEF1(NcoI), pKSLEF2(NcoI), and pKSLEF3(NcoI), respectively. The primers used for mutagenesis are shown in Table 4.1. Mutagenesis
changed the second amino acid of LEF-1 from leucine to valine and left LEF-2 and LEF-3 unchanged. pKSLEF1(Ncol), pKSLEF2(Ncol), and pKSLEF3(Ncol) functioned in transient replication assays as well as the parent clone, indicating that the sequence change had no effect on their ability to support transient DNA replication (data not shown).

Yeast two-hybrid clones

Full-length lef-1 and lef-3 were cloned into pAS1 and pACTII [gifts from Steve Elledge (Baylor College of Medicine, Houston TX)] (Durfee et al, 1993) by digesting pKSLEF1(Ncol) and pKSLEF3(Ncol) with Ncol-BamHl and ligating the fragment into pAS1 and pACTII cut with the same enzymes. C-terminal deletion constructs of LEF-3 (aa 1-370, and aa 1-314) were made by exonuclease III digestion (Henikoff, 1987), followed by subcloning into pAS1. LEF-3 N-terminal deletion constructs pASLEF3 (aa 311-385, aa 244-385, aa 165-385 and aa 77-385) were created by introducing Ncol sites into pKSLEF3(Ncol) at codon positions 244, 311, 165, and 77 by site-directed mutagenesis followed by subcloning of Ncol-BamHl fragments into pAS1. The primers used are shown in Table 4.1.
Table 4.1: Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Clone*</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pKSLEF-1 (Ncol @ codon 1)</td>
<td>GTTCAAAGGGCACCATGGTAGTGTCAGTTAT</td>
</tr>
<tr>
<td>2. pKSLEF-2 (Ncol @ codon 1)</td>
<td>AGAAGCCGCGAACCATGGCGAATGCA</td>
</tr>
<tr>
<td>3. pKSLEF-3 (Ncol @ codon 1)</td>
<td>TCGACAAACAGCAACCATGGCGACCCA</td>
</tr>
<tr>
<td>4. pKSLEF-3 (Ncol @ codon 1 and 244)</td>
<td>CGAGGCTAAAGAAAAACCATGGCATATCAATTTG</td>
</tr>
<tr>
<td>5. pKSLEF-3 (Ncol @ codon 1 and 311)</td>
<td>TCTACCTCTAGCCATGGCGAAATGGAA</td>
</tr>
<tr>
<td>6. pKSLEF-3 (Ncol @ codon 1 and 165)</td>
<td>CAACGACGATCCCATGGACGTGTTCAAG</td>
</tr>
<tr>
<td>7. pKSLEF-3 (Ncol @ codon 1 and 77)</td>
<td>GCAATCTTTCAAGCCATGGAGAAGGC</td>
</tr>
</tbody>
</table>

*Clones 4, 5, 6, and 7 were created using clone three.
GST fusions and transcription/translation (TnT) constructs

Glutathione S-transferase (GST) fusion proteins were constructed using pGEX CS-1 (a gift from Bill Dougherty) modified from pGEX (Pharmacia) such that it contained an Ncol site downstream of the glutathione S-transferase gene that allowed direct cloning of our constructs. C-terminal deletion constructs of LEF-3 (aa 1-370, and aa 1-314) were made as described above. lef-1, lef-2, and lef-3 (full length, aa 1-370, and aa 1-314) were subcloned into pGEX CS-1 using Ncol-BamHI (for pGEXLEF1, pGEXLEF2 and pGEXLEF3) or Ncol-Smal (for pGEXLEF3 aa 1-370 and pGEXLEF3 aa 1-314). The in vitro transcription and translation plasmid, pKSLEF3(TnT), was constructed by digesting pKSLEF3(Ncol) with Ncol-Apal, treating the digestion products with T4 DNA polymerase, and re-ligating the vector, thus eliminating the lef-3 promoter region and bringing the bacterial T3 promoter (from pKS-) within 40 bp of the translational start site for lef-3.

Seven-His fusion clone of lef-3

A seven-histidine N-terminal fusion construct of lef-3 was made using pTrc-7Hpro (a gift from Bill Dougherty) modified from pTrc 99A (Pharmacia Biotech) such that it contained an Ncol site downstream of seven histidine codons; this allowed direct cloning of our constructs using Ncol and BamHI.
MISCELLANEOUS PROCEDURES

Site-directed mutagenesis

Site directed mutagenesis was accomplished by following the procedure of T.A. Kunkel et al (Kunkel et al, 1987; Sambrook et al, 1989). The primers used to generate the clones in this study are shown in Table 4.1.

Yeast two-hybrid transfections and liquid assays, glutathione S-transferase fusion affinity assays, and transient DNA replication assays

These procedures were carried out as previously described (Evans et al, 1997).

LEF-3 PURIFICATION

Nuclear extracts

Nuclear extracts were prepared at 16 h post infection as previously reported by B. Glocke et al (Glocker et al, 1993).

Purification

All procedures were carried out at 4°C. Nuclear extract (15 ml from 1.5 liters of infected Sf-9 cells) was dialyzed against buffer E [20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 1.5 mM MgCl₂, 10 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] plus 100 mM NaCl and then clarified by centrifugation. The extract was passed over a 20 ml single-stranded DNA agarose (GIBCO-BRL) column (16 mm X 40 cm)
previously equilibrated in buffer E containing 100 mM NaCl (flow rate; 0.25 ml/min). The column was washed with 50 ml of equilibration buffer, and 100 ml of buffer E containing 500 mM NaCl. LEF-3 was eluted from the column with a 20 ml linear gradient from 500 mM to 1 M NaCl in buffer E. Fractions containing LEF-3 (determined by SDS-PAGE) were dialyzed against buffer E and concentrated in a Centriplus-10 concentrator (Amicon). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad).

The concentrated protein was diluted to an appropriate concentration with buffer E and 100 µl aliquots were applied to a prepacked Superose 12 HR 10/30 column (Pharmacia Biotech) preequilibrated in buffer E. The column was resolved at a flow rate of 0.05 ml/min and 100 µl fractions were collected. Peak fractions were pooled, concentrated in a centricon-10 microconcentrator (Amicon) and frozen at -80°C.

POLYACRYLAMIDE GEL ELECTROPHORESIS, WESTERN BLOTTING AND POLYCLONAL ANTISERUM PRODUCTION

SDS-PAGE (12%) was performed as described in Sambrook et al (Sambrook et al, 1989). Gels were either fixed and stained with 0.2% Coomassie brilliant blue in 50% methanol and 10% acetic acid (Bio-Rad) or used for Western blots as previously described (Quant et al, 1984; Quant-Russell et al, 1987). Western blots were probed with a 1:1000 dilution of rabbit polyclonal antiserum to LEF-3, washed three times with 10 ml of 0.5% blocking solution (Boehringer Mannheim), incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), and developed with a
chemiluminescence blotting substrate in accordance with the manufacturer's instructions (Boehringer Mannheim).

Rabbit polyclonal antiserum for LEF-3 was prepared by expressing an N-terminal seven His-tagged fusion construct of LEF-3 (see plasmid constructs) in *E. coli* DH5α followed by purification on Ni-nitrilotriacetic acid (NTA) resin (Qiagen) according to the manufacturer's instructions. Purified His tagged LEF-3 was subcutaneously injected into a New Zealand White rabbit with complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for subsequent injections (two to three weeks between injections). Rabbit antiserum was collected one week after the third injection and tested by Western blot analysis.

Native PAGE was performed on a Hoefer SE 600 gel electrophoresis unit using a 4-12% gradient polyacrylamide gel. Standards used in native PAGE were from Sigma (gel filtration molecular weight marker kit). Gels were fixed and stained with Coomassie brilliant blue.

**MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY (MALDI)**

MALDI was performed on a custom-built time-of-flight mass spectrometer (Cotter, 1992; Farmer and Caprioli, 1991; Hillenkamp *et al*, 1991). A voltage of 24 kV was used to accelerate ablated ions from the sample stage into the instrument's flight tube. Every mass spectrum was recorded as the sum of 30 consecutive spectra, each produced by a single pulse of 355 nM photons from a Nd:YAG laser (Spectra Physics). Matrix ions or ions from an added standard were used for mass calibration.
All samples were analyzed using a standard saturated solution of HCCA (\(\alpha\)-cyano-4-hydroxy cinnamic acid) (Aldrich) in a matrix of 33% acetonitrile and 10% formic acid. For each mass analysis, 0.5 \(\mu\)l of analyte solution (2.0 mg/ml LEF-3) was mixed in a 1:3 ratio with the matrix solution and 0.5 \(\mu\)l of this mixture was deposited on the sample stage. At the first sign of crystal formation (generally 10-15 seconds after deposition when viewed under a stereo microscope) the droplet was gently wiped off with a lab tissue, leaving a seed layer of crystallites on the surface of the sample stage. Another 0.5 \(\mu\)l portion of the analyte/matrix mixture was then deposited on top of the seed layer and allowed to dry. The crystals were washed with water and used for MALDI.

Cross-linked samples were prepared by adding glutaraldehyde directly to purified LEF-3 (2.0 mg/ml) to give final concentrations of 0.1-6% glutaraldehyde. Samples were left at 23°C for 1 h and immediately analyzed by MALDI.

**ANALYTICAL ULTRACENTRIFUGATION**

Purified LEF-3 was dialyzed overnight against buffer F (20 mM HEPES-KOH, 150 mM NaCl, 1 mM DTT, 0.5 mM PMSF) and adjusted to an OD 280 of 0.25 with the same buffer. Sedimentation equilibrium experiments were carried out with the Beckman Optima XL-A analytical ultracentrifuge. The rotor temperature was 20°C and the speed was fixed at 6,000 rpm. Equilibrium analysis was carried out after a 36 h run (equilibrium had been reached since no change in distribution occurred over a 6 h period). The analysis of the data used a nonlinear, least-squares fitting routine of the program, XL-A
version 2.41 (Malencik and Anderson, 1996). The solution density used for the equilibrium analysis was 1.00538 g/cm$^3$ and the partial specific volume for LEF-3 was calculated as 0.728 cm$^3$/g (Cohn and Edsall, 1943; Perkins, 1986).

Results

LEF-3 INTERACTION EXPERIMENTS

A yeast two-hybrid system based on the modular nature of the GAL4 DNA binding and activation domains (Chien et al, 1991; Estojak et al, 1995; Guarente, 1993; Keegan et al, 1986; Ma and Ptashne, 1987) was used to examine interactions between the nine baculovirus gene products previously identified as being involved in DNA replication (Kool et al, 1994a; Lu and Miller, 1995b). All nine of the replication genes were cloned into pAS1, containing the GAL4 DNA binding domain, and pACTII, containing the GAL4 activation domain (Durfee et al, 1993). An initial screening of these clones revealed several interactions, including an interaction involving LEF-3 with itself in both plate and liquid assays (Fig. 4.1, row 1). No β-galactosidase activity was detected when double recombinants were made containing the LEF-3 DNA binding domain or activation domain fusions and the control reciprocal vectors (data not shown). This indicated that the full length fusion proteins did not have any intrinsic capacity to transcriptionally activate the GAL4 promoter. The original pAS1 plasmid (without fusion protein) has some capability of transactivating GAL4 promoters when transfected alone. However, when an insert is introduced into the multiple
cloning site, this capability is eliminated. Fusions of LEF-1 were used as negative controls for these experiments because they did not interact with LEF-3 in the yeast two-hybrid system or GST fusion affinity assays (data not shown and Fig. 4.2, lane 2). Yeast transformed with LEF-3 (in pAS1 or pACTII) grew slowly on drop-out plates and in liquid cultures. Plates were incubated at 30°C for 5-6 days (3-4 days is normal) before plate assays and liquid cultures were grown for 24-36 hours (12-16 hours is normal) before liquid assays were performed. The slow growth of yeast transformed with LEF-3 did not affect the reproducibility of the plate or liquid assays.

Several N- and C-terminal deletion clones of LEF-3 were constructed [aa 1-370, aa 1-314, aa 311-385, aa 244-385, aa 165-385, aa 77-385 (Fig. 4.1, rows 2-7)], all of which failed to interact with full length LEF-3 in yeast two-hybrid assays. These data suggested that either the interaction domain in LEF-3 contains components of both the N- and C-terminal regions, or that the deletions we examined disrupt secondary structures required for interaction.

To confirm the LEF-3 interaction observed in the yeast two-hybrid system we examined the interaction between in vitro transcribed and translated LEF-3 and GST fusion proteins (Fig. 4.2). GST fusion constructs were made with lef-1, lef-2, lef-3, lef-3 aa 1-370, and lef-3 aa 1-314 (called pGEXLEF1, pGEXLEF2, pGEXLEF3, pGEXLEF3 aa 1-370, and pGEXLEF3 aa 1-314, respectively), expressed in E. coli, and whole cell extracts were prepared. The expression of a fusion protein of the expected size was confirmed by incubating the whole cell extract with glutathione Sepharose beads and washing with phosphate buffered saline to remove unbound
proteins. The retained proteins were eluted and sized by SDS-PAGE. All five constructs showed protein fusion products of the expected size (data not shown). Each GST fusion was bound to glutathione Sepharose beads and incubated with $[^{35}\text{S}]$methionine labeled LEF-3 generated by \textit{in vitro} transcription and translation reactions. After extensive washing, the bound proteins were eluted, separated by SDS-PAGE, and analyzed by autoradiography (Fig. 4.2). The input \textit{in vitro} transcribed and translated LEF-3 is shown in lane 1. A major band of 44 kDa is present which is consistent with initiation at the first ATG. Several minor bands of lower molecular weights are also present and may correspond to translation from several in-frame downstream ATGs. When the radiolabeled LEF-3 was incubated with the GST fusions bound to glutathione Sepharose beads, the following was observed (Fig. 4.2): LEF-3 did not interact with GST-LEF-1 (lane 2) or GST-LEF-2 (lane 3); LEF-3 did interact with GST-LEF-3 (lane 6), but did not interact with GST-LEF-3 aa 1-370 (lane 4) or GST-LEF-3 aa 1-314 (lane 5).
Figure 4.1. Diagram of full-length LEF-3 and deletion clones. On the left are diagrams showing the portions of LEF-3 present in the mutants analyzed. Clone #1 is the full-length LEF-3. In the middle column, the levels of lacZ expression from yeast that contained both the LEF-3 clones diagrammed (in pAS1) and the full-length LEF-3 cloned into pACTII. lacZ activity was determined for double recombinants which gave a blue color on plate assays; those which gave no blue color are indicated as -. All deletion clones (#2-7) were negative on plate assays. Full-length LEF-3 was positive on plate assays, and the number represents lacZ expression calculated from four liquid assays after two independent transformations (± standard deviation). Specific activity is expressed as nmoles/minute/mg protein. The right column shows the ability of each construct to function in transient replication assays.

Figure 4.2. Analysis of the interaction of GST fusions with in vitro transcribed and translated LEF-3. Lane 1 shows the input in vitro transcribed and translated ([35S]methionine labeled) LEF-3. The subsequent lanes show the labeled LEF-3 which was retained by glutathione Sepharose beads previously incubated with glutathione S-transferase fusions of LEF-1 (lane 2), LEF-2 (lane 3), LEF-3 aa 1-370 (lane 4), LEF-3 aa 1-314 (lane 5), or full-length LEF-3 (lane 6).
### Full length LEF-3 and deletions

<table>
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<tr>
<th>#</th>
<th>Deletion</th>
<th>Yeast two hybrid interaction with pACTLEF3</th>
<th>Transient DNA replication assay</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>aa1-aa85</td>
<td>5.2 ± 0.8</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>aa1-aa70</td>
<td>-</td>
<td>-</td>
</tr>
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<td>3</td>
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<tr>
<td>7</td>
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*Figure 4.1*
Figure 4.2
LEF-3 is required for transient replication of an origin-containing plasmid (Kool et al, 1994a; Lu and Miller, 1995b). To determine if the interaction between LEF-3 and itself was a prerequisite for its ability to function in baculovirus DNA replication, selected clones [aa 1-370, aa 1-314, aa 311-385, aa 244-385, aa 165-385, aa 77-385 (Fig. 4.1, rows 2-7)] were placed behind their native promoters and used in transient DNA replication assays (Kool et al, 1994a; Kool et al, 1994c). All of the deletion clones tested were negative for interaction with full length LEF-3 and failed to support DNA replication (data not shown and Fig. 4.1), suggesting either that LEF-3 oligomerization is required for the proper function of LEF-3 or that the deletions disrupted secondary structures required for both interaction and replication.

LEF-3 PURIFICATION AND OLIGOMERIC STRUCTURE DETERMINATION

Determination of the oligomeric structure of LEF-3 required isolation of the protein in a purified form. To accomplish this, 16 h post infection nuclear extracts were prepared (Glocker et al, 1993) and subjected to single-stranded DNA agarose chromatography using a modification of the method of Hang et al (Hang et al, 1995). LEF-3 eluted from the single-stranded DNA agarose column in 800 mM to 1 M NaCl resulting in greater than 80% purification of LEF-3 (Fig. 4.3a). A second protein of 140-150 kDa co-eluted with LEF-3 from the single-stranded DNA agarose column (Fig. 4.3a) suggesting that it interacted with LEF-3. Western blot analysis with anti-P143 (helicase) polyclonal antiserum identified this protein as P143.
(unpublished data). These two proteins were separated by Superose 12 gel filtration, resulting in a highly purified LEF-3 that migrated with an apparent molecular weight of 44 kDa on SDS-PAGE (Fig. 4.3b). While Superose 12 gel filtration was an adequate technique for purification, the LEF-3 complex that passed through the column eluted at variable apparent sizes ranging from 205 to 255 kDa depending upon the amount of LEF-3 loaded onto the column (25 μg to 200 μg). This variability made it difficult to assign an accurate molecular weight to the purified complex.

To confirm that the purified protein corresponded to the AcMNPV lef-3 gene, polyclonal antiserum was produced against an N-terminal histidine tagged LEF-3 which was expressed in E. coli and purified using Ni-NTA resin (Qiagen). The antiserum specifically recognized LEF-3 in 16 h post-infection nuclear extracts and the purified LEF-3 (Fig. 4.3c, lanes 1 and 3), but did not recognize any proteins from mock-infected 16 h nuclear extracts (lane 2). Purified LEF-3 was tested for SSB activity using gel shift assays with 32P labeled single-stranded DNA, resulting in observations similar to those previously reported (Hang et al, 1995) (data not shown).
Figure 4.3. Purification of LEF-3. (a) SDS-PAGE of peak fractions from single-stranded DNA agarose chromatography after concentration in a Centriplus-10 concentrator. The positions of the molecular weight markers (Gibco BRL), in (kDa), are shown on the left and the positions of helicase (P143) and LEF-3 are shown on the right. (b) SDS-PAGE of 100 µl fractions containing the LEF-3 protein peak from Superose 12 gel filtration. The numbers shown at the top represent fractions collected after 9 ml of buffer E passed through the column from the time of loading. (c) Western blot analysis of purified LEF-3 using anti-LEF-3 polyclonal antiserum; lane 1, 16 h post infection nuclear extracts; lane 2, 16 h mock-infected nuclear extracts; lane 3, purified LEF-3.
Figure 4.3
Figure 4.3 continued
Native PAGE (4-12% gradient PAGE) analysis of purified LEF-3 revealed a single band indicating that the majority of protein present was complexed as a single oligomeric structure and not as a mixture of different oligomeric forms (Fig. 4.4). The molecular weight of the complex was calculated to be about 131 kDa from standards run on the same gel. A standard curve was derived from 3 of 4 standards run on the same gel [β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa)] (Sigma). This data suggested that LEF-3 may form a homotrimer in solution.

To further investigate the oligomerization of LEF-3, sedimentation equilibrium of purified LEF-3 was carried out with a Beckman Optima XL-A analytical ultracentrifuge. The technique of sedimentation equilibrium depends upon the distribution of the solute in the sample well at equilibrium, where the distribution due to diffusion is balanced by the distribution due to sedimentation. This allows calculation of the average molecular weight of the solute (Van Holde, 1985). Figure 4.5 shows the distribution of LEF-3 in the sample well after 36 h at 6,000 rpm (20°C). Equilibrium analysis at 36 h showed that purified LEF-3 formed a complex of 132 kDa, indicating that LEF-3 is a homotrimer.
Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI) of Cross-Linked LEF-3

The oligomeric structure of LEF-3 was also examined by cross-linking the protein using 0.1 to 6% glutaraldehyde followed by MALDI analysis. MALDI can cause a slight oligomerization of proteins so it is necessary to compare cross-linked with non-cross-linked samples (relative to a standard in each run) when analyzing subunit structures (Farmer and Caprioli, 1991). Figure 4.6a shows non-cross-linked LEF-3 analyzed by MALDI. The 44.6 kDa single-charged monomer is the major species; however, the oligomerization caused by MALDI can be seen in the single- and double-charged species in the form of dimers, trimers, and tetramers. The double-charged dimer and tetramer peaks are masked by the single-charged monomer and dimer peaks.

MALDI analysis of LEF-3 cross-linked with 0.1% glutaraldehyde is shown in Figure 4.6b. This sample clearly shows an increase in the size of the single- and double-charged trimer peaks (141,300 Da and 70,700 Da, respectively) relative to the monomer peaks. Peak area analyses indicated that the cross-linked single- and double-charged trimer peaks were five and seven times larger, respectively, than the corresponding non-cross-linked peaks relative to the monomer peak in each MALDI run. The sizes of the dimer and tetramer peaks increased slightly in the cross-linked sample, but did not change significantly (less than one fold change) relative to the monomer peak in each of the MALDI runs. The increase in size of the trimer peaks in the cross-linked samples clearly indicates that LEF-3 is a homotrimer.
The increased molecular weights of the cross-linked complexes are due to the addition of the glutaraldehyde cross-linker. From the increase in molecular weights of the proteins crosslinked with 0.1% glutaraldehyde (100.1 molecular weight), about 25 molecules of glutaraldehyde were added per subunit of LEF-3. Although at higher concentrations of glutaraldehyde (0.2-6%), the size of the trimer peaks did not increase significantly, the molecular weight increased proportionally with the increasing concentration of glutaraldehyde (data not shown).
Figure 4.4. Native PAGE (4-12%) analysis of purified LEF-3. Purified LEF-3 was subject to native PAGE for 6 h at 150 V. The standards used were from Sigma (gel filtration molecular weight marker kit). The gel was stained with Coomassie brilliant blue (Bio-Rad). Molecular weight markers (Sigma), in kDa, are shown on the left.

Figure 4.5. Sedimentation equilibrium of purified LEF-3 after 36 h at 6,000 rpm (20°C). The absorbance was monitored at 280 nm and is plotted as a function of the radial position, where \( r^2 \) is the square of the distance from the axis of rotation (cm\(^2\)) and \( r_m^2 \) is the square of the distance of the meniscus from the axis (cm\(^2\)). Equilibrium analysis was carried out as described in Materials and Methods.

Figure 4.6. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) of purified LEF-3; (a) non-cross-linked LEF-3; (b) LEF-3 cross-linked with 0.1% glutaraldehyde.
Figure 4.4
Figure 4.5
Figure 4.6a
Uncrosslinked LEF-3

Figure 4.6b
Gluteraldehyde
Crosslinked LEF-3

Figure 4.6a, b
Discussion

In this report, we have demonstrated by both yeast two-hybrid analysis and GST fusion affinity experiments that LEF-3 interacts with itself. LEF-3 deletion constructs which disrupted this interaction also eliminated the ability of the protein to function in transient replication assays, suggesting that this interaction is essential for the proper function of LEF-3. It is also possible that these deletions disrupted secondary structures required for both interaction and replication.

We purified LEF-3 to apparent homogeneity and used native PAGE, analytical ultracentrifugation, and chemical cross-linking to determine the oligomeric structure of LEF-3. The purified LEF-3 monomer was determined to have an actual molecular weight of 44.6 kDa by MALDI, which is close to what would be predicted from the amino acid sequence (44.5 kDa). Native PAGE, analytical ultracentrifugation, and chemical cross-linking studies demonstrated that LEF-3 forms a homotrimer in solution.

SSB's have been reported to form monomers, homodimers, and homotetramers (Kornberg and Baker, 1992), and the human SSB RP-A forms a heterotrimer consisting of 70 kDa (Rpa1), 34 kDa (Rpa2) and 13 kDa (Rpa3) subunits (Kenny et al, 1989; Wold and Kelly, 1988).

During the purification of LEF-3 by single-stranded DNA agarose chromatography, a larger protein of approximately 140 to 150 kDa co-eluted with LEF-3 in 800 mM to 1 M NaCl (Fig. 4.3a). This protein followed the same elution profile as LEF-3, indicating that the two proteins may be interacting. Western blot analysis using anti-P143 (helicase) polyclonal antiserum indicated that the 140 to
150 kDa protein was P143 (unpublished data). P143 (helicase) has a calculated molecular weight of 143 kDa and has been identified as one of the six genes required for replication of an origin-containing plasmid (Kool et al, 1994a; Lu and Miller, 1995b). The interaction evidence presented here and previously reported for LEF-1 and LEF-2 (Evans et al, 1997) suggests that AcMNPV DNA replication involves a complex set of interactions between a viral DNA replication complex and both double- and single-stranded DNA. Further analysis of the interactions between baculovirus replication proteins should lead to an understanding of the roles each of these proteins plays in baculovirus DNA replication.

Acknowledgments

The assistance of Lilo Barofsky of the Mass Spectrometry Facility and Services Core Unit of the Environmental Health Science Center at Oregon State University and Drs. Dean Malencik and Sonia Anderson with analytical ultracentrifugation is gratefully acknowledged. We also thank Dr. Bill Dougherty and Dr. Steve Elledge for providing the plasmid vectors used in this study. We thank Dr. Douglas Leisy for suggestions and criticisms of this manuscript. This project was supported by grants from the NSF (MCB-9630769), ACS (SG-208), and NIEHS (ES 000210). The hospitality of Drs. M. Chamberlin, C. Kane and members of their laboratory to G.R. during his sabbatical leave is gratefully acknowledged. This is Technical Report No. 11,134 from the Oregon State University Agricultural Experiment Station.
CHAPTER 5

Characterization of the Interaction Between the Baculovirus Single-Stranded DNA Binding Protein (LEF-3) and Helicase (P143)

JAY T. EVANS, GREG S. ROSENBLATT, DOUG J. LEISY AND GEORGE F. ROHRMANN
Abstract

LEF-3 and P143 are two of six proteins encoded by the Autographa californica multinucleocapsid nuclear polyhedrosis virus genome which are required for DNA replication in transient replication assays. LEF-3 has the properties of a single-stranded DNA binding protein and P143 exhibits amino acid sequence homology to helicases. In this report we describe the interaction of LEF-3 and P143 by yeast two-hybrid analysis, immunoprecipitation experiments, and co-purification by single-stranded DNA agarose chromatography. Using the yeast two-hybrid system, we mapped the interaction domain of LEF-3 (385 amino acids) to amino acids between positions 1 and 165. Deletion analysis of P143 failed to reveal an interaction domain, suggesting that there were either multiple interaction domains or that the deletions disrupted secondary structures required for the interaction between LEF-3 and P143.

Introduction

The Baculoviridae are a large family of DNA viruses that are pathogenic predominantly for insects of the order Lepidoptera, but some strains infect species of Hymenoptera, Diptera, and Trichoptera. The most well-characterized baculovirus, Autographa californica multinucleocapsid nuclear polyhedrosis virus (AcMNPV), has a circular double-stranded DNA genome of 134 kb, which encodes approximately 150 genes (Ayres et al, 1994), and has eight homologous regions (hrs) which have been shown to act as early
gene transcriptional enhancers (Guarino and Summers, 1986b; Leisy et al, 1995; Rodems and Friesen, 1993; Rodems and Friesen, 1995) and may function as origins of DNA replication (Leisy et al, 1995; Leisy and Rohrmann, 1993; Pearson et al, 1992).

A transient DNA replication assay has been used to identify nine genes from AcMNPV which are involved in DNA replication (Kool et al, 1994a; Lu and Miller, 1995b). Six of these genes appear to be required for DNA replication and encode the following proteins: a DNA polymerase homolog (Tomalski et al, 1988); P143, a helicase homolog (Lu and Carstens, 1991); LEF-3, a homotrimeric single-stranded DNA binding protein (Evans and Rohrmann, 1997; Hang et al, 1995); IE-1, a transcriptional activator (Guarino and Summers, 1987) which also binds to hrs (Choi and Guarino, 1995; Guarino and Dong, 1991; Kovacs et al, 1992; Leisy et al, 1995; Rodems and Friesen, 1995); and two proteins, LEF-1 (a putative primase) and LEF-2, which have been shown to interact with each other (Evans et al, 1997). The three gene products which are stimulatory for DNA replication include two additional transcriptional activators, IE-2 (Carson et al, 1991a) and PE-38 (Krappa and Knebel-Mörsdorf, 1991), and P35, which blocks apoptosis (Clem et al, 1991; Herschberger et al, 1992) and therefore may influence DNA replication in an indirect manner. A DNA polymerase activity differing from the cellular enzyme has been described in baculovirus infected cells (Mikhailov et al, 1986a; Mikhailov et al, 1986b; Miller et al, 1981; Wang and Kelly, 1983; Wang et al, 1983) and is likely attributable to the DNA polymerase homolog. In addition, a ts mutation in p143 (helicase) was shown to block DNA replication (Gordon and Carstens, 1984; Lu and Carstens, 1991), and recently it
was demonstrated that P143 is capable of binding to double-stranded DNA (Laufs et al, 1997).

Initial attempts to characterize the interactions between the products of these nine genes using yeast two-hybrid assays revealed several interactions. We have previously described the interaction between LEF-1 and LEF-2 (Evans et al, 1997), and recently we provided evidence that LEF-3 (SSB) interacts with itself and forms a homotrimer in solution (Evans and Rohrmann, 1997). In this report we describe the interaction between AcMNPV LEF-3 (SSB) and P143 (helicase) by yeast two-hybrid analyses, immunoprecipitation experiments, and co-purification.

Materials and Methods

INSECT CELLS

*Spodoptera frugiperda* (Sf-9) cell (Vaughn et al, 1977) suspension cultures were maintained in serum free SF900 II SFM medium (GIBCO-BRL) supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) (BioWhittaker Inc.), and fungizone (375 ng/ml) (Gibco-BRL). Cell culture maintenance was carried out according to published procedures (Summers and Smith, 1987).

BACTERIAL AND YEAST CELLS

All bacterial plasmids were maintained in *Escherichia coli* (E. coli) DH5α. *Saccharomyces cerevisiae* Y166 (MATa; ura3-52; leu2-3,-112; his3Δ200; ade2-101; trp1-901; gal4Δ; gal80Δ;
RNR::GAL-URA3; LYS2::GAL-HIS3; GAL-\( \text{lacZ} \)) was used for the yeast two-hybrid assays.

PLASMID CONSTRUCTS

All baculovirus constructs were originally derived from the AcMNPV E2 strain (Smith and Summers, 1978). \( \text{lef}-3 \) was cloned as an \( \text{EcoRI}-\text{Apal} \) fragment (m.u. 42.8 to 44.5) into pUC19 and subcloned into pBluescript (pKS-) (Stratagene, Inc.) (Kool et al, 1994a; Kool and Vlak, 1993). \( p143 \) was cloned as an \( \text{EcoRI}-\text{Sspl} \) fragment (m.u. 59.9-63.5) into pBluescribe (pBS-) (Kool et al, 1994a; Kool and Vlak, 1993). \( \text{Ncol} \) sites were generated at the ATG start codon of each gene by site-directed mutagenesis (Sambrook et al, 1989) to form pKSLEF3(\( \text{Ncol} \)) and pKSP143(\( \text{Ncol} \)), respectively. Several cloning steps were required to mutagenize and transfer \( p143 \) from pBS- to pKS-. pBSP143 was digested with \( \text{HindIII} \) and the 5' leader sequence plus 459 base pairs (bp) of coding sequence from \( p143 \) was isolated and cloned into pKS- cut with \( \text{HindIII} \). This construct (pKSP143sH) containing the N-terminus of P143 was used for site-directed mutagenesis to produce an \( \text{Ncol} \) restriction site at the ATG start codon of \( p143 \) (pKSP143m). pKSP143m was then digested with \( \text{SphI} \) and re-ligated to remove the \( \text{PstI} \) site from the 5' polylinker. The resultant plasmid was digested with \( \text{EcoRI}-\text{XbaI} \), treated with T4 DNA polymerase and re-ligated, removing a \( \text{PstI} \) site from the 3' polylinker region of pKS- and producing pKSP143m(\( \Delta \text{SphI} \))(\( \Delta \text{EcoRI-XbaI} \)). pBSP143 was digested with \( \text{PstI}-\text{EcoRI} \) and a fragment containing the C-terminus of \( p143 \) was cloned into pKSP143m(\( \Delta \text{SphI} \))(\( \Delta \text{EcoRI-XbaI} \)) cut with the same enzymes. The primers used for mutagenesis of pKSLEF-3 and
pKSP143smH were TCGACAACAGCACCATGGCGACCAA and ACACGATAGCCACCATGGTTGACACACATTAC, respectively. The sequences of both pKSLEF-3(Ncol) and pKSP143(Ncol) were confirmed by DNA sequence analysis. Mutagenesis changed the second amino acid of P143 from isoleucine to valine and left the LEF-3 amino acid sequence unchanged. pKSLEF3(Ncol) and pKSP143(Ncol) functioned in transient replication assays as well as the parent clones, indicating that the sequence changes had no effect on their ability to support transient DNA replication (data not shown).

Yeast two-hybrid clones

Full-length lef-3 was cloned into pAS1 and pACTII [gifts from Steve Elledge (Baylor College of Medicine, Houston Texas) ] (Durfee et al, 1993) as previously described (Evans and Rohrmann, 1997). Full-length p143 was first cloned into pTrc-7Hpro (Evans and Rohrmann, 1997) by digesting pKSP143(Ncol) with EcoRI, treating with T4 DNA polymerase, digesting with Ncol, and ligating the p143 containing fragment into pTrc-7Hpro cut with Ncol and Smal creating pHTP143. pHTP143 was digested with Ncol-BamHI and the p143 containing fragment was cloned into pAS1 and pACTII digested with the same enzymes. N- and C-terminal deletion constructs of LEF-3 containing aa 1-370, aa 1-314, aa 311-385, aa 244-385, aa 165-385, and aa 77-385 were constructed as previously described (Evans and Rohrmann, 1997). The C-terminal deletion construct of LEF-3 containing aa 1-214 was constructed by digesting pKSLEF-3(Ncol) with Ncol-HincII followed by subcloning into pAS1 and pACTII digested with Ncol-Smal. The deletion
construct containing aa 49-385 was made by digesting pKSLEF-3 with ClaI, treating with T4 DNA polymerase, digesting with BamHI and the resulting fragment was cloned into pAS1 and pACTII cut with Smal-BamHI. The LEF-3 C-terminal deletion constructs aa 1-311, aa 1-244, aa 1-165, and aa 1-77 were constructed by introducing Ncol sites into pKSLEF3(Ncol) at codon positions 77, 165, 244, and 311 by site-directed mutagenesis, followed by subcloning of Ncol fragments into pAS1 and pACTII. The primers used for mutagenesis were described previously (Evans and Rohrmann, 1997).

C-terminal deletion constructs pASP143(aa 1-980) and pACTP143(aa 1-980) were made by digesting pKSP143(Ncol) with SphI, treating with T4 DNA polymerase, digesting with Ncol, and subcloning into pAS1 and pACTII cut with Ncol-Smal. pASP143(aa 1-738) was made by digesting pASP143 with SalI and re-ligating the vector. pACTP143(aa 1-738) was made by digesting pKSP143(Ncol) with SalI, treating with T4 DNA polymerase, digesting with Ncol and subcloning the fragment into pACTII cut with Ncol-Smal. N-terminal deletion constructs pASP143(aa 81-1221) and pACTP143(aa 81-1221) were constructed by digesting pKSP143 with Ncol-PstI, treating with T4 DNA polymerase, and re-ligating the vector, eliminating the Ncol-PstI fragment and reconstituting the Ncol site. This new construct, pKSP143(ΔPstI) was digested with Ncol-BamHI followed by subcloning into pAS1 and pACTII cut with the same enzymes.
MISCELLANEOUS PROCEDURES

Site-directed mutagenesis

Site directed mutagenesis was accomplished by following the procedure of T.A. Kunkel et al (Kunkel et al, 1987; Sambrook et al, 1989). The primers used to generate the clones in this study are shown above or were described previously (Evans and Rohrmann, 1997).

Yeast two-hybrid transfections and liquid assays

These procedures were carried out as previously described (Evans et al, 1997).

Immunoprecipitations

IgG from anti-LEF-3 polyclonal antiserum and pre-immunization antiserum were purified by protein A-Sepharose chromatography (Harlow and Lane, 1988) and coupled to CNBr-activated Sepharose 4B in accordance with the manufacturer's instructions (Pharmacia Biotech). Sepharose 4B-IgG was washed and re-suspended in NET gel buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% nonidet P40 (NP-40), 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide). Sepharose 4B-IgG (40 µl of 50% slurry) was incubated with 40 µl of 16 h post infection (pi) nuclear extracts (NE) or mock-infected NE for 1.5 h, and washed three times with NET gel buffer. The bound proteins were removed from the Sepharose beads by boiling in SDS polyacrylamide gel electrophoresis (PAGE) sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and subjected to 6% SDS PAGE.
followed by Western blot analysis with anti-P143 polyclonal antiserum.

LEF-3/P143 PURIFICATION

Nuclear extracts

Nuclear extracts were prepared at 16 h post infection as previously reported (Glocker et al, 1993).

LEF-3/P143 co-purification

Co-purification of LEF-3 and P143 was carried out as previously described (Evans and Rohrmann, 1997).

POLYCLONAL ANTISERUM PRODUCTION, POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

Preparation of rabbit polyclonal antiserum against LEF-3 was described previously (Evans and Rohrmann, 1997). To prepare antiserum against P143, a gene fusion expressing the C-terminal 777-1221 amino acids (SalI fragment) of P143 fused to maltose binding protein (pMal-cRI vector from New England Biolabs Inc.) was constructed. The fusion protein was expressed in E. coli DH5α and gel-purified by SDS PAGE. The purified fusion protein (150 μg) was subcutaneously injected into a New Zealand White rabbit with complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for subsequent injections (two to three weeks between injections). Rabbit antiserum was collected one week after
the third injection and tested by Western blot analysis using 16 h pi.

SDS-PAGE was performed as described in Sambrook et al. (Sambrook et al., 1989). Gels were either fixed and stained with Coomassie brilliant blue (Bio-Rad) or used for Western blots. For Western blots, proteins were electrophoretically transferred from polyacrylamide gels to a PVDF-plus transfer membrane (Micron Separations Inc.) for 4 h at 185 mA (Genie Electrophoretic Blotter, Idea Scientific). The membranes were then incubated in 1% blocking solution (Boehringer Mannheim) for 12 h at 4°C. Western blots were probed with a 1:2000 dilution of anti-P143 polyclonal antiserum, or a 1:7500 dilution of anti-LEF-3 polyclonal antiserum, washed three times with 0.5% blocking solution (Boehringer Mannheim), incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), and developed with a chemiluminescence blotting substrate in accordance with the manufacturer’s instructions (Boehringer Mannheim).

Results

INTERACTION OF FULL-LENGTH LEF-3 AND P143

The yeast two-hybrid system was used to identify interactions between the nine genes involved in AcMNPV DNA replication. In this system, DNA segments encoding the GAL4 DNA binding and activation domains have been separately cloned into two different plasmids (pAS1 and pACTII, respectively), each of which has different auxotrophic markers for selection of single or double recombinants.
in yeast (Chien et al., 1991; Fields and Song, 1989; Guarente, 1993; Keegan et al., 1986; Ma and Ptashne, 1987). Genes of interest are cloned into these vectors and expressed as fusion proteins containing the GAL4 activation or GAL4 DNA binding domains at their N-termini. When cotransfected into yeast, interaction of the fusion partners allows the GAL4 activation domain to be brought into proximity with the lacZ and ura promoters causing transactivation of these genes. Interacting partners can be detected by both β-galactosidase assays (colormetric colony lift assays and liquid assays) and by growth on plates lacking uracil.

Each of the nine genes involved in AcMNPV DNA replication were cloned into pAS1 and pACTII (Durfee et al., 1993) and double recombinants of all combinations were tested for interaction. Initial screening of these clones revealed several interactions, including an interaction between LEF-1 and LEF-2 (Evans et al., 1997) and LEF-3 and itself (Evans and Rohrmann, 1997). In addition, an interaction was detected between LEF-3 and P143 in both orientations of the yeast two-hybrid system (Table 5.1). In these experiments, pASLEF1 was used as a negative control because the vector, pAS1, alone (without an insert) results in low levels of transactivation (Evans and Rohrmann, 1997). pASP143 expressed with pACTLEF3 resulted in a transactivation of 2.4 ± 0.4 nmols/min/mg, whereas control crosses between pASP143 and pACTII and pACTLEF3 with pASLEF1 showed no transactivation (Table 5.1). When pACTP143 was expressed with the control pASLEF1, a transactivation of 4.3 ± 0.3 nmols/min/mg (Table 5.1) was observed. This level of activation was much less than that seen
Table 5.1: Yeast Two-Hybrid Liquid Assays

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<th>PACTLEF3</th>
<th>PACTP143</th>
<th>PACTII</th>
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<tr>
<td>pASLEF3</td>
<td>35.3 ± 9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 ± 3.4</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>pASP143</td>
<td>2.4 ± 0.4</td>
<td>No Growth</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>pASLEF1</td>
<td>&lt; 1</td>
<td>4.3 ± 0.3</td>
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<sup>a</sup> lacZ activity on ONPG in nmoles/minute/mg protein
when pACTP143 was crossed with pASLEF3 (17 ± 3.4 nmols/minute/mg) (Table 5.1) and was the result of transactivation by pACTP143 alone. We confirmed that pACTP143 alone exhibits transactivation (3-5 nmoles/minute/mg) by producing double recombinants of it with a variety of pAS1 constructs that by themselves show no transactivation. Because pACTP143 was capable of transactivation by itself (although at much lower levels than when in the presence of pASLEF3), this activation was taken as background and subtracted from the liquid assay results of all crosses involving pACTP143 in Figure 5.2. Yeast transformed with P143 (in pAS1 or pACTII) grew very slowly on plates and in liquid culture used for the selection of the appropriate auxotrophic markers. In addition, no recombinants were found when P143 was crossed with itself, suggesting that the P143 fusions have an inhibitory effect on yeast growth which becomes lethal in double recombinants in which P143 is present in both vectors (Table 5.1).

To confirm the interaction between LEF-3 and P143 observed in the yeast two-hybrid system, immunoprecipitation experiments were performed using anti-LEF-3 polyclonal antiserum (Fig. 5.1). IgG from pre-immune or LEF-3 polyclonal antiserum were purified by protein A-Sepharose chromatography and linked to CNBr-activated Sepharose 4B. The Sepharose beads (with bound IgG) were then incubated with 16 h pi NE or mock-infected NE, washed, and the retained proteins were visualized by Western blot analysis using anti-P143 polyclonal antiserum. When Sepharose-LEF-3 antiserum was used for immunoprecipitation with 16 h pi NE, Western blot analysis with anti-P143 polyclonal antiserum revealed a band of approximately 143 kDa (lane 1) which co-migrated to the same
location as P143 from 16 h pi NE (lane 4). Immunoprecipitation of mock-infected NE with Sepharose-LEF-3 antiserum, or 16 h pi NE with Sepharose-pre-immune serum gave no bands corresponding to the size of P143 (lanes 2 and 3). Reciprocal immunoprecipitations with anti-P143 polyclonal antiserum and subsequent analysis with LEF-3 polyclonal antiserum were not possible due to the cross reaction of the secondary goat anti-rabbit IgG horseradish peroxidase conjugate with the IgG heavy chain from the P143 polyclonal antibody used in the immunoprecipitations. The IgG heavy chain has a molecular weight of approximately 50 kDa and produces a strong signal that obscures the reaction of the LEF-3 antiserum with LEF-3.
Figure 5.1. Western blot analysis of immunoprecipitation of P143 by LEF-3 antiserum bound to CNBr-activated Sepharose 4B. Lane 1 shows the immunoprecipitation of P143 from 16 h pi NE with Sepharose-LEF-3 antiserum. Lanes 2 and 3 are negative controls with 16 h pi NE immunoprecipitated using Sepharose-pre-immune sera (lane 2) and mock-infected NE immunoprecipitated with Sepharose-LEF-3 antiserum (lane 3). Lane 4 shows the position of P143 from 16 h pi NE. The Western blot was developed with P143 antiserum as described in materials and methods. The position of molecular weight markers (Gibco BRL), in kDa, are shown on the left.
Figure 5.1

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<tr>
<td>16 h pi NE</td>
<td>+</td>
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<td>anti-LEF-3</td>
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<td>pre-immune sera</td>
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- P143 (143 kDa)
INTERACTION OF DELETION CLONES WITH FULL-LENGTH LEF-3 AND P143

To map the interaction domains of LEF-3 and P143, a set of N- and C-terminal deletions were constructed in the yeast two-hybrid vectors pAS1 and pACTII (Durfee et al, 1993). These deletions were transfected into yeast with either full length LEF-3 or P143 in the reciprocal vector. None of the N- or C-terminal deletions of P143 (aa 1-980, aa 1-738, aa 81-1221) interacted with full length LEF-3 in pAS1 or pACTII, indicating that either multiple interaction domains exist, or the deletions we constructed disrupted secondary structures required for the interaction between P143 and LEF-3 (data not shown). When N- and C-terminal deletions of LEF-3 fused to the GAL4 DNA binding domain (pAS1) were tested for interaction with full length P143 fused to the GAL4 activation domain (pACTII), the C-terminal deletions retaining amino acids 1-165 interacted with full length P143 (Fig. 5.2, rows 2-7), whereas a deletion containing only amino acids 1-77 failed to interact with full length P143 (Fig. 5.2, row 8). None of the LEF-3 N-terminal deletions tested were able to interact with full length P143 (Fig. 5.2, rows 9-13). The same results were obtained when the LEF-3 deletions were tested as GAL4 activation domain fusions (pACTII) interacted with P143 fused to the GAL4 DNA binding domain (pAS1) (data not shown).

There are a number of variables that can affect the interpretation of liquid assay results in the yeast two-hybrid system and prevent the direct conversion of the levels of lacZ activity into relative strengths of interaction. These include mRNA stability, protein stability, protein folding, the stability of the
plasmid construct, and toxicity of the construct to the host cell (Estojak et al, 1995). Therefore, the values shown in Table 5.1 and Figure 5.2 demonstrate the ability of fusion partners to interact, but do not necessarily represent relative interaction strengths between fusion partners.

LEF-3 AND P143 CO-PURIFICATION

The partial purification of LEF-3 from 16 h pi NE using a single-stranded DNA agarose resin has been previously reported (Evans and Rohrmann, 1997; Hang et al, 1995). LEF-3 elutes from the single-stranded DNA agarose resin in 700 mM to 1000 mM NaCl, resulting in greater than 80% purification of LEF-3. A second protein of approximately 140 kDa co-elutes with LEF-3 from the single-stranded DNA agarose column (Fig. 5.3a). Concentration of peak fractions from the single-stranded DNA agarose column using a Centriplus 10 concentrator (Amicon) clearly demonstrated that these two proteins are the major products eluting from the column in 700 mM to 1000 mM NaCl (Fig. 5.3b). Western blots of peak fractions from the single-stranded DNA agarose column, using either anti-LEF-3 or anti-P143 polyclonal antiserum, identified the 44 kDa band as LEF-3 (Fig. 5.4a) and the 140 kDa band as P143 (Fig. 5.4b). Since purified P143 does not bind to single-stranded DNA (Laufs et al, 1997), this data suggests that the presence of P143 in these fractions is due to its interaction with LEF-3.
Figure 5.2. Identification of LEF-3 domains that interact with full-length P143. The diagrams on the left show the portions of LEF-3 present in the mutants analyzed. Clone 1 is the full-length LEF-3 (aa 1-385). The two right-hand columns show the levels of lacZ expression from yeast that contained both the lef-3 deletion clones diagrammed (in pAS1) and the full-length p143 gene cloned into pACTII or pACTII with no insert (negative control). lacZ expression was calculated from three to four liquid assays from independent transformations (± standard deviations). Specific activity is expressed as nmoles/min/mg of protein. The transactivation from pACTP143 (4.3 nmoles/min/mg) (Table 5.1) has been subtracted from the pACTP143 crosses as background. Protein expression was confirmed by Western blot analysis using a monoclonal antibody (Babco) to the HA epitope.

Figure 5.3. Co-purification of LEF-3 and P143 by single-stranded DNA agarose chromatography. a) SDS PAGE (10%) of 20 μl aliquots from 1 ml fractions eluted from the single-stranded DNA agarose column in 700 mM to 1000 mM NaCl  b) SDS PAGE of peak fractions (fractions 3 to 10 from figure 5.3a) from the single-stranded DNA agarose column concentrated in a Centriplus-10 concentrator (Amicon). The position of molecular weight markers (Gibco BRL), in kDa, are shown on the left. The gels were stained with Coomassie brilliant blue (Bio-Rad)

Figure 5.4. Western blot analysis of LEF-3 and P143 co-purified by single-stranded DNA agarose chromatography. a) Anti-LEF-3 polyclonal antiserum. b) Anti-P143 polyclonal antiserum. Lanes 1, 16 h pi NE; lanes 2, 16 h mock-infected NE; lanes 3, peak fraction from single-stranded DNA agarose column (Fig. 5.3a, fraction 7). The position of molecular weight markers (Gibco BRL), in kDa, are shown on the left.
Figure 5.2

LEF-3 Deletions in pASI

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LacZ Expression

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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>29.7 ± 3.5</td>
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<td>4</td>
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<td>5</td>
<td>11.4 ± 1.2</td>
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<td>6</td>
<td>20.8 ± 2.4</td>
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Figure 5.3
Figure 5.4
Discussion

We have used the yeast two-hybrid system to identify several interactions between proteins required for baculovirus DNA replication (Evans et al, 1997; Evans and Rohrmann, 1997). In this report we describe an interaction between the baculovirus single-stranded DNA binding protein (LEF-3) and a putative baculovirus helicase (P143). This interaction was shown using the yeast two-hybrid system, immunoprecipitations, and co-purification from single-stranded DNA agarose. Previous studies by S. Laufs et al (1997) have shown that purified P143 binds non-specifically to double-stranded DNA, but has very little affinity for single-stranded DNA (Laufs et al, 1997). This supports our evidence that P143 is eluting from the single-stranded DNA agarose column as a complex with LEF-3, and not because of an ability to bind single-stranded DNA. Deletion analysis using the yeast two-hybrid system enabled us to map the interaction domain of LEF-3 to the region between amino acids 1 and 165. Deletions of P143 revealed no discrete interaction domain, suggesting that either there were multiple domains required for the interaction with LEF-3, or that the deletions we constructed disrupted secondary structures required for the interaction.

We have previously reported that LEF-3 interacts with itself to form a homotrimer in solution (Evans and Rohrmann, 1997). In addition, the complete set of LEF-3 deletions shown in this report were tested for interaction with full length LEF-3 (Evans and Rohrmann, 1997; data not shown). None of the deletions were able to interact with full length LEF-3. However as described above, the
deletions containing amino acids 1 to 165 of LEF-3 are able to interact with full-length P143. These data indicate that LEF-3 interaction with itself (trimerization) is not required for the interaction between LEF-3 and P143.

In several other viral and non-viral systems (both prokaryotic and eukaryotic), helicases and SSB's have been shown to have a functional interaction in which the activity of the helicase is either stimulated or inhibited by the SSB (Biswas et al, 1995; Hamatake et al, 1997; Le Gac et al, 1996; Matson, 1991; Matson and Kaiser-Rogers, 1990; Seo and Hurwitz, 1993; Seo et al, 1991; Tsaneva and West, 1994; Umezu and Nakayama, 1993). Direct interaction of helicases and SSB's have been reported for HSV-1, where the viral origin-binding protein (UL9), which also has helicase activity, has been shown to tightly associate with the HSV-1 SSB (ICP8) (Boehmer et al, 1994; Boehmer and Lehman, 1993) and in other systems where the heterotrimeric SSB, replication protein A (RP-A), interacts with the SV40 large tumor antigen (Dornreiter et al, 1992) and co-purifies with the calf thymus DNA helicase F (Georgaki et al, 1994). Indirect interactions between SSB's and helicases have also been observed in the bacteriophage T4, where the gene 59 protein interacts with both the T4 helicase (gene 41 protein) and SSB (gene 32 protein) (Barry and Alberts, 1994a; Barry and Alberts, 1994b; Morrical et al, 1994; Yonesaki, 1994). The interaction of AcMNPV LEF-3 and P143 is consistent with the interactions observed in these other systems and may be required for the proper function of these proteins in DNA replication. The function of this interaction may be to assist in loading the helicase onto a partially melted origin of replication, or in the binding of the SSB to single-stranded
DNA after helicase denaturation, followed by coating of the single-stranded DNA by cooperative binding of additional SSB. Future investigations into the functional significance of this interaction will be important for understanding the roles of these two proteins in baculovirus DNA replication.

Acknowledgments

The authors thank Joel Funk for his suggestions and criticisms of this manuscript, and the Central Services Laboratory at OSU for assistance with this project. We would also like to thank Dr. Bill Dougherty and Dr. Steve Elledge for providing plasmid vectors used in this study. This project was supported by grants from the NSF (MCB-9630769) and ACS (SG-208).
CHAPTER 6

Conclusions and Future Directions
Conclusions

The use of DI particles and infection-dependent replication assays to identify possible origins of DNA replication, was a pivotal point in the progress towards understanding the process of baculovirus DNA replication (Ahrens and Rohrmann, 1995b; Kool et al, 1995; Kool et al, 1993a; Lee and Krell, 1992; Lee and Krell, 1994; Pearson et al, 1992). The presence of hr and non-hr origins suggests there might be multiple initiation events by one or more initiation mechanisms. The use of multiple origins may be of little consequence if baculovirus DNA replication proceeds by a rolling circle mechanism. The identification of concatemeric intermediates, and the enzymes involved in the resolution of these structures, is needed to confirm that baculovirus DNA replication proceeds by a rolling circle mechanism.

The identification of putative replication origins led to the development of a transient DNA replication assay and ultimately to the identification of genes involved in baculovirus DNA replication. A core set of replication proteins was identified in AcMNPV and OpMNPV as being required in a transient DNA replication assay (Ahrens and Rohrmann, 1995a; Ahrens and Rohrmann, 1995b; Ahrens and Rohrmann, 1996; Ahrens et al, 1995a; Kool et al, 1994a; Lu and Miller, 1995b). This conserved set of proteins include a DNA polymerase, P143 (putative helicase), single-stranded DNA binding protein (LEF-3), LEF-1 (putative primase), LEF-2 (unknown function), and a transcriptional activator, IE-1, which also binds putative replication origins. In other well characterized replication systems such as E. coli, T4, T7, SV40, and HSV-1, a similar set of core replication proteins is highly conserved (Kornberg and Baker, 1992).
The identification of protein-protein interactions between these six proteins is a key step in understanding the roles of these proteins in DNA replication.

This thesis describes the identification and characterization of protein-protein interactions between the six proteins required in transient DNA replication assays. Chapter 1 describes the interaction of baculovirus replication factors LEF-1 and LEF-2. In addition, the interaction domain in LEF-2 was mapped to amino acids between positions 20 and 60. LEF-1 has limited amino acid homologies to a conserved domain found in other known primases. When this primase motif was mutated by a single amino acid change (WVVDAD to WVVQAD), the new clone was unable to substitute for LEF-1 in a transient DNA replication assay (Evans et al., 1997). A similar mutation in HSV-1 UL52 eliminated the primase activity associated with the UL5-UL8-UL52 helicase/primase complex (Klinedinst and Challberg, 1994). These data suggest that LEF-1 may function as a primase.

The baculovirus single-stranded DNA binding protein, LEF-3, was found to interact with itself and with P143, a putative helicase (Chapter 4, 5) (Evans and Rohrmann, 1997). LEF-3 was purified and the oligomeric structure was identified as a homotrimer. The ability of LEF-3 to form trimers is consistent with other published reports of SSB’s forming oligomers. SSB’s have been reported to form monomers, dimers, and tetramers, and the human replication protein A (RPA) forms a heterotrimer consisting of 70, 34 and 13 kDa subunits (RPA1, 2 and 3 respectively) (Kenny et al., 1989; Kornberg and Baker, 1992; Wold and Kelly, 1988).
Deletion analysis of LEF-3 identified a set of C-terminal deletions which were able to interact with full length P143, but unable to interact with LEF-3, indicating that the interaction between LEF-3 and P143 is not dependent upon the interaction of LEF-3 with itself (Chapter 5). The interaction of LEF-3 and P143 is consistent with the interactions seen in other replication systems. The human heterotrimeric SSB, RPA, interacts with the SV40 large tumor antigen (Dornreiter et al, 1992) and co-purifies with DNA helicase F from calf thymus extracts (Georgaki et al, 1994). In HSV-1, the viral origin binding protein (UL9), which also has helicase activity, interacts with the HSV-1 SSB (ICP8) (Boehmer et al, 1994; Boehmer and Lehman, 1993). In most systems where no direct interaction has been found between helicases and SSB's, a functional interaction between these two replication factors has been identified (Biswas et al, 1995; Boehmer et al, 1994; Boehmer and Lehman, 1993; Hamatake et al, 1997; Le Gac et al, 1996; Matson, 1991; Matson and Kaiser-Rogers, 1990; Seo and Hurwitz, 1993; Seo et al, 1991; Tsaneva and West, 1994; Umezu and Nakayama, 1993).

Baculovirus DNA Replication Model

The first step in most origin specific DNA replication systems is recognition and binding of an origin binding protein to one or more origins of DNA replication. In baculoviruses, IE-1 has been shown to bind hrs (putative origins) as a dimer (Rodems and Friesen, 1995) and is required in transient DNA replication assays (Kool et al, 1994a; Leisy et al, 1995; Lu and Miller, 1995b). The requirement of
IE-1 in these assays and the identification of hrs as possible origins indicates that IE-1 may function as an origin binding protein to initiate the assembly of a DNA replication complex. Binding of IE-1 to an hr may destabilize the DNA double helix allowing the SSB, LEF-3, to cooperatively bind to single-stranded DNA tracts and further destabilize the DNA. SSB interacts with the baculovirus helicase, P143, bringing it into contact with the newly forming replication fork and causing further melting of the DNA double helix. The destabilization of the origin by P143 and LEF-3 may allow access to the baculovirus primase, LEF-1, and primase accessory factor, LEF-2. The primase complex could then synthesize a primer at the origin, allowing the baculovirus DNA polymerase to initiates replication from the primer terminus. DNA replication most likely proceeds by a rolling circle mechanism resulting in the production of concatemeric viral genomes that are resolved into unit length viral particles, re-circularized and packaged by an unknown mechanism.

Future Directions

The identification of putative origins and viral genes which are involved in baculovirus DNA replication has opened multiple avenues of research. Future investigations into the identification of in vivo origins (within the context of the complete viral genome) and further characterization of IE-1 binding to hrs will provide many helpful incites into the initiation of baculovirus DNA replication.

Several of the proteins involved in baculovirus DNA replication (DNA polymerase, helicase, and primase) have been assigned functional identities by sequence homology to other known
replication factors. Purification of these proteins and characterization of their biochemical activities is required to confirm the function of these proteins in DNA replication and to identify other roles which may be associated with these factors (host range, possible roles in early and late transcription, repair functions, etc.). In addition, the purification of replication factors may lead to the development of an in vitro replication system which will allow a more detailed look at the process of DNA replication. Further characterization of protein-protein interactions between individual proteins and protein-protein and/or protein-origin complexes are needed to reconstruct the cascade of events which occur at a replication fork.

The identification of baculovirus genes which are involved in both replication and host range (p143, hcf-1, p35, iap, lef-7, etc) (Croizier et al, 1994; Kamita and Maeda, 1996; Maeda et al, 1993; Lu and Miller, 1995a), and the ability of host DNA polymerases to partially substitute for the viral DNA polymerase in transient DNA replication assays (Lu and Miller, 1995b), provides evidence that host proteins play an important role in baculovirus DNA replication. Complete understanding of this system will most likely involve the identification and characterization of multiple cellular factors from a variety of insect hosts.
BIBLIOGRAPHY


