Identification of Six *Autographa californica* Multicapsid Nucleopolyhedrovirus Early Genes That Mediate Nuclear Localization of G-Actin

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Nuclear filamentous actin (F-actin) is required for nucleopolyhedrovirus (NPV) progeny production in NPV-infected, cultured lepidopteran cells. We have determined that monomeric G-actin is localized within the nuclei of host cells during the early stage of infection by *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). With a library of cloned AcMNPV genomic fragments, along with a plasmid engineered to express enhanced green fluorescent protein–*Bombyx mori* G-actin in transient transfection experiments, we identified six AcMNPV early genes that mediate nuclear localization of G-actin in TN-368 cells: *ie-1*, *pe38*, *he65*, *Ac004*, *Ac102*, and *Ac152*. Within this subset, *ie-1* and *pe38* encode immediate-early transcriptional transactivators, *he65* encodes a delayed-early product, and the products encoded by *Ac004*, *Ac102*, and *Ac152* have not been characterized. We found that when driven by foreign promoters, *ie-1*, *pe38*, and *Ac004* had to be expressed prior to *Ac102* or *he65* for nuclear G-actin to accumulate and that expression of *Ac152* was no longer required. These results and others suggested that the product of *Ac152* was a transactivator (directly or indirectly) of both *Ac102* and *he65* and that recruitment of G-actin to the nucleus was a temporally regulated process. Determining the functions of each of the six AcMNPV gene products with respect to our assay should provide valuable clues to basic cellular mechanisms of actin regulation and how AcMNPV infection affects them.

Members of the *Nucleopolyhedrovirus* genus of the *Baculoviridae* are divided into two groups based on the phylogenetic relatedness of their double-stranded DNA genomes (16). Members of both groups, however, are dependent upon nuclear filamentous (F) actin for progeny production, suggesting that this unique trait is ancestral to the nucleopolyhedrovirus (NPV) lineage (19). The best-studied baculovirus is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), a widely used expression vector and the type species of the NPVs (4, 21, 39, 51). Not surprisingly, AcMNPV has led the way in studies of baculovirus manipulation of the actin cytoskeleton, and these studies have brought to light a number of features regarding this process.

The budded form of AcMNPV (AcMNPV BV) is taken into cells by adsorptive endocytosis, and nucleocapsids penetrate the cytoplasm when the viral envelope fuses with the endosomal membrane (53). Fusion is triggered by the decreasing pH of the endosome and is mediated by the BV envelope protein, gp64 (6). Immediately following penetration, F-actin cables can be detected within the cytoplasm, often in association with viral nucleocapsids (10). These actin cables are thought to facilitate transport of the nucleocapsids to the nucleus (29), where genome replication and nucleocapsid morphogenesis take place (12). Following uncoating within the nucleus and expression of early viral genes, the actin cytoskeleton assumes a second configuration, with the formation of F-actin aggregates at the plasma membrane (9, 48). This second change is

mediated by the product of a single early viral gene, *arif-1*, which is a ~47-kDa phosphoprotein that colocalizes with the F-actin aggregates (11).

During late gene expression, a third and more dramatic shift in the actin cytoskeleton takes place: F-actin appears within the nucleus, both within the central virogenic stroma, where viral DNA synthesis occurs (58), and also (and more prominently) within the surrounding “ring zone,” juxtaposed to the inner nuclear membrane (9, 52, 54). Within the nucleus, p78/83, a minor capsid protein, colocalizes with the F-actin (28), and p39, the major capsid protein, partially colocalizes with it (9). Both p78/83 and p39 are actin-binding proteins; p78/83 appears to bind F-actin preferentially, while p39 binds both F- and G-actin (29).

Nuclear F-actin is essential for nucleocapsid morphogenesis (38, 52). Accordingly, cytochalasin D and latrunculin A, F-actin-disrupting drugs, prevent progeny virus production (19). In the presence of cytochalasin D, viral DNA is not packaged properly, nor are capsids produced in unit lengths (52). Instead, long and predominantly empty tubules, replete with p39, accumulate, and they are not associated with basal structures. In contrast, normal AcMNPV nucleocapsid morphogenesis begins with the formation of cylindrical capsid sheaths (presumably composed of the major capsid protein, p39) in association with basal structures (12). Basal structures of *Orgyia pseudotsugata* MNPV have been reported to contain the *O. pseudotsugata* MNPV homologue of p78/83 (49), and by inference, the basal structures of AcMNPV should contain p78/83. If so, then it is plausible that F-actin is involved in the assembly process during the earliest stages of nucleocapsid morphogenesis. The recent finding that p78/83 and its homologues contain putative Wiskott-Aldrich syndrome protein sequence motifs that bind

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and activate the Arp2/3 complex (a eukaryotic multifunctional nucleator and organizer of actin [25]) supports this speculation (35).

Whatever the mechanism, in order for actin to facilitate nucleocapsid morphogenesis within the nucleus, it first must be recruited there. Here we report that monomeric G-actin accumulated within nuclei of AcMNPV-infected cells during the early stages of infection and that expression of six AcMNPV early genes was sufficient for this to occur in transiently transfected TN-368 cells. Two of the six genes involved in the nuclear localization of G-actin, *he65* and *Ac102*, were found to have overlapping functions, and the product of a third gene, *Ac152*, was needed to activate expression of *he65* and *Ac102*. When driven by foreign promoters, expression of only four of the nuclear localization of G-actin genes, *ie-1*, *pe38*, *Ac004*, and either *Ac102* or *he65*, resulted in the accumulation of G-actin within nuclei of TN-368 cells.

While the existence of actin, actin-related proteins, and actin-binding proteins within the nuclei of metazoan cells has historically been controversial, it is now widely accepted, but the roles of these proteins within nuclei are still unclear (43). Identification of the AcMNPV nuclear localization of G-actin genes described herein not only advances our understanding of baculovirus biology but also may help to elucidate the cellular processes involved in the transport, retention, regulation, and function of nuclear actin.

MATERIALS AND METHODS

Cells and viruses. Sf21 cells (13) were cultured at 27°C in Grace's medium (JRH Biosciences) supplemented with 10% fetal bovine serum (Gibco-BRL). TN-368 cells (17) were cultured at 27°C in Grace's medium supplemented with 10% fetal bovine serum, lactalbumin (Gibco-BRL), and yeastolate hydrolysate (Gibco-BRL). Media for cells grown in suspension culture were supplemented with 0.1% pluronic F-68 (Sigma). The E2 strain of AcMNPV was used in this study (50).

Plasmids. An AcMNPV E2 genomic plasmid library was assembled with the help of colleagues at Texas A&M University, Max Summers and Linda Guarino. *EcoRI*-A (in pUC19) was obtained from Max Summers, and *EcoRI*-B (in pUC8), *HindIII*-B (in pUC8), *HindIII*-F (in pUC8), *PstI*-C (in pUC8), *PstI*-F'-E' (in pUC8), and *hsp70 ie-1* (in pHEH) were obtained from Linda Guarino. We cloned *EcoRI*-J into pBSKS+ (Stratagene).

Expression vector pIE1/153A (33) was supplied to us by Kostas Iatrou (University of Calgary). *Bombyx mori* cytoplasmic A4 actin DNA (38) was obtained from Nicole Mounier (University of Lyon), and pBSHsp was provided by Gerald Rubin (University of California). *eGFP*, used for the *eGFP*-A4 gene fusion, originated from pEGFP-Actin (Clontech). pBSKS+ (Stratagene) and pUC18 (Pharmacia) were used for subcloning fragments of the AcMNPV genome.

Plasmid constructions. Expression plasmids driving enhanced green fluorescent protein (eGFP)-*B. mori* A4 actin were constructed in several steps. First, the human actin sequence in the commercial vector pEGFP-Actin was replaced with the *B. mori* A4 actin gene. For in-frame fusion to *eGFP*, *B. mori* A4 actin was removed from a previously constructed pAcMP3-based plasmid (39) with *XbaI* and *BglII*, blunted, and inserted into the *XhoI*- and *BamHI*-blunted pEGFP-Actin vector. The orientation of the insert was determined by restriction analysis and DNA sequencing. The *eGFP*-*B. mori* A4 actin sequence was removed subsequently with *NheI* and *XbaI* and inserted into pIE1/153A cleaved with *XbaI* to form pIE1actGA.

The *ie-1* gene was excised from the pIE1/153A-based plasmids that contained the NLA genes to create pact plasmids with *NheI* and *ApaI* followed by blunting and religating (Fig. 1). The pIE1/153A and the pact-based plasmids therefore differ functionally from one another in that the latter do not express IE-1 in addition to eGFP-actin (Fig. 1). Finally, PCR-amplified NLA genes were blunt ended and ligated into *HindIII*-cleaved, blunted pBSHsp to create plasmids containing NLA genes driven by the *Drosophila melanogaster hsp70* promoter. Orientation of the inserts in the plasmids was determined by restriction analysis and DNA sequencing. We found that when *eGFP*-*B. mori* actin was expressed in cultured insect cells, regardless of the vector, it polymerized and produced no

FIG. 1. Vector constructs used in transfection experiments. Common plasmid vector backbone details are omitted. pIE1/153A and pIE1actGA contain the *ie-1* open reading frame driven by the *ie-1* promoter (*ie-1 p*). *hr3*, *B. mori* NPV homologous region 3; *act p*, *B. mori* A3 actin promoter; MCS, multiple cloning site; poly A, polyadenylation signal; eGFP-actin, fused open reading frames of enhanced green fluorescent protein and actin.

apparent abnormal cellular effects, suggesting that the GFP-tagged actin was functional.

Subcloning of AcMNPV genomic plasmids. *HindIII*-F was subcloned into smaller fragments *PstI*-N (2.7-kb *PstI* fragment of *HindIII*-F cloned into pBSKS+), *SphI*-J (5.4-kb *SphI* fragment of *HindIII*-F, cloned into pUC18), *HindIII*-FES (2.9-kb *EcoRI*/*SalI* fragment of *HindIII*-F, cloned into pBSKS+), and *HindIII*-FXH (2.4-kb *XhoI*/*HindIII* fragment of *HindIII*-F, cloned into pBSKS+). *PstI*-C was subcloned into smaller fragments *PstI*-CH1 (5.5-kb *PstI*/*HindIII* fragment of *PstI*-C, cloned into pBSKS+), *KpnI*-E (2.0-kb *KpnI* fragment of *PstI*-C, cloned into pBSKS+), *KpnI*-D (5.8-kb *KpnI* fragment of *PstI*-C, cloned into pBSKS+), *EcoRI*-L (4.0-kb *EcoRI* fragment of *PstI*-C, cloned into pBSKS+), and *PstI*-CE4 (4.3-kb *EcoRI* fragment of *PstI*-C, cloned into pBSKS+).

PCR amplification of AcMNPV open reading frames. *Ac001*, *Ac002*, *Ac004*, *Ac101*, *Ac102*, *Ac149*, *Ac150*, *Ac152*, and *pe38* were PCR amplified from *PstI*-C or *HindIII*-F with 5' primers with an 8-nucleotide sequence containing a *BamHI* site followed by 20 nucleotides of gene-specific sequence immediately preceding the start codon and 3' primers with an 8-nucleotide sequence containing an *XbaI* site followed by 20 nucleotides of (complementary) gene-specific sequence which ended 50 nucleotides downstream of the stop codon. *he65* and *Ac107* were PCR amplified from *PstI*-C with 5' primers with an 8-nucleotide sequence containing a *BamHI* site followed by 20 nucleotides of gene-specific sequence immediately preceding the start codon and 3' primers with an 8-nucleotide sequence containing a *BamHI* site followed by 20 nucleotides of (complementary) gene-specific sequence which ended 50 nucleotides downstream of the stop codon. Amplified genes were then cloned into *BamHI*-cleaved pIE1/153A or *BamHI*- and *XbaI*-cleaved pIE1/153A and verified by DNA sequencing.

Transfections. Cells (1.9×10^5 Sf21 or 8×10^4 TN-368) were plated onto 22-mm² coverslips (in six-well plates) in a volume of 250 μ l and allowed to settle for 30 min. DNA for transfection was prepared with Endofree maxi-prep kits (Qiagen or Sigma). In infection experiments, medium was removed, and cells were infected at a multiplicity of infection of 10 for 1 h (with or without 5 μ g of aphidicolin per ml), then transfected at 2 h postinfection. Transfections were performed with Cellfectin (Gibco-BRL) by the manufacturer's protocol, with 0.5 μ g of pIE1actGA and 5 μ g of viral fragments (in plasmids) per coverslip.

In experiments with aphidicolin, 5 μ g/ml was added to transfection media. Coverslips were fixed 2 to 3 days posttransfection for 10 min in 2% paraformaldehyde in PHEM buffer (60 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9), washed once in PHEM, stained with 4',6'-diamidino-2-phenylindole (DAPI) (Sigma; 1 μ g/ml in PHEM) for 10 min, washed twice more in PHEM, and mounted on microscope slides in Prolong antifade mounting medium (Molecular Probes). In some experiments, cells were solubilized following DAPI treatment with 0.15% Triton X-100 and stained with tetramethylrhodamine isothiocyanate-phalloidin (Sigma). Coverslips were viewed with a Zeiss Axiophot photomicroscope equipped for fluorescence microscopy or a Zeiss confocal laser scanning microscope.

RESULTS

Nuclear G-actin accumulation is mediated by early viral genes. To determine whether the G-actin that polymerizes within the nuclei of AcMNPV-infected cells during late gene

FIG. 2. Fluorescence microscopy images of Sf21 cells infected with AcMNPV at a multiplicity of infection of 10 in the presence of 5 μ g of aphidicolin per ml and then transfected with pIE1actGA at 2 h postinfection. Cells were fixed and stained at 48 h postinfection. (A) DAPI-stained DNA. (B) eGFP-*B. mori* G-actin. (C) Tetramethylrhodamine isocyanate-phalloidin staining of filamentous actin.

expression was recruited to the nucleus during early gene expression, we transfected AcMNPV-infected Sf21 cells with pIE1actGA at 2 h postinfection in the presence and absence of 5 μ g of aphidicolin per ml, a drug that prevents AcMNPV late gene expression (45). Sf21 cells infected in the presence of aphidicolin showed eGFP-actin in the nucleus (Fig. 2A and B). In contrast to cells infected in the absence of aphidicolin, this nuclear eGFP-actin did not stain with rhodamine phalloidin, indicating that it was present in its unpolymerized, monomeric state (Fig. 2C). These results showed that early gene expression was sufficient for the nuclear localization of G-actin and confirmed the requirement of late gene expression for subsequent polymerization of nuclear G-actin (not shown) (9).

Transfection of pIE1actGA into TN-368 cells along with mixtures of viral fragments covering the AcMNPV genome. The finding that AcMNPV early gene expression was sufficient to localize G-actin within the nucleus made it possible to carry out transient expression of AcMNPV genomic fragments along with pIE1actGA in order to identify early viral factors involved in this process. Baculovirus early genes are expressed exclusively by host factors alone (immediate-early genes) or by host factors in combination with immediate-early virus-encoded transactivators (delayed-early genes) (for a review, see reference 47).

We began with three mixtures of plasmids that contained between 24 and 36% of the AcMNPV genome (Fig. 3). These mixtures were transfected along with pIE1actGA into TN-368 cells, which were subsequently processed at 3 days posttransfection. Use of pIE1actGA eliminated the need to add a separate plasmid encoding IE1, a well-characterized transactiva-

tor of a number of AcMNPV delayed-early genes. We used TN-368 cells rather than *Spodoptera frugiperda*-derived cell lines for these experiments because TN-368 cells do not undergo IE1-induced apoptosis as readily as do cells in the *S. frugiperda* lineage (41).

The mixtures containing either the *HindIII*-F, *PstI*-C, and *HindIII*-B fragments or only the *HindIII*-F and *PstI*-C fragments (covering approximately 20% of the genome) showed a pattern of eGFP-actin localization distinct from all other transfection mixtures (Fig. 4A and B). The majority of these transfected cells showed a high level of eGFP-actin signal that made it difficult to distinguish subcellular structures, but eGFP-G-actin was clearly localized within the nuclei of approximately 10% of the cells. In addition, cells showing obvious cytoplasmic but no nuclear signaling, such as those shown in Fig. 4C and D, were not found in this positive transfection mixture.

Subcloning of the *PstI*-C and *HindIII*-F fragments to identify the viral genes involved in the nuclear localization of G-actin. To identify specific viral genes involved in the nuclear localization of G-actin, we subcloned the *PstI*-C fragment into the *PstI*-CH1, *KpnI*-E, *KpnI*-D, *EcoRI*-L, and *PstI*-CE4 fragments (Fig. 5). We subsequently transfected each of these subclones in combination with *HindIII*-F and pIE1actGA into TN-368 cells and found that either the *PstI*-CH1, *KpnI*-E, or *KpnI*-D fragment gave a strong positive signal in combination with *HindIII*-F (on the order of 10% clearly positive cells and few to no clear negatives). *KpnI*-D and *KpnI*-E are distinct clones, and therefore we reasoned that these clones each contained at least one gene with apparent overlapping function that could complement *HindIII*-F in our assay.

FIG. 3. Genomic locations of plasmid DNAs used in transfections with pIE1actGA. Three separate mixtures, represented on three separate lines, are shown beneath the *EcoRI* map of AcMNPV.

To specify which specific open reading frames (ORFs) were involved, we PCR amplified ORFs *Ac101*, *Ac102*, *Ac103*, *Ac105* (*he65*), and *Ac107* (Fig. 5) and inserted each into the pIE1/153A expression vector. Subsequent transfections of individual ORFs with *HindIII*-F and pIE1actGA showed that either *Ac102* or *Ac105* (*he65*) could act in combination with *HindIII*-F to induce the nuclear localization of G-actin.

Relative to *PstI*-C, the subcloning of the *HindIII*-F fragment proved to be more challenging. We subcloned *HindIII*-F into the *PstI*-N, *SphI*-J, *HindIII*-FES, and *HindIII*-FXH fragments, but no combination (even combinations with apparent complete coverage of *HindIII*-F) resulted in a clear positive signal when transfected with *PstI*-C. One possible explanation for this result was that a homologous region present within *HindIII*-F was lacking in some of the subclones containing ORFs involved in the nuclear localization of G-actin. The lack of a homologous region may have resulted in lower relative expression of those ORFs because homologous regions bind IE-1 and act as transcriptional enhancers (8, 15, 30, 37, 46).

Candidate ORFs, therefore, were PCR amplified from *HindIII*-F and cloned into pIE1/153A. AcMNPV ORFs *Ac149*, *Ac150*, *Ac151* (*ie-2*), *Ac152*, *Ac153* (*pe-38*), *Ac002*, and *Ac004* were amplified and tested in combination with *PstI*-C. A clear positive result was obtained when the *PstI*-N fragment was transfected along with *pe-38*, *Ac004*, and *PstI*-C or when *PstI*-N, *HindIII*-FES, and *pe-38* were transfected along with *PstI*-C. Further transfections showed that ORFs *Ac152* and *Ac004* in combination with *pe-38* and *PstI*-C were sufficient for nuclear localization of G-actin (Fig. 6B).

Timing of expression of genes involved in nuclear localization of G-actin. Once *Ac102* and *he65* from *PstI*-C and *Ac152*, *Ac004*, and *pe-38* from *HindIII*-F were identified as nuclear localization of G-actin (NLA) genes, we cloned each of them into pIE1/153A and subsequently transfected these plasmids into TN-368 cells. We were disappointed to find that this transfection mixture did not give a positive signal. Previous experiments in which *PstI*-C or *HindIII*-F was transfected with individual ORFs cloned into pIE1/153A had suggested that timing of expression of NLA genes was important. For example, we

had noted that *PstI*-C along with *Ac152*, *pe-38*, and *Ac004* gave a positive signal at 3 but not 2 days posttransfection unless *ie-2*, an immediate-early transactivator gene, was included in the mix.

We carried out a series of experiments, therefore, in which we transfected subsets of various combinations of the NLA genes 1 day in advance of the remainder and then processed the cells 1 or 2 days following the second transfection. We found that when *Ac152*, *pe-38*, and *Ac004* were transfected 1 day prior to either *Ac102* or *he65* and the cells were processed 2 days after the second transfection, nuclear G-actin was clearly detected (Fig. 6E and F).

Reduced number of NLA genes required when driven by a foreign promoter. The five NLA genes cloned into pIE1/153A yielded a clear positive signal when the appropriate timing was observed. Having established this, we conducted experiments to determine whether all five of the gene products were needed when NLA gene expression was driven by the cellular actin promoter in the pIE1/153A vector (31). We noted that mixtures containing pIE1actGA and *PstI*-C along with *pe-38* and *Ac004* (but not *Ac152*) cloned into pIE1/153A did not give a positive signal, whereas mixtures of pIE1actGA and *pe-38*, *Ac004*, and either *Ac102* or *he65* cloned into pIE1/153A did give a positive signal. These results indicated that the *Ac152* gene product served to activate and/or enhance expression of both *Ac102* and *he65* when these genes were driven by their native viral promoters. Hence, expression of a minimum subset of three NLA genes cloned into pIE1/153A, *pe38*, and *Ac004* plus *Ac102* or *he65* was sufficient for mediating G-actin accumulation within the nucleus (Fig. 5).

In order to determine whether IE1 was necessary when expression of the NLA genes was driven by the actin promoter in pIE1/153A, we deleted *ie-1* from the pIE1/153A vector (creating pact [Fig. 1]), inserted each of the NLA genes and *eGFP-B. mori A4* actin into the modified vector, and transfected TN-368 cells. We found that in the absence of IE1, we did not get a positive nuclear signal, but we were able to rescue the signal when we added a single vector that expressed *ie-1* to the mix (e.g., pIE1actGA). These results suggested that the

FIG. 4. Fluorescence microscopy images of TN-368 cells transfected with cloned fragments of AcMNPV DNA, at 3 days posttransfection. (A) eGFP-*B. mori* G-actin in cells transfected with pIE1actGA and the *Pst*I-C and *Hind*III-F fragments of AcMNPV. (B) DAPI staining of cells shown in panel A. (C) eGFP-*B. mori* G-actin in cells transfected with pIE1actGA and the *Eco*RI-A, *Eco*RI-J, and *Pst*I-F'-E' fragments of AcMNPV. (D) DAPI staining of cells shown in panel C.

nuclear localization of G-actin activity depended on either enhanced expression of one or more of the NLA genes through the interaction of IE1 with the homologous region 3 (*hr3*) sequence in pIE1/153A (Fig. 1) (31), or on an interaction of IE1 with a cellular factor(s), or both.

To distinguish among these possibilities, we next cloned the NLA genes behind the *Drosophila hsp70* promoter in the plasmid pBS_{hsp}. We transfected TN-368 cells sequentially with pactGA on day 1, pBS_{hsp}/*ie-1*, pBS_{hsp}/*pe38*, and pBS_{hsp}/*Ac004* on day 2, and pBS_{hsp}/*Ac102* on day 3; when we examined those cells on day 5, we found that G-actin had been recruited to the nucleus. In control experiments in which pBS_{hsp}/*ie-1* was not added, no nuclear localization of G-actin was evident, indicating that IE1 was needed for interaction with cellular factors in our assay.

DISCUSSION

Actin is an abundant protein in all eukaryotes and is the major component of the microfilament network of the cytoskeleton. This protein has been studied for decades and has many well-defined roles, all of which are executed within the

cytoplasm. Many viruses use actin in their transport, assembly and replicative processes, but with the exceptions of AcMNPV nucleocapsid assembly (38) and the export of human immunodeficiency virus type 1 RNAs (18, 20), these activities all occur within the cytoplasm as well. Our discovery that nuclear F-actin was required for nucleocapsid morphogenesis therefore suggested that AcMNPV gene products were involved in the recruitment and/or retention of actin within the nucleus. Here we report that localization of G-actin within the nucleus occurs during early viral gene expression and that the products of six early viral genes, *ie-1*, *pe38*, *he65*, *Ac004*, *Ac102*, and *Ac152*, are involved in this activity.

Among the products of these genes, IE1 is the best characterized. It is highly conserved among baculoviruses and is a transcriptional transactivator of several early, late, and very late genes (1, 5, 15, 30, 40), including an NLA gene, *he65* (26). Our finding that IE1 was essential for nuclear localization of G-actin, even when expression of the other required NLA genes was independent of its presence, suggested that IE1's role in our assay was mediated through interactions with host factors. It is known that IE1 has the capability of interacting

FIG. 5. Map showing subcloned fragments of *PstI*-C and *HindIII*-F. The locations of the ends of the fragments within the AcMNPV genome are indicated by the numbers flanking *PstI*-C and *HindIII*-F. Predicted open reading frames (2) are indicated below the *PstI*-C and *HindIII*-F fragments by number (and gene name if previously characterized), with the predicted direction of transcription indicated by arrowheads.

with host factors because its expression can induce apoptosis in Sf21 cells (41). Interestingly, IE1's ability to induce apoptosis is augmented by another NLA gene product, PE38 (42). It is possible, therefore, that initiation of the apoptotic process is part of the viral strategy for recruiting actin to the nucleus. In support of this idea, actin is recruited to nuclei of HL60 human promyelocytic cells induced to undergo apoptosis (34). In addition, during apoptosis, chromatin undergoes characteristic morphological changes involving margination toward the nuclear periphery, similar to chromatin changes that occur in baculovirus-infected cells (1, 56). Moreover, it has been postulated that in apoptosing cells, nuclear actin may play a role in chromatin margination (34).

IE1 and PE38 also have been implicated in viral DNA synthesis. In plasmid-based assays, IE1 is essential for DNA synthesis, and PE38 is stimulatory (22). In addition, IE1 binds to homologous regions which serve as origins of replication in plasmid-based assays (31). In infected cells, however, it is not known whether IE1 is needed for DNA replication beyond its role as an activator of required early genes, or if

homologous region sequences act as origins of replication. Even so, existing data are consistent with the hypothesis that IE1 has a direct role in origin binding and replication complex formation (31). If so, then PE38 may also participate in this activity.

Not only does PE38 stimulate DNA synthesis in plasmid-based assays, but AcMNPV *pe38* deletion mutants have been found to be defective in viral DNA synthesis and BV progeny production (D. Theilmann, personal communication; M. L. Milks, J. O. Washburn, L. G. Willis, L. E. Volkman, and D. Theilmann, submitted for publication). In addition, PE38 has two putative DNA-binding motifs, a RING motif, a leucine zipper, and a nuclear translocation signal (23, 24). A consistent theme among proteins with RINGs is their ability to mediate protein associations that lead to the formation of large multi-protein complexes (7), such as chromatin remodeling complexes (44). It is possible that IE1, PE38, and F-actin participate in such a complex. Consistent with this idea, parental and progeny AcMNPV DNAs assume chromatin-like structures early during infection (57), and nuclear actin and actin-related

FIG. 6. Confocal fluorescence microscopy images of TN-368 cells transfected with pIE1actGA alone or with cloned genes or genomic fragments of AcMNPV. Cells were fixed 3 days posttransfection and stained with DAPI. (A) *Pst*I-C and *Hind*III-F fragments of AcMNPV. (B) *Ac*004, *Ac*152, and *pe*-38 and 24 h later *Pst*I-C. (C) *Hind*III-F and 24 h later *Ac*102. (D) *Hind*III-F and 24 h later *he*65. (E) *Ac*004, *Ac*152, and *pe*-38 and 24 h later *Ac*102. (F and G) *Ac*004, *Ac*152, and *pe*-38 and 24 h later *he*65. (H) pIE1act GA alone.

proteins (arps) have been identified as components of chromatin remodeling complexes (44).

We determined that, when transfected into TN-368 cells, a mixture of *Ac*004, *pe*38, and *Pst*I-C was insufficient for induction of nuclear localization of G-actin. Transfection with ORFs *Ac*152, *Ac*004, and *pe*38 together with *Pst*I-C, however, or *pe*38,

*Ac*004, and either *Ac*102 or *he*65 cloned into pIE1/153A was sufficient for nuclear localization of G-actin (Fig. 6B). These results provided evidence that the product of *Ac*152 transactivated both *Ac*102 and *he*65. If this result is confirmed, then the products of *Ac*152 and *ie*-1 are both transactivators of the early gene *he*65 (3). Why both would be needed in the context of our

assay is not apparent, but another AcMNPV delayed-early gene, *p143*, has also been found to be positively regulated by two different AcMNPV-encoded transactivators, IE1 and PE38 (30).

The finding that the products of *Ac102* and *he65* can function individually in combination with the other NLA gene products to recruit G-actin to the nucleus indicates that the products of these genes have overlapping functions, at least in TN-368 cells. The product of *he65* is predicted to have zinc finger and DNA ligase motifs (not shown), but these motifs were not found in the *Ac102* sequence, suggesting that the two gene products may function through different pathways or by different modes of action. AcMNPV has an extremely wide host range (14), and it is possible that these products have various degrees of activity in different hosts and/or host tissues. Species-specific effects and tissue-specific functions of AcMNPV gene products have been reported previously (5, 27, 32, 41).

Once the NLA genes were cloned into expression vectors, it became evident that timing of their expression was important for a clear nuclear signal to appear. We found that *ie-1*, *pe38*, and *Ac004* had to be expressed prior to *he65* or *Ac102*. The reasons for this are not clear at present, in part because the functions of the NLA gene products are largely unknown. Moreover, few clues to their function were gleaned through scanning the DNA and protein databases because none of the NLA sequences showed significant sequence identity beyond zinc finger and leucine zipper motifs. One activity that might be expected for members of the complex, however, is the masking of the two leucine-rich nuclear export signal sequences recently identified on actin (55). Whether or not the NLA gene products retain nuclear G-actin in this manner, the determination of their roles in our assay will lead to a better understanding of the cellular processes involved in the transport, retention, regulation, and function of nuclear actin.

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