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Characterization of a baculovirus nuclear localization signal domain in the late expression factor 3 protein

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ABSTRACT

The baculovirus Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) single-stranded DNA binding protein LEF-3 is a multi-functional protein that is required to transport the helicase protein P143 into the nucleus of infected cells where they function to replicate viral DNA. The N-terminal 56 amino acid region of LEF-3 is required for nuclear transport. In this report, we analyzed the effect of site-specific mutagenesis of LEF-3 on its intracellular distribution. Fluorescence microscopy of expression plasmid-transfected cells demonstrated that the residues 28 to 32 formed the core nuclear localization signal, but other adjacent positively-charged residues augmented these sequences. Comparison with other group I *Alphabaculoviruses* suggested that this core region functionally duplicated residues including 18 and 19. This was demonstrated by the loss of nuclear localization when the equivalent residues (18 to 20) in Choristoneura fumiferana nucleopolyhedrovirus (CfMNPV) LEF-3 were mutated. The AcMNPV LEF-3 nuclear localization domain was also shown to drive nuclear transport in mammalian cells indicating that the protein nuclear import systems in insect and mammalian cells are conserved. We also demonstrated by mutagenesis that two conserved cysteine residues located at 82 and 106 were not essential for nuclear localization or for interaction with P143. However, by using a modified construct of P143 that localized on its own to the nucleus, we demonstrated that a functional nuclear localization domain on LEF-3 was required for interaction between LEF-3 and P143.

Introduction

The family *Baculoviridae* consists of rod-shaped, enveloped viruses with double-stranded circular DNA genomes ranging from about 80 to 180 kbp. Baculoviruses are arthropod-specific and found in over 600 insect species mainly from the orders Lepidoptera, Hymenoptera and Diptera. This host specificity has made baculoviruses popular for use as protein expression vectors and as non-chemical biopesticides, and this in turn, has stimulated research into the molecular biology of the baculovirus replication cycle. Members of the species *Autographa californica multiple nucleopolyhedrovirus* exhibit a wide host range with the ability to infect many species of Lepidopteran and also to replicate to high titers in insect cell cultures. Most other species are of lower virulence and higher degrees of host specificity. It is likely that the determinants of host specificity are closely related to the regulation of the major steps in the baculovirus life cycle, yet the mechanism preventing the virus from crossing host species barriers remains unclear (Cory et al., 1997). Studies on the baculovirus replication cycle have shown that gene expression is a temporally regulated cascade divided into early, late and very late stages post-infection. The early stage takes place before viral DNA replication initiation so the early gene products are involved in establishing the proper context for viral DNA replication (Friesen, 1997; Lu and Miller, 1997). The late and very late stages are dependant on viral DNA replication and occur concurrently or after the initiation of this process, resulting in the synthesis of viral structural proteins as well as proteins involved in the occlusion process (Lu and Miller, 1997). This cascade of gene expression appears to be regulated at the level of transcription where one group of proteins has an essential role in regulating the subsequent expression of other genes (Passarelli and Guarino, 2007).

Many genes essential for transcription and DNA replication in Autographa californica nucleopolyhedrovirus (AcMNPV), the prototype baculovirus, have been identified and characterized. Transient expression assays in cell culture have identified genes, collectively known as late expression factors (*lefs*), which are required for recognition and stimulation of some early and late viral promoters (Todd et al., 1995). A subset of these *lefs* have been identified by genetic analysis and transient replication assays to be essential or stimulatory for AcMNPV DNA replication. The essential replication genes include *ie-1*, *p143*, *dnapol*, *lef-1*, *lef-2*, and *lef-3* (Kool et al., 1994; Lu and Carstens, 1991; Lu and Miller, 1995). Although most of the genes essential for replication are conserved between different baculovirus

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species (*ie-1* and *lef-3* are the exceptions), the gene requirements for optimal virus specific DNA replication can vary in different host cell lines (Lu and Miller, 1995). The switching of various *lefs* involved in DNA replication and late gene expression has been attempted between virus species to study replication in non- or semi-permissive cells and to alter the host range of the baculovirus (Thiem, 1997). One of the genes identified as a factor influencing baculovirus host range is the helicase, *p143* (Croizier et al., 1994; Maeda et al., 1993). P143 is capable of binding non-specifically to double-stranded (ds) and single-stranded (ss) DNA (Laufs et al., 1997; McDougal and Guarino, 2001) but must be transported into the nucleus by a single-stranded DNA binding protein, LEF-3, in order to function (Chen and Carstens, 2005).

Homologues of LEF-3 are found in all baculovirus species except the Hymenopteran and Dipteran viruses (Vanarsdall et al., 2007). While its specific role in replication and gene expression is not known, some properties of LEF-3 have been identified. LEF-3 functions as an homo-oligomer (Mikhailov et al., 2008), binds ssDNA (Hang et al., 1995; Mikhailov et al., 2006), transports P143 to the nucleus (Wu and Carstens, 1998) and interacts with a viral alkaline nuclease (Mikhailov et al., 2003). Protein–protein interactions between IE-1, LEF-3 and P143 as a complex on viral DNA have been demonstrated (Ito et al., 2004). These properties suggest that LEF-3 is an important component for the assembly and function of the viral replisome. These data also suggest that LEF-3 has several functional domains including those responsible for self-interaction, ssDNA binding, interaction with host karyopherins for nuclear localization, interaction with P143, and interaction with alkaline nuclease. The C-terminus is thought to be involved in protein–protein interactions (Evans et al., 1999). The domain shown to bind ssDNA is large and centrally located within the protein (Mikhailov et al., 2006). A region within the N-terminal 125 amino acids of LEF-3 is required for interaction with P143, while the N-terminal 56 amino acids are responsible for nuclear localization (Chen and Carstens, 2005). Studies on nucleo-cytoplasmic transport in eukaryotic organisms have identified several highly regulated mechanisms for intracellular trafficking pathways involving different karyopherins that interact with a nuclear localization signal (NLS) (Tran et al., 2007). Classical NLSs consist of short stretches of positively charged basic amino acids flanked by prolines (monopartite) or a short cluster of lysine/arginine residues separated from the monopartite region by a 10 to 12 amino acid linker (bipartite) (Cook et al., 2007). In this study, we describe mutagenic analysis of the previously identified LEF-3 NLS domain. We constructed a number of deletions and site-specific mutations within the LEF-3 N-terminal 56 amino acids and investigated their effect on intracellular localization to identify the critical amino acids for this function. We also characterized the role that the NLS and adjoining sequences have on P143 interaction and nuclear localization.

Results

LEF-3 NLS function is conserved between AcMNPV and CfMNPV

LEF-3 is translocated into the nucleus shortly after expression in the cytoplasm and is also the only gene product required to transport P143 into the nucleus (Wu and Carstens, 1998). The N-terminal 56 amino acid domain of AcMNPV LEF3 includes the region required for active transport of LEF-3 into the nucleus (Chen and Carstens, 2005). This region is much larger than many of the known classical NLSs including those identified in baculovirus. For example, AcMNPV polyhedrin contains a NLS consisting of only four amino acids, KRKK (Jarvis et al., 1991) while IE-1 requires a short sequence containing the essential amino acids KxxRR that functions as a dimer for its NLS function (Olson et al., 2002).

An alignment of the N-terminal 56 amino acids of AcMNPV LEF-3 with eleven other group I *Alphabaculovirus* sequences revealed a high degree of amino acid conservation, except for a stretch of nine amino acids (MAMASSPKK). This region was conserved in AcMNPV, BmNPV, RoMNPV, PlxyMNPV and MaviNPV but was not found in other group I baculovirus species including CfMNPV LEF-3 (Fig. 1). Because CfMNPV LEF-3 was transported to the nucleus on its own, and was also responsible for transporting CfMNPV P143 to the nucleus (Chen et al., 2004), we predicted that these nine amino acids were not essential for NLS function.

To confirm the role of the N terminal 48 amino acid region of CfMNPV LEF-3 (equivalent to amino acids 2–56 in AcMNPV LEF-3) in nuclear transport, this region was fused with the GFP reporter gene [pHSEHCfLEF3-(3–48)-GFP]. Following transfection of this plasmid into Sf21 cells, nuclear GFP fluorescence was observed (Fig. 2A, demonstrating that this region contained the CfMNPV LEF-3 NLS, and that the absence of the MAMASSPKK domain in this protein did not restrict nuclear transport. Deleting amino acids 20 to 28 from the AcMNPV LEF-3 (pHSEHAcLEF3-Δ20–28) did not disrupt its nuclear localization, demonstrating that amino acids 20 to 28 are also not essential for AcMNPV LEF-3 NLS function (Fig. 2A). However, this region includes two basic amino acids (KK) that could be viewed as a duplication of two conserved basic amino acids at positions 18 and 19 (KR) because these represent basic amino acids conserved in the other group I viruses. This led us to further investigate this region by constructing other deletions.

LEF-3 amino acids 14 to 32 contain core NLS sequence

We investigated whether regions within the AcMNPV LEF-3 N-terminal 56 amino acids containing conserved basic amino acids

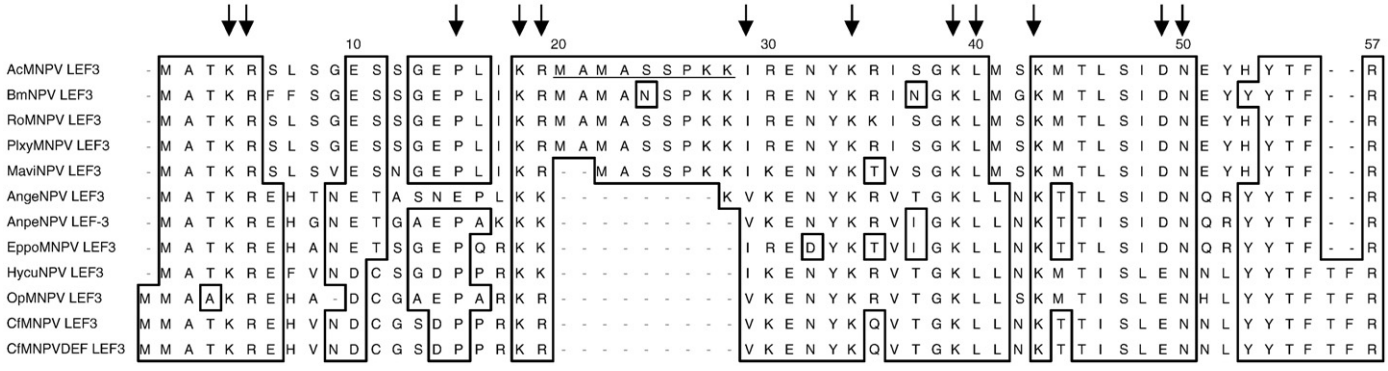
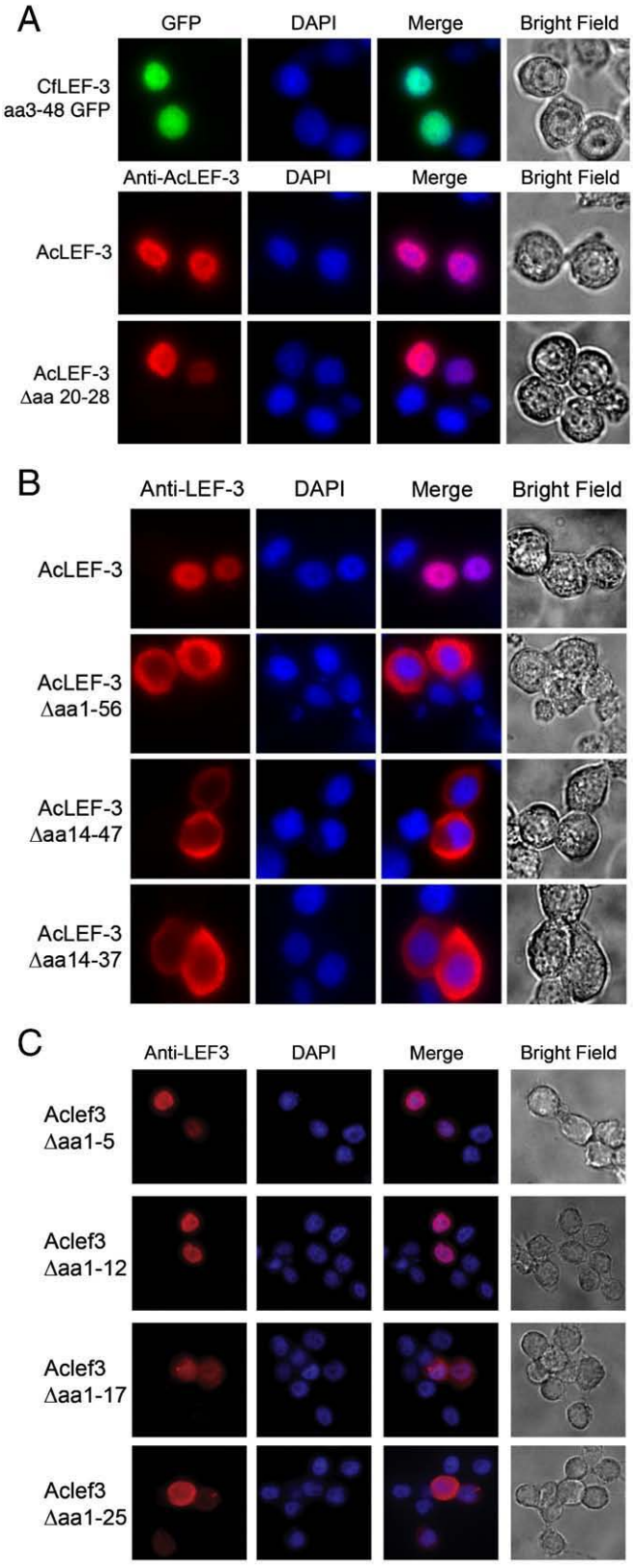


Fig. 1. Alignment of group 1 Alphabaculovirus LEF-3 amino acid sequences. The LEF-3 N-terminal amino acid region of 12 Alphabaculovirus isolates was analyzed by ClustalW alignment. Conserved amino acids are boxed, revealing the high degree of sequence conservation in this region of all of these viruses. The amino acid domain MAMASSPKK, which was targeted for deletion in AcMNPV, is underlined. Conserved AcMNPV amino acids, targeted for mutagenesis, are identified above with arrows. The numbers refer to the amino acids in AcMNPV LEF-3. Autographa californica MNPV (AcMNPV), Bombyx mori NPV (BmNPV), Rachiplusia ou MNPV (RoMNPV), Plutella xylostella MNPV (PlxyMNPV). Anticarsia gemmatalis NPV (AngeNPV), Antheraea pernyi NPV (AnpeNPV), Epiphyas postvittana MNPV (EppoMNPV), Hyphantria cunea NPV (HycuNPV), Orgyia pseudotsugata MNPV (OpMNPV), Choristoneura fumiferana MNPV (CfMNPV), Choristoneura fumiferana DEF NPV (CfMNPVDEF).

were essential for nuclear localization. Deleting amino acids 14 to 47 or 14 to 37 of AcMNPV LEF-3 produced proteins that remained cytoplasmic, results identical to those seen when the complete 1 to 56 amino acids region was deleted (Fig. 2B). Deleting amino acids 1 to 5 or 1 to 12 did not alter the NLS function but deleting 1 to 17 or 1 to 25 resulted in diffused LEF-3 throughout the cells (Fig. 2C), suggesting that important components of the NLS domain were located between amino acids 12 and 17. In order to determine the C-terminal region of the NLS domain, we constructed a number of fusion proteins between specific AcMNPV LEF-3 amino acids and GFP. As a control, a construct of AcMNPV LEF-3 amino acids 5 to 56 fused with GFP was included. Constructs of amino acids 5 to 47 and 5 to 37 fused with GFP were largely transported to the nucleus with weak cytoplasmic signal (Fig. 3). We confirmed these results using a construct of CfMNPV LEF3 in which amino acids 6 to 39 (similar to AcMNPV LEF-3 amino acids 5 to 47) were fused with GFP and tested for intracellular localization. The GFP signal was nuclear supporting the conclusion that this region carries a NLS sequence. A pattern of strong nuclear and weak cytoplasmic signal was also observed when constructs of AcMNPV LEF-3 amino acids 14 to 37 or of amino acids 14 to 19 plus 29 to 37 (removing the 20 to 28 nonessential region) were fused with GFP. When region 26 to 32 (PKKIREN) was fused to GFP, although there was some GFP accumulation in the nucleus, the fluorescence in the cytoplasm was stronger than seen with the other fusion constructs. These results suggest that the core LEF-3 NLS includes amino acids 26 to 32 but that adjoining sequences are important for efficient nuclear localization. These data also supported the suggestion that amino acids 27 and 28 (KK) could be substituted by the basic residues 18 and 19 (KR) implying that there is a duplication of the AcMNPV NLS core sequences.



Point mutations of AcMNPV LEF-3 identify regions essential for nuclear transport

Comparison of the group I *Alphabaculovirus* LEF-3 sequences revealed several conserved charged amino acids within the N-terminal 38 to 56 amino acids. To determine whether any of these were essential for NLS activity, a series of site-specific point mutations including K39A/L40S, K43I/T45A/E51G and D49I/N50I were introduced within a construct of LEF-3 amino acids 2 to 56 fused to GFP. All of these mutant proteins localized to the nucleus (data not shown), suggesting that these amino acids had little or no influence on NLS function.

We also targeted conserved basic amino acids and a conserved proline in the NLS region of the full length LEF-3 including K4E/R5G, P15A, and K18E/R19F. All of these mutant proteins localized to the nucleus (Fig. 4A), suggesting that the conserved amino acids chosen for mutagenesis were not essential for this function. However, when we made a second set of constructs with the same point mutations in AcMNPV LEF-3 where amino acids 20 to 28 had been deleted, all of these mutant proteins remained cytoplasmic. Therefore, neither the deletion of amino acids 20 to 28 or the point mutations alone destroyed the NLS function, but the combination of removing 20 to 28 and mutating conserved amino acids dramatically affected nuclear transport. When we mutated the CfMNPV LEF-3 amino acids in its predicted core NLS sequence equivalent to AcMNPV LEF-3 17 to 19 (R18A/K19A/R20A), the LEF-3 signal was cytoplasmic (Fig. 4B). This strongly supports our hypothesis that the structure formed by this region is the key factor required for nuclear transport, and that

Fig. 2. Intracellular localization of LEF-3 deletion mutants in Sf21 cells. Sf21 cells were transfected with LEF-3 expressing plasmids. At 24 h post transfection and heat shock, cells were fixed and either examined directly for GFP expression or stained with polyclonal anti-AcMNPV LEF-3 (1:250) primary antibody and detected with goat anti-rabbit IgG conjugated with Alexa Fluor 568 (1:1000) secondary antibody. Cells were also stained with DAPI to highlight the nucleus. Bright field images were included to reveal the whole cells. (A) pHSEHCfLEF3aa3–48-GFP, pHSEHAcLEF3 or pHSEHAcLEF3del (20–28) all expressed proteins which localized to the nucleus, demonstrating that the 20–28 domain, present in AcMNPV but not CfMNPV, was not essential for nuclear localization. (B) pHSEHAcLEF3del(1–56), pHSEHAcLEF3del(14–47), or pHSEHAcLEF3del (14–37) transfected cells revealed only cytoplasmic LEF-3, indicating that critical NLS sequences are located within the region of 14 to 37. (C) Sequential deletion of amino acids from the LEF-3 N-terminal (1 to 5, 1 to 12, 1 to 17 or 1 to 25) resulted in disruption of nuclear localization only when 17 or 25 amino acids were removed from the N-terminus of AcMNPV LEF-3.

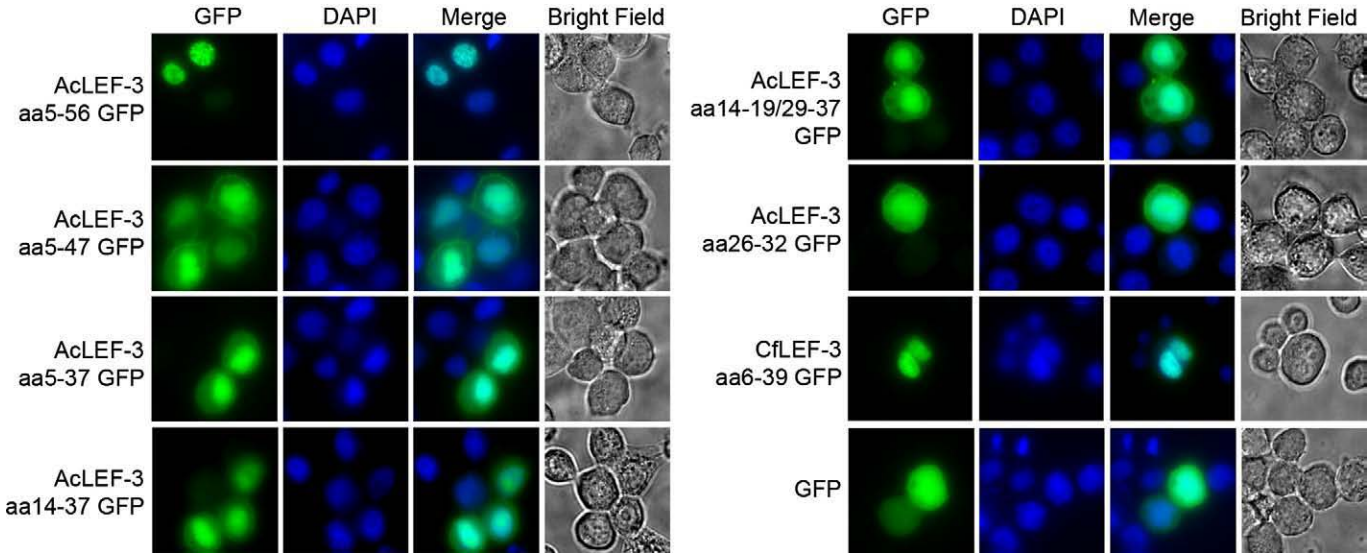


Fig. 3. Fusion of LEF-3 sequences to GFP reveals core NLS domain. Sf21 cells were transfected with a series of plasmids expressing amino acids within the LEF-3 amino acids 5 to 56 region fused with GFP, then examined for GFP expression (GFP). Cells were also stained to reveal the location of nuclei (DAPI). Combining GFP and DAPI staining revealed the intracellular location of GFP (Merge). Fusion of amino acids 5 to 56, 5 to 47, 5 to 37, 14 to 37 and 26 to 32 to GFP all produced proteins capable of nuclear localization. Deleting amino acids 20 to 28 from the 14 to 37 fusion protein did not disrupt nuclear localization. A plasmid expressing GFP on its own without an NLS (GFP) revealed GFP signal distributed throughout the cell. A plasmid expressing the CfMNPV LEF-3 (CfLEF-3aa-6–39 GFP) amino acids 6 to 39 fused with GFP resulted in nuclear fluorescence, demonstrating that this domain also carries sequences necessary for nuclear localization.

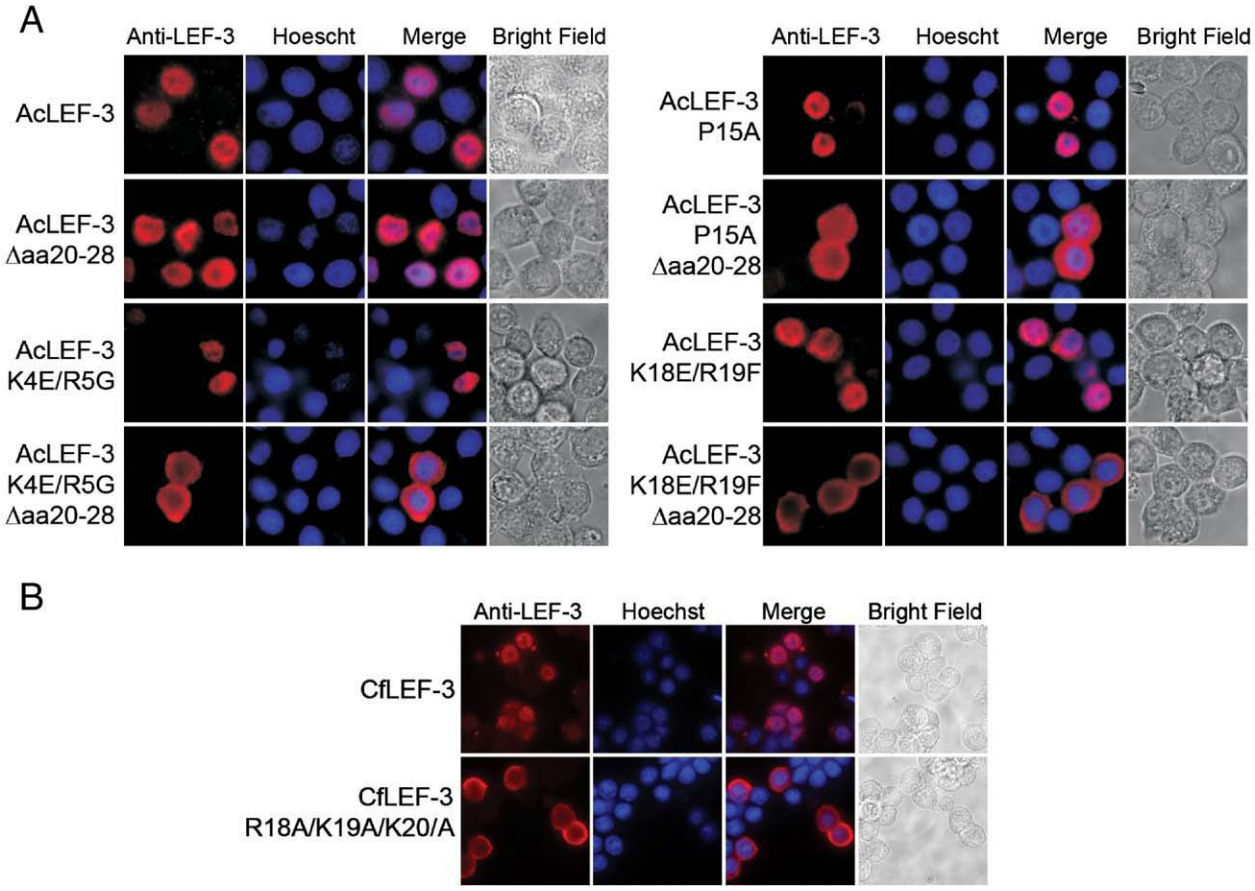


Fig. 4. Intracellular localization of AcMNPV LEF-3 point mutations combined with the deletion of amino acids 20 to 28. Sf21 cells were transfected with a series of plasmids expressing LEF-3 constructs including point mutations within conserved amino acids (K4R5, P15 or K18R19) or the same point mutations combined with deletion of amino acids 20 to 28. Cells were stained with rabbit anti-LEF-3 antibody (Anti-LEF-3) and nuclei were revealed by staining with Hoescht stain (Hoescht). Specific intracellular localization of LEF-3 was determined by combining the fluorescence and Hoescht staining patterns (Merge). Plasmids expressing AcMNPV LEF-3 or LEF-3 with amino acids 20 to 28 deleted were included. All point mutations did not affect nuclear localization but when combined with the additional deletion of amino acids 20 to 28, LEF-3 remained cytoplasmic (A). Mutation of the CfMNPV LEF-3 core basic amino acids R18K19K20 to alanines (CfLEF-3 R18A/K19A/K20A) resulted in cytoplasmic localization (B).

AcMNPV LEF-3 carries a duplication of basic amino acids in the two domains that can separately act as NLS sequences.

LEF-3 NLS functions in mammalian cells

Because it seemed likely that nuclear localization of LEF-3 required cellular factors, we investigated the ability of the LEF-3 NLS to function in non-insect cells. A plasmid expressing the AcMNPV LEF-3 amino acids 1 to 56 fused to GFP from a chicken beta-actin/rabbit beta-globin hybrid promoter (AG) and the cytomegalovirus-IE enhancer was constructed (pCAGGSAcLEF3NLS-GFP). When this plasmid was transfected into Vero cells (derived from African green monkey kidney cells) or HEK293 T cells (derived from human embryonic kidney cells), GFP localized exclusively to the nucleus (Fig. 5), indicating that the group I *Alphabaculovirus* LEF-3 NLS is probably universally recognized by conserved cellular systems in both invertebrate and vertebrate cells.

Interaction between LEF-3 and P143

The domain of AcMNPV LEF-3 responsible for localizing P143 to the nucleus includes amino acids 2 to 125 while amino acids 2 to 83 are not sufficient for this activity (Chen and Carstens, 2005). We previously noted two conserved cysteine residues in AcMNPV LEF-3, located at positions 82 and 106, and suggested they might play a role in LEF-3/P143 interaction. To test this hypothesis, these cysteines were targeted for site-directed mutagenesis. When plasmids expressing either mutant, AcMNPV LEF-3 C82A or C106L, were transfected into Sf21 cells, in both cases, LEF-3 was nuclear demonstrating that mutating these residues did not disrupt LEF-3 nuclear localization (Fig. 6A). When plasmids expressing either mutant were cotransfected with a plasmid expressing AcMNPV P143 fused with GFP, the GFP signal was nuclear indicating that these cysteine residues were also not essential for the P143 nuclear transport function of LEF-3 (Fig. 6B).

We have previously shown that when the LEF-3 NLS domain (amino acids 1 to 56) was fused in-frame with P143, P143 localized on its own to the nucleus (Chen and Carstens, 2005). We took advantage of this observation to examine the ability of P143 to interact with LEF-3, reasoning that if there was any interaction between LEF-3 and P143, then constructs of LEF-3 missing the NLS but expressing the domain required for P143 interaction would translocate to the nucleus along with P143. To test this, plasmids expressing AcMNPV LEF-3 with the NLS deleted (pHSEHAcLEF3Δ1–56) and P143 carrying the LEF-3 NLS [pHSEH-(1–56)P143-GFP] were co-transfected into Sf21 cells and localization of LEF-3 and P143 was investigated by fluorescence microscopy. As expected, P143 was found in the nucleus of co-transfected cells. However, LEF-3 remained cytoplasmic when amino acids 1 to 56, 14 to 47 or even 14 to 37 were deleted, even in the presence of nuclear P143 (Fig. 7A). Because the deletion mutants might have removed essential components for LEF-3/P143 interactions, we also tested various LEF-3 point mutant constructs unable to localize to the nucleus because of amino acid substitutions (K4E/R5G, P15A, and K18E/R19F) combined with the deletion of amino acids 20–28. However, all of the mutant LEF-3 proteins remained cytoplasmic (Fig. 7B). We also did co-transfections with the LEF-3 N-terminal deletion mutants (Δ1–5, Δ1–12, Δ1–17, and Δ1–25). However, only those constructs that localized to the nucleus on their own (Δ1–5 and Δ1–12) were found in the nucleus (data not shown). Therefore, it is apparent that a fully functional NLS within LEF-3, in addition to sequences extending to amino acid 125, are essential for the ability of LEF-3 to interact with and transport P143 to the nucleus.

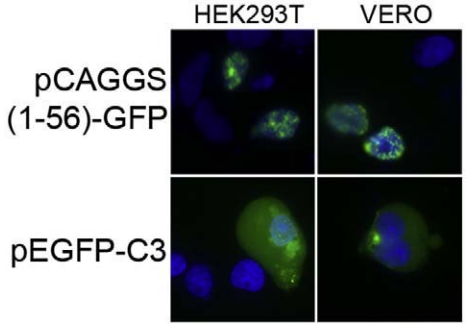


Fig. 5. AcMNPV LEF-3 NLS functions in mammalian cells. HEK293 and Vero cells, transfected with a plasmid expressing GFP fused with the AcMNPV LEF-3 NLS (amino acids 1 to 56) [pCAGGS(1–56)-GFP], were stained with Hoescht dye to reveal the location of nuclei. GFP fluorescence merged with Hoescht revealed nuclear fluorescence. Control cells, transfected with a GFP-expressing plasmid without the NLS (pEGFP-C3) revealed cytoplasmic fluorescence.

Discussion

Specific intracellular trafficking of proteins into the nucleus of eukaryotic cells is an important mechanism for gene regulation and for correct formation of protein complexes during DNA replication. Due to regulation of transport by the nuclear pore complex (NPC), many proteins destined to function in the nucleus are required to carry specific amino acid domains that form a nuclear localization signal (Cook et al., 2007). In the classical nuclear transport pathway, the NLS must interact with various cellular import and export carrier proteins in order to translocate through the NPC. The two main proteins in this import system are importin α, an adaptor, and importin β, a transport factor (Goldfarb et al., 2004; Lange et al., 2007). Importin α contains the NLS binding sites that interact with and recognize the cargo protein destined for the nucleus. It in turn binds to importin β, a protein responsible for shuttling the protein complex into the nucleus through direct interactions with the NPC. Structural analysis of importin α reveals two functional domains, a N-terminal importin-β binding (IBB) domain and a large, curved NLS binding domain consisting of ten armadillo (ARM) repeats, each composed of three α helices. These ARM motifs form a long configuration that allows for the binding of both monopartite and bipartite NLS motifs (Conti and Kuriyan, 2000). The different binding sites also make it possible for multiple regions of a NLS to interact with importin α, thus relaxing the requirements for target recognition. There is little known about the transport mechanisms and proteins used in insect cells but it is likely that proteins similar to importin α and β are involved.

Previous results from our lab have shown that the N-terminal 56 amino acids are required for nuclear transport of AcMNPV LEF-3. In this study, by using deletion mutagenesis, in addition to fusing specific amino acids with GFP, we have identified AcMNPV LEF-3 amino acids 26 to 32 (PKKIREN) as the likely core NLS. However, these amino acids appear in a nine amino acid stretch of AcMNPV as well as in the homologues of RoMNPV, BmNPV, PlxyMNPV and MaviNPV, that may functionally duplicate a domain including the proline at position 15 and two basic amino acids located at positions 18 and 19 of AcMNPV LEF-3. This duplication of function was demonstrated in three ways. First, when amino acids 20 to 28 were deleted from AcMNPV LEF-3, the protein still localized to the nucleus demonstrating that on their own, amino acids 28 and 29 were not required for nuclear localization. Secondly, CfMNPV LEF-3, which does not carry this region, localizes to the nucleus. Thirdly, when two basic amino acids at position 18 and 19 of the AcMNPV LEF-3, which are conserved in all group I *Alphabaculoviruses*, were mutated, this inhibited NLS activity only when amino acids 20 to 28 were also deleted. We confirmed this hypothesis by mutating the core basic amino acids in the CfMNPV LEF-3 NLS (amino acids 18 to 20, RKR). Changing these three amino acids to alanines completely abolished the NLS function, and resulted in cytoplasmic LEF-3. These results suggest that viruses expressing a protein similar to AcMNPV LEF-3 have evolved a novel NLS that may

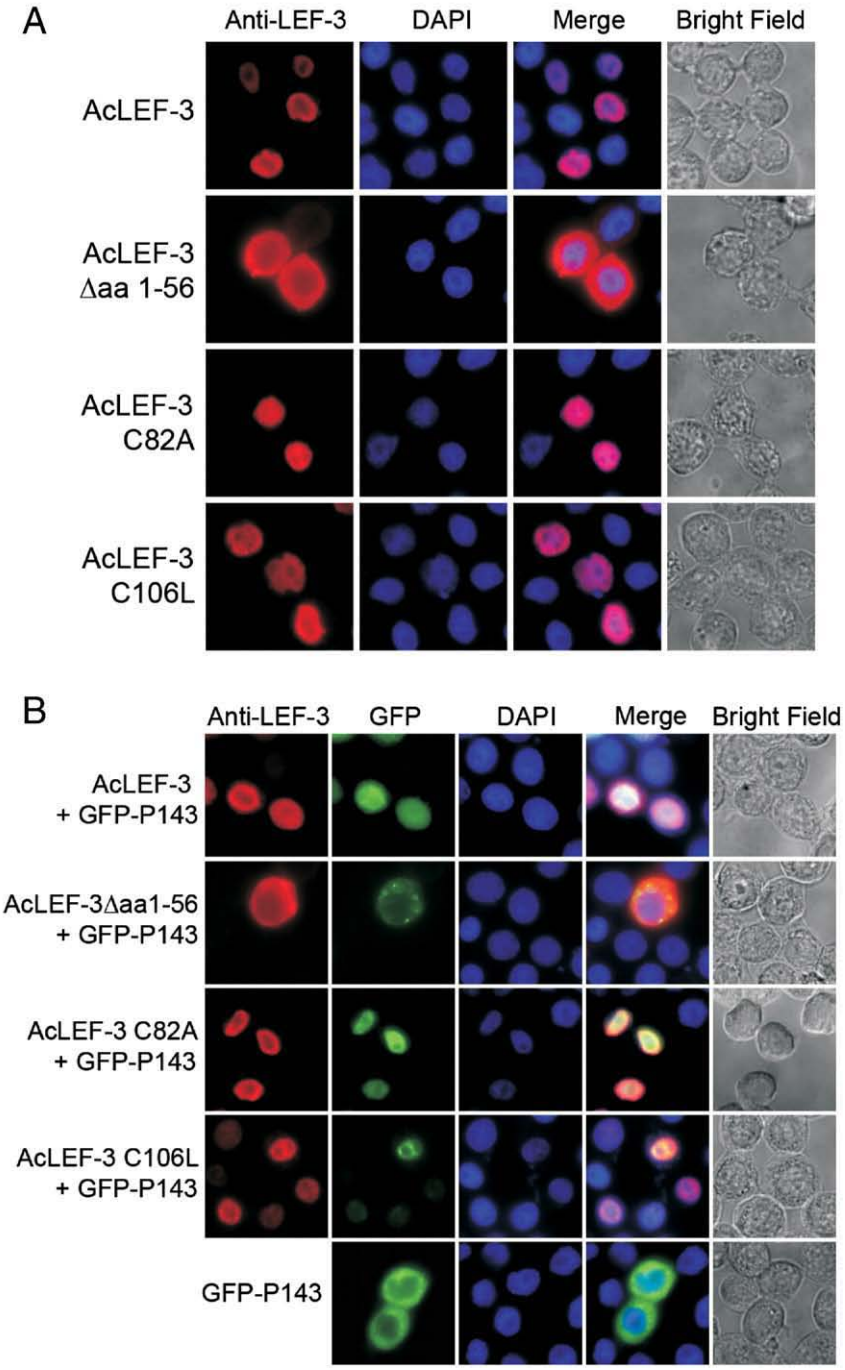


Fig. 6. Mutation of conserved cysteine residues does not alter LEF-3 nuclear localization or its interaction with P143. Plasmids expressing LEF-3 point mutations C82A or C106L were transfected into Sf21 cells, alone (A) or co-transfected with a plasmid expressing AcMNPV P143 fused in frame with GFP (B). LEF-3 was detected by immunofluorescence (Anti-LEF-3) and GFP-tagged P143 was detected by fluorescence (GFP). Nuclei were stained with DAPI. Intracellular localization of fluorescence was detected by merging the antibody and/or GFP fluorescence with DAPI (Merge). LEF-3 expressing either mutation (C82A or C106L) was capable of transporting P143 to the nucleus. Controls of intact AcMNPV LEF-3 (nuclear), LEF-3 minus amino acids 1 to 56 (cytoplasmic), and AcMNPV P143 fused with GFP on its own (cytoplasmic) were included.

better interact with the host importin machinery. Duplication of a core NLS sequence may lead to more efficient transport of LEF-3 to the nucleus and subsequent increased replication efficiency. This enhancement of transport efficiency may be in the rate of transport, strength of interaction with importins, with P143, or the flexibility of binding to multiple import factors. The N-terminal 19 amino acids of LEF-3 seems to adopt an open configuration compared with the rest of the protein (Mikhailov et al., 2006). We have repeated those experiments using limited trypsin digestion of purified AcMNPV LEF-3 and conducted a mass spectrometry analysis of the resulting peptides. Our data confirm that amino acids 1 to 19 are much more susceptible to trypsin digestion than the major domain of amino acids 20 to 326 (unpublished data), indicating that the NLS domain likely forms a separate open domain available for interaction with transport proteins. The similarities in the LEF-3 NLS regions of all group I *Alphabaculoviruses* suggest that a common host mechanism is used to recognize this domain and contribute to nuclear transport. Our data also demonstrated that this mechanism is conserved between insect and mammalian cells.

Crystallography studies on the mechanism of NLS binding to importin α show that there are two sites for NLS binding, with the large basic cluster of monopartite and bipartite NLS binding to the

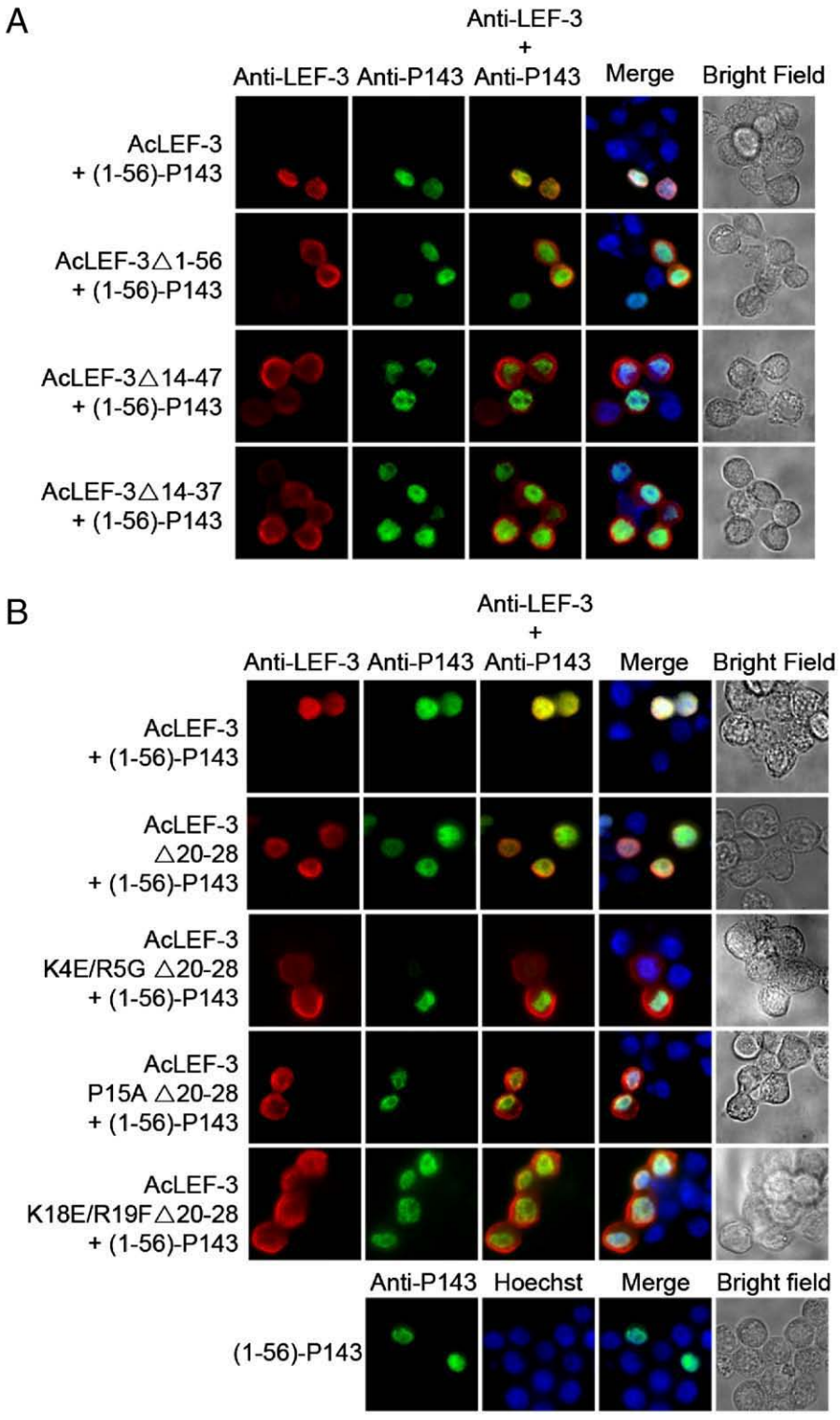


Fig. 7. NLS-deleted LEF-3 does not interact with P143. Sf21 cells were co-transfected with plasmids expressing LEF-3 carrying specific deletions or point mutations within the LEF-3 N-terminal region and a plasmid expressing P143 fused with LEF-3 amino acids 1 to 56. The first column shows cells stained for LEF-3 (Anti-LEF-3), the second column shows cells stained for P143 (Anti-P143), the third column shows a merge between columns one and two (Anti-LEF-3 + Anti-P143), column four shows a merge of column three plus Hoechst staining (Merge) and column five shows direct bright field cells (Bright Field). Deletion of amino acids 1–56, 14–47 or 14–37 resulted in cytoplasmic LEF-3, while P143 was nuclear (A). Mutation of amino acids K4R5, P15 or K18R19 from LEF-3 together with deletion of LEF-3 amino acids 20 to 28 resulted in cytoplasmic LEF-3, while P143 was nuclear (B). The bottom row shows the nuclear signal of cells transfected with a plasmid expressing P143 fused with LEF-3 amino acids 1 to 56, stained for P143 (Anti-P143), nuclei (Hoechst), a merge of P143 and Hoescht staining (Merge), and a normal bright field view (Bright field).

major site and the smaller basic cluster of the bipartite binding to the minor site (Fontes et al., 2003). Hence, a bipartite NLS can compensate for less efficient binding of its basic cluster at either site by binding to both. AcMNPV LEF-3 contains several pairs of conserved basic amino acids that could function as bipartite sites including K4R5, K18R19, K27K28, and K34R35 in the N-terminal 56 amino acid region. Mutagenesis of one pair at a time did not destroy the NLS suggesting that other basic residues compensated and acted as importin binding

sites. However, mutating K4R5 or K18R19 in addition to removing amino acids 20–28 removed more than one cluster of basic residues, apparently leaving LEF-3 with insufficient contacts for efficient binding to the importin protein. CfMNPV LEF-3 is missing the region including AcMNPV K27K28, but does have the R18K19R20 basic region. These basic amino acids likely form the core NLS for CfMNPV LEF-3 since mutating them completely inactivated nuclear transport. We therefore suggest that the consensus sequence of the group I *Alphabaculovirus* LEF-3 NLS is a proline followed within two amino acids by K K/R I/V K/R E N.

LEF-3 is also required to transport P143 to the nucleus (Wu and Carstens, 1998). We previously demonstrated that amino acids 1 to 125 of LEF-3 were sufficient for this function and noted that there were two conserved cysteine residues in this region of all group 1 *Alphabaculovirus* LEF-3 (Chen and Carstens, 2005). Disulfide bonds, formed by two cysteine residues can play an important role in protein conformation and may be involved in protein folding pathways. We tested whether these conserved cysteines might play a role in LEF-3 structure or in its ability to interact with P143 by mutating amino acids C82 and C106. Both mutated proteins localized to the nucleus and were also capable of interacting with P143 and transporting it to the nucleus. This demonstrated that at least for these specific functions, these two cysteines were not critical. However, these conserved amino acids could be important for other LEF-3 functions, for example, for binding to single strand DNA. It has been suggested that there are two separate single strand DNA binding domains in LEF-3, only one of which overlaps these conserved cysteine residues (Mikhailov et al., 2006).

We also investigated whether we could detect an interaction between LEF-3 and P143 by carrying out a reciprocal nuclear transport assay, using an engineered copy of P143 carrying the LEF-3 NLS. We used this protein to test for interaction with NLS-depleted LEF-3. However, none of the LEF-3 constructs tested were transported into the nucleus in the absence of the core NLS, even though P143 was completely nuclear. This suggests that there is specificity to the interaction between LEF-3 and P143 that includes both the NLS and other sequences within the 1–125 amino acid domain that are essential for transporting P143 to the nucleus. An interaction between LEF-3 and P143 was previously demonstrate by co-elution of P143 and LEF-3 from ssDNA-affinity columns loaded with infected cell extracts (Evans et al., 1999; Wu and Carstens, 1998). A weak interaction between LEF-3 and P143 was also demonstrated by a yeast two-hybrid screen (Evans et al., 1999). It is possible that such interactions were not detectable by our immunofluorescence screen. It is also possible that a direct binding interaction between P143 and LEF-3 does not occur but that P143 piggybacks onto the LEF-3 protein only when it is bound to the cellular nuclear importin complex. Such a bridging interaction has been predicted for herpes simplex virus 1 (HSV-1) UL37, which interacts in the cytoplasm of infected cells with the HSV-1 ORF29p homologue ICP8. UL37 is transported into the nucleus with ICP8 via a classical NLS in the C terminus of ICP8 as a result of this interaction (Stallings and Silverstein, 2005). It is also possible that the interaction with a karyopherin causes a conformational change in LEF-3 that then allows it to interact with P143.

While LEF-3 is predicted to be essential for baculovirus replication, little is known about its function in this process. LEF-3 must transport P143 and be translocated into the nucleus for replication to occur but P143 in the nucleus on its own is not sufficient for viral replication (Chen and Carstens, 2005). Our results show that the NLS domain, including the core sequence, is conserved within the group I *Alphabaculoviruses* and represents a functionally essential region. The AcMNPV LEF-3 NLS resembles both monopartite and bipartite transport systems, depending on the presence or absence of amino acids 20–28. However, this domain is not present in several other group 1 viruses including CfMNPV LEF-3. This suggests that AcMNPV and its close relatives have evolved a more flexible NLS system for increased efficiency in nuclear transport that may contribute to its ability to replicate faster than CfMNPV in cell culture (Liu and Carstens, 1993). It may also contribute to the higher virulence of AcMNPV than CfMNPV. It is evident that the NLS region is also essential for interaction of P143. Further characterization of the functional domains of AcMNPV LEF-3 and comparison to other baculovirus species will provide a greater understanding of its role in baculovirus DNA replication and host range determination. The fact that the AcMNPV LEF-3 NLS domain also functions in mammalian cells indicates that LEF-3 should provide a useful model system for studying the similarities between invertebrates and vertebrate intracellular transport systems.

Materials and methods

Cells and viruses

The *Spodoptera frugiperda* continuous cell line IPLBSF-21 (Sf21) was maintained in TC100 medium supplemented with 10% fetal calf serum (FCS) at 28 °C. AcMNPV (strain HR3) was prepared and titrated as previously described (Lu and Carstens, 1991).

Plasmids

Plasmid constructs of point mutations or deletions within the N-terminal region of AcMNPV LEF-3 were derived from pHSEHLEF3 (Chen and Carstens, 2005) where protein expression was driven by a *Drosophila melanogaster* heat shock 70 promoter (Rapp et al., 1998). The CfMNPV LEF-3 gene (Chen et al., 2004) was subcloned into the same expression vector to produce pHSEHCfLEF3. Specific deletions and mutations within the *lef-3* open reading frame were generated by PCR-fusion mutagenesis as described previously (Chen and Carstens, 2005). Briefly, two nested primers were designed to be complementary and carried two segments corresponding to flanking sequences upstream and downstream of the region to be mutated. The sequences of the nested primers used in the PCR mutagenesis reactions are summarized in Table 1S. Each of the primers was used in reactions with upstream or downstream outside primers C-22910 or C-22911. The products were then mixed, denatured, and used as template in a third PCR amplification using only the outside primers. The final PCR products were digested with XbaI and NotI, and cloned into XbaI–NotI digested pHSEH so that each coding sequence was fused in-frame with the influenza virus haemagglutinin (HA) epitope and a six-histidine tag at the N terminus.

Plasmid constructs of LEF-3/GFP fusion proteins and point deletions within amino acids 2–56 of AcMNPV were derived from pHSEHAcLEF3(2–56)-GFP (Chen and Carstens, 2005). To generate GFP fusion protein, PCR was used to amplify different region of AcMNPV *lef-3* (Aclef3) or CfMNPV *lef-3* (Cflef3). Forward primers contained a PstI site and reverse primers contained a SmaI site. The PCR products were digested with PstI and SmaI and cloned into pHSEHGFP (Chen and Carstens, 2005) also digested with PstI–SmaI. Site-directed mutagenesis within the LEF-3(2–56)GFP fusions was done using two complementary mutated nested primers coding for specific amino acid changes in their sequences. The sequences of the nested primers used in these PCR mutagenesis reactions are summarized in Table 2S. Each of the primers was used in reactions with upstream or downstream outside primers C-22910 or C-22911. The final PCR products were digested with XbaI and NotI, and cloned into XbaI–NotI digested pHSEH. All plasmids were confirmed by restriction enzyme digestion and nucleotide sequence analysis. We also confirmed that each plasmid expressed the expected product by preparing western blots of transfected cells extracts (Fig. 1S).To clone the AcMNPV LEF-3 NLS into a plasmid vector capable of expression in mammalian cells, the vector pCAGGS (Niwa et al., 1991) was digested with XhoI, and ligated to a XhoI digested PCR product of

pHSEHAclef3(2–56)-GFP amplified with C-27471 and C-27472 generating pCAGGSAclef3NLS-GFP.

Transfections, immunoblotting and immunofluorescence microscopy

Sf21 cells (3×10^6) in 35-mm dishes (with or without coverslips) were transfected with 2 μg of plasmid DNA as previously described (Chen et al., 2004) and incubated at 28 °C for 22 h. The cells were heat shocked at 42 °C for 30 min at 22 h post transfection, and returned to 28 °C. At 24 h post transfection, cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were washed three times with PBS and then permeabilized with 0.1% Triton X-100 in PBS with 1% normal goat serum (PBS/NGS) for 3 min at room temperature. Following three washes with PBS, the cells were blocked for 5 min in PBS/NGS and then incubated with a primary rabbit polyclonal anti-AcMNPV LEF-3 antibody and/or mouse monoclonal anti-AcMNPV P143 antibody (1:250 dilution in PBS/NGS) for 1 h at room temperature. Following three washes with PBS/NGS, the coverslips were incubated with secondary goat anti-rabbit IgG conjugated with Alexa Fluor 568 antibody and/or goat anti-mouse IgG conjugated with Alexa Fluor 488 antibody (1:1000 dilution in PBS/NGS) (Molecular Probes) for 1 h at room temperature. Following three washes with PBS/NGS, the cells were stained with 1% (v/v) DAPI (4′, 6-diamidino-2-phenylindole) (Molecular Probes) or Hoechst stain (2 μg/ml bisBenzimide H33342, Sigma) for 3 min at room temperature. Following three washes with PBS, coverslips were mounted on glass slides with one drop of hydromount aqueous non-fluorescing mounting media (National Diagnostics) and air dried for 15 min. Cells were viewed using a 60× oil immersion lens (NA 0.45) on a Nikon TE200 epifluorescence microscope. Images were captured and compiled with a cooled CCD camera (Roper Scientific, Cool Snap HQ) using Metamorph software (Molecular Devices).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.10.051.

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