Expression and Mutational Analysis of *Autographa californica* Nucleopolyhedrovirus HCF-1: Functional Requirements for Cysteine Residues

Joyce A. Wilson, Scott D. Forney, Alessandria M. Ricci, Emily G. Allen, Kathleen L. Hefferon, and Lois K. Miller

Departments of Entomology and Genetics, University of Georgia, Athens, Georgia 30602

Received 22 March 2005/Accepted 22 August 2005

The host cell-specific factor 1 gene (*hcf-1*) of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus is required for efficient virus growth in TN368 cells but is dispensable for virus replication in SF21 cells. However, the mechanism of action of *hcf-1* is unknown. To begin to understand its function in virus replication we have investigated the expression and localization pattern of HCF-1 in infected cells. Analysis of virus-infected TN368 cells showed that *hcf-1* is expressed at an early time in the virus life cycle, between 2 and 12 h postinfection, and localized the protein to punctate nuclear foci. Through coprecipitation experiments we have confirmed that HCF-1 self-associates into dimers or higher-order structures. We also found that over-expression of HCF-1 repressed expression from the *hcf-1* promoter in transient reporter assays. Mutagenesis of cysteine residues within a putative RING finger domain in the amino acid sequence of HCF-1 abolished self-association activity and suggests that the RING domain may be involved in this protein-protein interaction. A different but overlapping set of cysteine residues were required for efficient gene repression activity. Functional analysis of HCF-1 mutants showed that the cysteine amino acids required for both self-association and gene repression activities of HCF-1 were also required for efficient late-gene expression and occlusion body formation in TN368 cells. Mutational analysis also identified essential charged and hydrophobic amino acids located between two of the essential cysteine residues. We propose that HCF-1 is a RING finger-containing protein whose activity requires HCF-1 self-association and gene repression activity.

The family *Baculoviridae* is a diverse group of viruses that infect primarily insects. Baculoviruses are used routinely as expression vectors but are also interesting because of their potential use as biological insecticides (32). Most baculovirus species infect a narrow range of host insects. The type baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is unusual in that it has a host range that spans many insect genera (9). It is important to understand the molecular biology of baculovirus host range and cell type-specific restriction so that it may be possible to predict the effects of genetically modified viruses on nonhost species.

The host range of baculoviruses is restricted at a stage subsequent to virus entry into cells. Free virus particles are capable of entering a wide variety of permissive and nonpermissive insect and mammalian cells, however, levels of early gene expression, DNA replication, and late-gene expression vary depending on the cell line (34, 35). In non- and semipermissive cells, including human liver cells, DNA is released into the nucleus in an expressible form (21, 35). However, in nonpermissive cells no DNA replication or late-gene expression occurs. Thus, the block to virus replication in nonpermissive insect cells appears to occur subsequent to early-gene expression.

To date, six baculovirus genes with the ability to alter the growth of AcMNPV in a cell line-specific manner have been identified (*p143*, *p35*, *lef-7*, *hcf-1*, *hrf-1*, and *ie2*) (2, 7, 8, 10, 26, 29, 33, 43). Three of the six genes (*p143*, *p35*, and *hrf-1*) have also been shown to alter the ability of the virus to infect the corresponding larval insect species. By changing a few or even a single amino acid in the sequence of the baculovirus helicase protein, P143, replication levels were significantly enhanced in *Bombyx mori* larvae and Bm1 cells (derived from *B. mori*), a host in which the virus does not normally grow well (2, 10, 26, 33). Data suggests that P143 may interact with viral and cell factors in a virus- and host cell-specific manner (4).

Baculoviruses with roles in counteracting apoptosis also play a role in host range. Wild-type AcMNPV replicates efficiently in both SF21 and TN368 cells. However, in the absence of the antiapoptotic gene *p35*, AcMNPV is not able to replicate efficiently in SF21 cells or in *Spodoptera frugiperda* larvae, but is still capable of replicating in TN368 cells and larvae (8). It is possible that the expression of an endogenous *Trichoplusia ni* *iap* (inhibitor of apoptosis) gene protects TN368 cells from apoptotic death (41). The *Lymantria dispar* nucleopolyhedrovirus gene *hrf-1* (host range factor 1) can alter the host range of the type baculovirus AcMNPV to include the cell line L6d52Y. Wild-type AcMNPV infections of L6d52Y cells are nonproductive. Viral DNA replication and late-gene transcription appear to be normal but there is a complete shutdown of all host and viral protein synthesis (6, 12, 13, 24, 25, 42, 43). Expression of the *Lymantria dispar* nucleopolyhedrovirus gene *hrf-1* in L6d52Y cells counteracts the translational arrest induced by the virus (42). The molecular mechanisms by which these genes affect host specificity appear to be varied and

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* Corresponding author. Mailing address: Ontario Cancer Institute, 620 University Ave., Suite 706, Toronto, Ontario, Canada M5G 2C1. Phone: (416) 918-3054. Fax: (416) 204-2278. E-mail: jwilson@uhnres.utoronto.ca.

† Deceased.
determining the nature of their activities is ongoing, and will likely require a deeper understanding of the nature of the virus’s ability to avoid host defense mechanisms and take over the protein expression and replication machinery of the host cell.

Host cell-specific functions of certain viral genes have also been identified through the use of a transient late-gene expression assay. Nineteen AcMNPV genes are required for transient late-gene expression in SF21 cells (28, 39, 44). Interestingly, the group of genes required for transient late-gene expression in another permissive cell line, TN368, differed from those required in SF21 cells. In TN368 cells, three of the 19 genes, p35, lef-7, and ie2, were expendable, and one additional gene, hcf-1, was required for reporter gene expression (27). The cell-specific effects of each of these genes were also apparent when viruses containing null mutations in these genes were tested in each cell line. Viruses containing a null mutant lef-7 gene produced fewer budded viruses and occlusion bodies in SF21 cells but appeared normal in TN368 cells (7). SF21 cells infected with ie2 null mutant viruses produced fewer occlusion bodies per infected cell and the rate of infection was slower than with the wild-type virus (38).

Similar experiments were done using a recombinant AcMNPV carrying a null mutant hcf-1 gene. The mutant viruses grew normally in SF21 cells but showed a mutant phenotype in TN368 cells and in T. ni larvae (29). hcf-1 null viruses were defective in DNA replication, late-gene transcription, and occlusion body production, and showed a 100-fold decrease in budded virus production in TN368 cells. In addition, by 18 h postinfection, both host and viral protein synthesis appeared to be completely ablated in mutant virus-infected cells. In infections of T. ni larvae, the hcf-1 null mutant virus showed lower virulence (longer time to 50% lethality) but similar infectivity than wild-type virus when infected via the oral route. However, the mutant viruses were 50-fold less infectious than the wild type if infected by intrahemocoelic injections.

The role that HCF-1 plays in an AcMNPV-infected cell is unknown. In a previously published report, HCF-1 was found to be expressed as an early protein in the nucleus of infected cells (19). In addition, HCF-1 was found to be capable of self-association. Mutational analysis using HCF-1 truncations showed that activity in transient late expression assays was abolished by deletion of any region of HCF-1 with the exception of the C-terminal 33 amino acids. In addition, self-association activity was found to be located at the N terminus of the protein (19).

We have expanded on these preliminary analyses to confirm that both the HCF-1 transcript and protein are expressed early in the virus infection cycle in both SF21 and TN368 cells. We also show that the hcf-1 promoter activity is consistent with that of an early gene. We have also confirmed the nuclear localization of HCF-1 and further determined that virus infection induces HCF-1 into punctuate nuclear structures. We have also confirmed the self-association activity of HCF-1, and have identified that HCF-1 has gene repression activity on the hcf-1 promoter when overexpressed.

Results published previously (19) indicated that regions essential to the function of HCF-1 reside throughout the protein. We have therefore performing a detailed mutagenic analysis of the protein to identify important amino acid residues. The most notable feature of the hcf-1 amino acid sequences is the abundance of cysteine residues, several of which are present in CxxC motifs (where C represents cysteine residues and x indicates the positions of any amino acid). The presence of CxxC motifs suggests that HCF-1 may coordinate metal ions to form a zinc finger-like structure. We have identified a region of HCF-1 that is predicted to form a RING finger structure, and have determined through mutagenesis that all of the cysteine residues within the predicted RING motif are required for activity in transient late-gene expression assays, for HCF-1 self-association, and for efficient occlusion body production in TN368 cells. In addition, we have determined that different but overlapping subsets of cysteine residues are required for HCF-1 gene repression activity. Using scanning charged-to-alanine or hydrophobic-to-alanine mutagenesis of the protein sequence, we have also identified two other groups of amino acids that are required for HCF-1 activity in transient late-gene expression assays. Finally, we have made several viruses having HCF-1 point mutations and show that mutants deficient in self-association, gene repression, or both had abnormal occlusion body production in infected TN368 cells.

MATERIALS AND METHODS

Cell and virus culture. All viruses were propagated and titrated in Spodoptera frugiperda IPBL-SF-21 (SF21) cells (45) using standard methods (36). SF21 and Trichoplusia ni TN368 cells (20) were cultured at 27° in TC100 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 0.2% tryptose broth.

Plasmid construction. All primers used in plasmid construction are described in Table 1. Several constructs expressing HA11-, Flag-, and hexahistidine-tagged versions of hcf-1 from the Drosophila melanogaster heat shock protein 70 (HSP70) promoter were constructed. Plasmid pPHS-HA-Hishcf-1V1+ contains an N-terminal HA11 epitope and hexahistidine tag fused to the hcf-1 gene under control of the HSP70 promoter and was made by amplifying the hcf-1 open reading frame using primers 5′HCF1BGL and 3′HCFPSP2 as previously described (39). Plasmid pH5c-HA-Hishcf-1 contains a C-terminal HA11 epitope and hexahistidine-tagged hcf-1 gene under control of the HSP70 promoter and was constructed by amplifying the hcf-1 open reading frame (minus the stop codon) using primers 5′HCFBGL and 3′HCFPSPNS and inserting the product into the vector pH5c-HA-Hishcf-1V1+ in frame with the C-terminal HA11 tag sequences in the plasmid (18).

Several plasmids containing tagged hcf-1 genes were made in the context of the virus genomic subclone pXABgE3.6. This plasmid contains hcf-1 and upstream and downstream flanking DNA so that it may be used to transfer the tagged hcf-1 gene into the virus. Plasmid pXAC-hcHishcf-1 contains a C-terminally HA11- and hexahistidine-tagged hcf-1 gene and was made by excising tagged hcf-1 from pH5c-HA-Hishcf-1 using NheI and BglII and then inserted into pXABgE3.6 which had also been digested with NheI and BglII. Plasmid pXAH-Ahcf-1, which contains an N-terminal HA11 epitope-tagged hcf-1, was made by inserting the annealed primers VEHCF1T and CEHCF1B into the NheI site of plasmid pXABgE3.6.

Plasmid pXAFgHishcf-1Bsu2 contains N-terminal Flag and hexahistidine tags, and a Bsu36I site inserted into the hcf-1 coding sequence at an internal Nhel site. The tags and Bsu36I site were added by insertion of a synthetic double-stranded DNA fragment with flanking Nhel sticky ends made by annealing the complementary oligonucleotides BGFGBOT and BGFGGTOP into plasmid pXABgE3.6 (27). The Nhel site is located 12 base pairs downstream of the hcf-1 start codon and the insertion results in fusion of the tags after the fourth amino acid of HCF-1. In addition, this plasmid contains a second Bsu36I site that was added just downstream of the hcf-1 termination codon by site-directed mutagenesis using mutagenic primer BGE-C-BSU and selection primer BGE-RV-AT.

Plasmid pXAnFgHishcf-1Bsu2 was used for all subsequent oligonucleotide-directed point mutations made in the hcf-1 gene sequence. Point mutations were introduced into the hcf-1 gene in plasmid pXAnFgHishcf-1Bsu2 by using the transformation mutagenesis kit (Clontech Laboratories, Inc. Palo Alto, CA), one of the mutagenic primers (HMT1nn, Table 1), and the selection primer BGE-AT-RV. A negative control plasmid, pXAnFgHisCATBsu2, was made by remov-
Table 1. Oligonucleotides

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<th>Type</th>
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<td>HCFIPRBBG</td>
<td>GGAACATCTTGTGATAGCTGACGTCGTGAGTGGTAGG</td>
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* Introduced restriction sites or nucleotide mutations are underlined. A nucleotide insertion is bolded.
buffer. DNA replication was inhibited by the addition of 2 μg/ml aphidicolin (Sigma) and cells were harvested at 12 h postinfection to distinguish between virus early and late transcription.

**RNA gels and Northern blots.** Total cellular RNA from mock and wild-type virus-infected SF21 and TN368 cells was isolated using the Micro RNA isolation kit (Stratagene Cloning Systems, La Jolla, CA); 15 μg of RNA from each sample was glyoxylated, electrophoresed through a 1.2% agarose gel and transferred to nylon membrane (Zeta-probe, Bio-Rad Laboratories, Hercules, CA) as described previously (36). The blots were probed with an hcf-1 strand specific riboprobe made using plasmid pSHSCLNX and the riboprobe system-T3 (Promega Corporation, Madison, WI).

**Primers.** Total RNA from SF21 and TN368 cells infected with wild-type AcMNPV-L1 virus was purified using the micro-RNA isolation kit (Stratagene cloning systems, La Jolla, CA). Primer extension analysis of hcf-1 transcripts was done using the primer extension system with avian myeloblastosis virus reverse transcriptase (Promega Corporation, Madison, WI) and the manufacturer’s protocol. A 32P-end-labeled oligonucleotide, Hcf-1pox, which is complementary to a region between 87 and 108 ribonucleotides downstream of the hcf-1 ATG codon was used to prime the reaction.

**Transfection, coprecipitation, and Western blot.** TN368 cells (1.5 10^6 in 60-mm tissue culture plates) were transfected with 5 μg of plasmid expressing HCF-1, mutant HCF-1, or CAT from the HSP70 promoter using standard methods (36). The following day the cells were infected with either wild-type virus or virus expressing Flag-tagged HCF-1 or CAT. At 1 h postinfection cells were heat shocked at 42°C for 30 min. Cells from each plate were harvested at 6 and 11 h postinfection or 2.5 or 7.5 h post heat shock into 200 μl of cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 100 mM NaCl, 1x protease inhibitor cocktail [Pharmingen]). Cells were lysed by agitation for 30 min. The lysates were centrifuged for 10 min; 10 μl of the cleared extract was reserved to analyze protein expression levels. The rest of the extract was mixed with 10 μl of anti-Flag M2-agarose affinity gel (Sigma-Aldrich, St. Louis, MO) for 4 h and then washed five times with 500 μl of NP-40 lysis buffer.

Proteins were eluted from the affinity gel by using SDS-PAGE sample buffer. Proteins were separated on a 12% SDS-polyacrylamide gels and proteins were transferred onto a nitrocellulose membrane (Hybond-C extra, Amersham Life Science Inc., Arlington Heights, Ill.). The membranes were blocked in a 5% nonfat milk solution and then probed with anti-HA.11 mouse monoclonal antibody at a dilution of 1:500 in blocking buffer for 2 h. Cells were washed four times in PBS, pH 7.2, and incubated for 1 h with Alexa 488 goat anti-mouse immunoglobulin G (Molecular Probes Inc. Eugene, OR) diluted 1:2,000 in blocking buffer containing 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Cells were washed three times with PBS, pH 7.2, and mounted in gel mount buffer, pH 7.2 (60% glycerol, 0.4× PBS, pH 7.2).

**RESULTS**

**Expression pattern of HCF-1 in infected cells.** The protein and mRNA expression patterns of hcf-1 were determined in infected cells by Western blot, Northern blot, and primer extension analyses (Fig. 1). Western blot analysis of total protein from a time course of cells infected with a virus expressing a C-terminally HA.11-tagged hcf-1 gene, v-c-HAHishcf-1, showed that HCF-1 was expressed from 2 to 12 h postinfection, with peaking HCF-1 protein levels at 6 h postinfection (Fig. 1A). Expression of HCF-1 was not inhibited by the presence of aphidicolin (a drug that inhibits viral DNA replication and thus abolishes viral late-gene expression), suggesting that hcf-1 is expressed as an early gene (Fig. 1A, top and bottom panels, lanes Ap). The pattern of HCF-1 expression in SF21 cells was similar to that seen in TN368 cells. These results were confirmed in several experiments, including time course infections of SF21 and TN368 cells using a virus carrying an N-terminally HA.11 epitope-tagged hcf-1, suggesting that the presence of the epitope tags did not alter the expression pattern.

**Northern blot analysis of hcf-1 mRNA expression in SF21 and TN368 cells showed similar patterns in the two cell lines (Fig. 1B). Expression of hcf-1 in both cell lines occurred from 2 to 12 h postinfection, however, transcript levels in TN368 peaked at 2 h postinfection and continued at the same level until 12 h, while transcript levels in SF21 cells were lower at 2 and 12 h postinfection and peaked at 6 h (Fig. 1B, lanes 6). In addition, SF21 cells showed a 5.9-kb RNA species from 12 to 24 h postinfection that was absent in infected TN368 cells. This transcript did not appear to be used to translate HCF-1 since the protein was not detected in SF21 cells at these time points (Fig. 1A). As expected for an early gene, aphidicolin failed to inhibit hcf-1 transcription (Fig. 1B, Ap). Primer extension analysis confirmed that hcf-1 is expressed as an early gene (Fig. 1C). The major transcription start site of hcf-1 is 24 and 35 base pairs downstream from a pair of TATA box motifs and 11 base pairs upstream from the hcf-1 start codon in both cell lines (Fig. 1D) and two minor start sites are located one and three nucleotides downstream.
acetyltransferase (cat) (Fig. 2) and cotransfection of SF21 cells with a variety of AcMNPV subclones. Transient expression assays established that the baculovirus early-gene transactivator gene ie-1 is essential for transient cat gene expression from the hcf-1 promoter in SF21 cells (Fig. 2, alone) and in TN368 cells (Fig. 9, alone) since no cat gene expression was seen in the absence of ie-1, cat gene expression was stimulated by cotransfection with the AcMNPV subclone AIE15 or pBSPΔxhoI (Fig. 2B), a plasmid subclone of AIE15 that contains the baculovirus trans-activator genes ie-1, ie-2, and pe38.

Smaller subclones of pBSPΔxhoI were used to identify that ie-1, ie-2 and pe38 are responsible for the trans-activation of hcf-1. It appeared that ie-2 augmented expression of CAT from the hcf-1 promoter when transfected in conjunction with ie-1 (Fig. 2A and C, ie2) since a plasmid construct containing ie-2 enhanced transient cat gene expression but a plasmid containing frame shift mutant version of ie-2 could not (Fig. 2A and B, pie2fs). The data suggest that pe38 is also able to trans-activate hcf-1 since plasmid pBSPΔΔPstI, which contains pe38 but not ie-2, induced CAT expression to levels greater than those observed in the presence of ie-1 alone (Fig. 2A, pie1 versus pBSPΔΔPstI). A subclone of pBSPΔΔPstI lacking open reading frame 152 and pe38 (Fig. 2B, pBSPΔΔHpaEsp3I) did not give activity greater than that seen with ie-1 alone (Fig. 2A) and suggested that either orf152 or pe-38 was the source of the additional stimulation.

Transfection of pie2fs, which expresses only orf152, did not show any stimulation of CAT expression, indicating that pe38 was responsible for the observed trans-activation. Thus, ie-1 has a stimulatory effect on transient cat gene expression from the hcf-1 promoter and, and ie2 and pe38 augment this effect. Neither ie2 nor pe38 had any effect on the hcf-1 promoter in the absence of ie1 (Fig. 2, pie2 and pBSPΔΔPstI) and whether the effects of ie2 or pe-38 are exerted directly in the hcf-1 promoter or indirectly through stimulation of ie1 expression has not been resolved. Orf121, which was reported to be an AcMNPV trans-activator (15), did not show any effect on the expression from the hcf-1 promoter (data not shown).

Cellular localization of HCF-1. HCF-1 was localized to the nucleus of both transfected and infected TN368 cells (Fig. 3H...
and I). TN368 cells were transfected with a plasmid expressing either an N- or C-terminally HA-tagged HCF-1 and stained for HA-tagged proteins at 24 h posttransfection and 2 h post-heat shock (Fig. 3B and C). Staining for HCF-1 clearly showed localization to the nucleus as shown by DAPI staining of the same cells (Fig. 3E and F). However, virus infection had a striking influence on the staining pattern and induced HCF-1 aggregation into nuclear foci. Figures 3H and I show duplicate experiments in which TN368 cells were infected with a virus that expressed an N-terminally tagged HCF-1 protein. The cells were stained at 12 h postinfection and show a punctate nuclear staining pattern. Controls of untransfected or wild-type AcMNPV L1-infected cells showed no significant staining with anti-Flag antibody (Fig. 3A and G).

HCF-1 mutagenesis and functional analysis in a transient late-gene expression assay. hcf-1 is required for transient late-gene expression in TN368 cells (27). To analyze amino acids in HCF-1 that are required for activity in transient assays we made a panel of hcf-1 genes containing point mutations (Fig. 4B). HCF-1 is a cysteine-rich protein in which several of the cysteine residues reside in CxxC motifs, where C represents a cysteine residue and x represents any other amino acid. These motifs are often involved in structural stability of the protein through zinc coordination. In addition, the arrangement of several of the cysteine residues is consistent with that of a specific type of RING finger structure.

The initial consensus sequence defined for a RING motif was C3HC4 where the first four cysteines coordinate one metal ion and the histidine and the three remaining cysteines coordinate a second zinc ion. Later studies expanded the definition of the RING motif to include C3HHC3 and C4C4 sequences (16). C4C4 RING domains have been characterized in the NOT4 protein component of the CCR-NOT4 complex and in the p44 subunit of the TFIIB eukaryotic transcription factors (1, 16, 23). HCF-1 has a motif that resembles that of a C4C4 RING consensus and is shown aligned with other confirmed C4C4 RING containing proteins in Fig. 4A. Seven hcf-1 point mutants were made by changing the second cysteine in each CxxC motif to alanine (Fig. 4B, mutants C30A, C47A, C52A, C125A, C154A, C164A, and C215A). Cysteine residues at amino acid numbers 125, 154, 164, and 215 constitute the putative RING domain. When the hcf-1 mutant genes were substituted for hcf-1 in the transient late-gene expression assays, several of the cysteine to alanine mutations caused decreases in CAT expression levels. Mutants C52A, C125A, C164A, and C215A showed background levels of cat gene expression (about 10% of wild-type HCF-1) and were similar to the negative control (lef library without the addition of hcf-1) and C154A had about 20% activity in transient assays (Fig. 4C). Mutant C30A showed no significant difference in activity compared to that seen for wild-type hcf-1 (Fig. 4B and C, mutant C30A) and mutant C47A had about 50% activity. This suggests that CxxC motifs located at amino acids 49 to 52, 122 to 125, 151 to 154, 161 to 164, and 212 to 215 (Fig. 4A, mutants C52A, C125A, C164A, and C215A, respectively) are necessary for efficient hcf-1 activity in the transient late-gene expression assay, and the motif at amino acids 44 to 47 has a supporting role.

All of the cysteine residues predicted to form a RING finger had strong effects on transient late-gene expression, which supports the notion that they may form a metal-coordinated structure that is required for the activity of HCF-1. It is also possible that some of the point mutations may have destabilized the protein, leading to inactivity through degradation.
In addition to the cysteine residues, we also analyzed 17 charged-to-alanine substitutions. Mutations were made to charged residues in regions that had at least two charged amino acids within a window of five (Fig. 4B, mutants 1 to 17). In transient late-gene expression assays most of the hcf-1 mutants showed over 50% activity, suggesting that the groups of charged amino acids did not form essential structures. Two charged-to-alanine mutations, 11 and 13 (having charged-to-alanine mutations at amino acids 173 to 175 and 191 to 193, respectively), showed significant decreases in activity (35 and 12%, respectively). A hydrophobic-to-alanine mutation, 28, was made by mutating four hydrophobic amino acids between amino acids 192 and 197 to alanine (Fig. 4B, mutant 28). This mutant also showed background levels of activity in transient late expression assays.

Point mutations 11, 13, and 28 lie between the third and fourth CxxC motifs of the putative RING domain and thus may form a loop or finger of the zinc finger. The activity of zinc-coordinated structures is often influenced by the amino acid residues within the loop regions of RING structures, and our results indicate the amino acids mutated in mutants 11, 13, and 28 represent important sites of activity within a putative loop structure.

One additional hcf-1 mutant which had a C-terminal truncation, was made by introducing a frameshift at amino acid 185 (Fig. 4B, mutant 29). Truncated hcf-1 (Fig. 4C, mutant 29) also showed background levels of cat gene expression in transient late-gene expression assays. This is not unexpected since this mutant is missing an essential CxxC motif located at amino acids 212 to 215.

**Functional analysis of HCF-1 mutants for occlusion body production.** Several of the hcf-1 mutations were inserted into the viral genome and assessed for activity in the context of a viral infection. Viruses were scored for their ability to produce polyhedral occlusion bodies in infected TN368 cells at 24, 48, and 72 h postinfection (Fig. 5). The results supported those seen in the transient late-gene expression assay. hcf-1 mutant C30A, which showed full activity in the transient late-gene expression assay was able to support occlusion body production in 100% of infected TN368 cells at 24, 48, and 72 h postinfection (Fig. 5, mutant C30A) and the infection was indistinguishable from that of a wild-type virus (Fig. 5, AcMNPV). In infections with hcf-1 null mutant viruses vhcf-1del and vXAnFgCATBsu2, most cells failed to show occlusion body formation. However, the block was not complete and a few cells contained a small number of occlusion bodies at 48 or
72 h postinfection. We have defined this phenotype as hcf-1 null. The reason that we observed polyhedral, albeit very few, in vHcF-1del virus-infected TN368 when a previous report observed a lack of occlusion body production (29) has not been resolved, but may be the result of differences in the characteristics of TN368 cells that may happen over time and several passages.

Mutants C52A, C125A, C164A, C215A, and 29 were inactive in transient late-gene expression assays (Fig. 4, mutants C52A, C154, C125A, C164A, C215A, and 29), and in the context of a viral infection showed an hcf-1 null phenotype in TN368 cells (Fig. 5, C52A, C125A, C164A, C215A, and 29). Mutant C154A, which showed 20% activity in transient late-gene expression assays (Fig. 4, mutant C154A), had a phenotype in TN368 cells in which the infected cells had more occlusion bodies than an hcf-1 null phenotype, but far fewer cells produced small numbers of occlusion bodies and the infection was impaired compared to infections with wild-type viruses (Fig. 5, mutant C154A versus AcMNPV and vXAFgHcF-1BsU2). Mutants C47A, which had 50% activity in the transient late-gene expression assay (Fig. 4, mutant C47A), had an intermediate phenotype in infected cells. Specifically, it showed a delay in occlusion body production in TN368 cells, but by 72 h postinfection, the level of occlusion body production was indistinguishable from that of a wild-type virus infection (Fig. 5, mutant C47A).

Substitutions of cysteine residues and truncation of proteins can have severe effects on protein conformation, protein stability, and protein expression levels. When we analyzed HCF-1 protein levels by immunoprecipitation from mutant virus-infected TN368 cells, the inactive or partially active hcf-1 mutants (C52A, C125A, C154A, C164A, C215A, and 29) showed lower expression levels than viruses expressing nonmutant Flag-tagged HCF-1 species (Fig. 6A). Thus, it is possible that the mutant hcf-1 gene products are inactive due to reduced HCF-1 protein levels. Alternatively, the low HCF-1 expression may have been due to an impaired virus infection, leading to poor expression from the hcf-1 promoter.

To address this issue, we examined the ability of overexpressed HA.11 epitope-tagged HCF-1 protein to complement an infection with the hcf-1 null mutant virus vHcF-1del and induce occlusion body. HA-tagged protein levels were analyzed by Western blot at 2.5 and 8.5 h post-heat shock (5 and 11 h postinfection, respectively) (Fig. 6B, top and middle, respectively) in pHShAHis-hcF-1 wild-type- and mutant-transfected TN368 cells. HCF-1 protein levels were equal to or greater than those seen in cells infected with a virus that expressed wild-type-tagged HCF-1 (Fig. 6A). Thus, it is possible that the mutant hcf-1 gene products are inactive due to reduced HCF-1 protein levels. Alternatively, the low HCF-1 expression may have been due to an impaired virus infection, leading to poor expression from the hcf-1 promoter.

In complementation experiments, cells were transfected with the reporter plasmid pCAPCAT, the late-gene expression library, and a plasmid containing the wild-type hcf-1 gene, pXAbgE36, a Flag- and hexahistidine-tagged hcf-1, pXAmFgHshcF-1Bsu2, or a tagged and mutated hcf-1. The mutants fall into several categories. Mutants 1 to 17 are charged-to-alanine mutations, mutant 28 contains four hydrophobic-to-alanine mutations, mutant 29 contains a frame shift (FS) at amino acid 185 and the rest are cysteine-to-alanine substitutions, CnA, where n is the position of the mutated cysteine resi-
FIG. 5. Micrographs of TN368 cells infected with HCF-1 wild-type and mutant viruses taken at 24, 48, and 72 h postinfection. The time that the photo was taken is listed at the top of each column, and the virus used in the infection is listed beside each row. The row labeled AcMNPV shows wild-type virus-infected cells and vhec-1del are cells infected with an hcf-1 null mutant virus. Micrographs labeled vXAFgCATBsu2 were infected with an hcf-1 null virus in which hcf-1 has been removed and replaced with N-terminally tagged cat. Micrographs labeled vXAFghcf-1Bsu2 are cells infected with a recombinant virus containing N-terminally tagged unmutated hcf-1. The rest of the micrographs are of cells infected with mutant viruses that are identical to vXAFghcf-1Bsu2 with the addition of the indicated point mutation.
The results for presence or absence of cells having wild-type levels of occlusion body production, was able to complement vhcf-1del and restore wild-type occlusion body production in some cells. These results suggest that the mutant phenotype of vxA-FLAG-C47A and vxA-FLAG-C154A may have been due to reduced levels of HCF-1 protein expression. Alternatively, the complementation assay may not have been sensitive enough for detection of intermediate phenotypes and temporal differences.

**HCF-1 self-association.** The HCF-1 amino acid sequence contains a motif suggesting that it forms a RING finger. Since RING-containing proteins are often involved in protein-protein interactions, we analyzed the possibility that HCF-1 may bind to other late expression factors. We and others did not detect significant binding of HCF-1 to other late expression factors using the conditions described (19; data not shown), we have observed significant binding of HCF-1 with itself (19). We transfected cells with plasmids that express either N-terminally HA.11 epitope-tagged versions of CAT or HCF-1 or a C-terminally HA.11-tagged HCF-1 and subsequently infected with a virus that expresses either a Flag-tagged version of HCF-1 or a Flag-tagged version of CAT.

We immunoprecipitated the Flag-tagged proteins with Flag resin and found that Flag-tagged HCF-1 could coprecipitate HA-tagged HCF-1 proteins (Fig. 7A, lanes 3 and 4) but failed to coprecipitate HA-tagged CAT proteins (Fig. 7A, lane 2). In other negative control samples HA-tagged HCF-1 was not coprecipitated with Flag-tagged CAT (Fig. 7A, lanes 5 and 6). Similar results were observed in coinfection experiments in which cells were infected with two viruses, each expressing either Flag-tagged HCF-1 or Flag-tagged CAT and HA.11-tagged HCF-1 or HA.11-tagged CAT (Fig. 7A, lanes 7 to 10). The interaction of HCF-1 with itself was also seen when proteins were expressed by cells cotransfected with plasmids expressing Flag-tagged and HA.11-tagged HCF-1 or HA.11-tagged CAT (Fig. 7B); thus, virus infection is not required for HCF-1 self-association.

In addition, self-interaction of HCF-1 was shown in vitro. Glutathione S-transferase (GST)-tagged HCF-1 was grown in bacteria, and GST and GST-HCF-1 were purified from cell extracts (expression levels in extracts are shown in Fig. 7C, lanes 7 and 8) using GST-Sepharose beads (Fig. 7C, lanes 5 and 6). Extracts from cells infected with vXAc-a-HAHCf-1 (HA.11-tagged HCF-1 levels shown in Fig. 7C, lanes 3 and 4) were subsequently added to the GST-HCF-1 beads. A greater amount of HA.11 epitope-tagged HCF-1 was eluted from the Sepharose beads that had been bound to GST-HCF-1 (Fig. 7C, lane 2) than the background amount that bound to GST alone (Fig. 7C, lane 1).

**Self-association activity of hcf-1 requires cysteine residues implicated in RING finger formation.** The role of cysteine residues in HCF-1 self-association activity was also analyzed (Fig. 8). Coprecipitation experiments showed that HA.11-tagged versions of mutants C30A and C47A were able to bind to unmutated Flag-tagged HCF-1 expressed from the recombinant virus vXA-n-FlagHisHcf-1 (Fig. 8A, lanes 5 and 6). However, very little HA.11-tagged HCF-1 mutants C52A, C125A, C154A, C164A, C215A, and 29 were coprecipitated by Flag-tagged HCF-1. (Fig. 8A, lanes 7, 8, 17, 18, and 19). Coprecipitation of a reduced quantity of the mutant HCF-1 proteins was not a result of poor expression or instability since the
FIG. 7. Coprecipitation of HA.11-tagged HCF-1 with Flag-tagged HCF-1. Viruses and plasmids were used to transfect and then infect TN368 cells shown in panel A, and plasmids were used to cotransfect TN368 cells shown in panel B. In both A and B the top blot shows the HA.11-tagged coprecipitated proteins. Flag-tagged proteins were immunoprecipitated from the cell extracts by using anti-Flag resin and probed with anti-HA.11 monoclonal antibody. (A, middle blot) and (B, middle blot) show the expression levels of HA.11-tagged proteins in 10% of the cell extracts. The blots shown on the top row were stripped and reprobed with anti-Flag antibody to show the precipitated Flag-tagged proteins (A and B, bottom blots). (C) In vitro precipitation of HA.11-tagged HCF-1 from transfected cell extracts by using Sepharose beads bound to bacterially expressed GST-HCF-1. Western blot of HA.11-tagged HCF-1 precipitated with Sepharose beads that had been bound to GST alone (1) or to GST-HCF-1 (2); 10% of the vXA-c-HAHishcf-1-infected cell extract was probed with anti-HA.11 antibody (lanes 3 and 4) to show expression of HA.11-tagged HCF-1. Expression of GST and GST-HCF-1 in 10% of the bacterial cell extracts is shown in lanes 7 and 8, and lanes 5 and 6 show GST and GST-HCF-1 bound to Sepharose. The locations of the GST, GST-HCF-1, and HA-11-tagged HCF-1 bands are indicated.

FIG. 8. Coprecipitation of HA.11-tagged HCF-1 with Flag-tagged mutant HCF-1. The combinations of plasmids and viruses used to transfect and then infect cells are indicated at the top of the figure. The top blots (A) show the anti-Flag resin precipitated proteins probed with anti-HA.11 antibody and the coprecipitated proteins. The middle blots (B) show the expression levels of HA.11-tagged protein in 10% of the extracts before precipitation and the bottom blots (C) show the precipitated Flag-tagged proteins. The locations of the HA.11- and Flag-tagged HCF-1 and CAT proteins are marked with arrows.
levels of HA.11- and Flag-tagged HCF-1 protein in the extracts were comparable (Fig. 8B and C). Significantly, all of the cysteine residues within the predicted RING finger motif were required for HCF-1 self-association. However, both self-association and cysteine 154 were required for HCF-1 gene repression activity but not for self-association and cysteine 154 was required for self-association but not for gene repression activity. Thus, self-association is not required for gene repression activity and gene repression activity is not required for HCF-1 self-association. However, both self-association and promoter repression activities are required for the full activity of HCF-1 in AcMNPV-infected TN368 cells.

Mutants C52A, C125A, C164A, and C215A were unable to perform either function, and viruses that expressed these hcf-1 mutants produced hcf-1 null levels of occlusion bodies in infected TN368 cells. HCF-1 mutants that were able to either self-associate or repress gene expression had a less severe phenotype in infected cells than those that could do neither. Mutant C47A failed to repress expression from phcf-1CAT, which expresses the lacZ gene. Levels of cat gene expression in transfections in which 10 μg of the balancer plasmid was added were defined as 100% (Fig. 9, pHSPlacZ).

Overexpression of HCF-1 from the HSP70 promoter led to a 75% reduction in CAT expression from the hcf-1 promoter (Fig. 9, pHSHAHishcf-1). Other constructs expressing tagged wild-type hcf-1 gave similar results (Fig. 9, pHSflagHishcf-1 and pHS-c- HAHishcf-1). The repression activity of hcf-1 was titratable, having a more pronounced effect when 10 μg of plasmid expressing HCF-1 was transfected than when 5 μg of HCF-1 expressing plasmid plus 5 μg of balancer plasmid pHSPlacZ was added (Fig. 9, pHSflagHishcf-1 and pHS-c- HAHishcf-1, histogram 10 versus 5). Overexpression of HCF-1 did not repress CAT expression from several other viral promoters, specifically ie1, lef-3, and lef-7 (data not shown), indicating that the effect of HCF-1 on promoter activity is not due to a generalized effect on viral promoters.

The ability of the mutant hcf-1 genes to trans-repress the hcf-1 promoter was analyzed. HCF-1 cysteine mutants C30A and C154A were able to repress CAT expression approximately 60% (Fig. 9, mutants C30A and C154A). However, many of the hcf-1 mutants (C47A, C52A, C125A, C164A, and C215A) were unable to repress cat gene expression (Fig. 9, C47A, C52A, C125A, C164A, and C215A). Interestingly, overexpression of HCF-1 truncation mutant 29 led to an activation of transient expression from the hcf-1 promoter (Fig. 9, mutant 29).

Many of the cysteine residues that are required for HCF-1 directed gene repression were also required for self-association and occlusion body production. Table 2 shows the composite functional analyses results for each cysteine residue mutation. Cysteine residues at positions 52, 125, 164, and 215 were required for both self-association and gene repression activity. However, cysteine at position 47 was required for HCF-1 gene repression activity but not for self-association and cysteine 154 was required for self-association but not for gene repression activity. Thus, self-association is not required for gene repression activity and gene repression activity is not required for HCF-1 self-association. However, both self-association and promoter repression activities are required for the full activity of HCF-1 in AcMNPV-infected TN368 cells.

\begin{table}
\centering
\caption{HCF-1 characteristics}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{HCF-1} & \textbf{Self-association activity} & \textbf{Repression activity} & \textbf{Occlusion body phenotype}\textsuperscript{a} \\
\hline
Wild type & + & + & Wt \\
C30A & + & + & Wt \\
C47A & + & - & 24-h delay \\
C52A & - & - & Null \\
C125A & - & - & Null \\
C154A & - & + & Impaired \\
C164A & - & - & Null \\
C215A & - & - & Null \\
29T & - & - & Null \\
\hline
\end{tabular}
\textsuperscript{a} Wt, wild type.
\end{table}
but was capable of self-association. In infected cells, viruses bearing this hcf-1 mutation showed a delay in occlusion body production of up to 24 h. Conversely, mutant C154A was able to repress transient cat gene expression but showed impaired self-association. Again, a less severe phenotype was observed in viruses carrying hcf-1 with this mutation since occlusion bodies were observed but in only 20 to 40% of infected cells, compared to 100% in wild-type virus-infected cells.

**DISCUSSION**

The baculovirus protein HCF-1 is required for replication of the virus in TN368 cells but is dispensable for replication in SF21 cells (27, 29). In TN368 cells hcf-1 mutant viruses are defective in replication and virus production. In addition, there is an apparent total shutdown of host and viral protein synthesis by 18 h postinfection (29). The total shutdown of host protein synthesis is reminiscent of that seen in SF21 cells infected with a virus lacking the antiapoptosis gene p35 and thus hcf-1 may play a role in regulation of the host cell, perhaps through counteracting a host defense mechanism. To obtain insight into the function of HCF-1, we have investigated the expression pattern and localization of HCF-1 in AcMNPV-infected and uninfected SF21 and TN368 cells.

An understanding of the functional role of the hcf-1 gene product in TN368 cells during a baculovirus infection requires a detailed knowledge of the expression pattern and regulation of hcf-1 in infected cells. The hcf-1 gene is expressed early in the infection cycle of AcMNPV in TN368 and SF21 cells and was present in infected cells from 2 to 24 h postinfection. Expression of hcf-1 early in the virus life cycle is consistent with a role for HCF-1 in late-gene transcription and viral DNA replication. Like many early baculovirus genes, transient expression from the hcf-1 promoter was stimulated by the expression of the viral trans-activators IE1, PE38, and IE2. Primer extension analysis showed that hcf-1 transcription initiates at a CA-C sequence located 24 and 35 base pairs downstream of two TATA sequence elements.

The hcf-1 gene product has significant sequence similarity with only one homologous gene present in a virus variant of AcMNPV called *Rachiplusia ou* multiple nucleopolyhedrovirus (RoMNPV) (AY145471) (17). RoMNPV and AcMNPV hcf-1 share 84% amino acid sequence identity and 91% conservation and notably share a prevalence of cysteine residues, several of which are present in CxxC motifs. We suggest that the important cysteine residues in HCF-1 may form a RING finger-like structure. The original motifs to define RING finger domains were C3HC4 and C3HHC3 (RING H2) (17) and were later expanded to include C4C4 sequences (16). HCF-1 does not contain a C3HC4 or C3HHC3 RING motif since it lacks a suitably located histidine residue. However, a search of the Superfamily database (36) predicted that amino acids 122 to 168 may form a RING/U-box domain (E-value 0.11).

Alignment of two available HCF-1 protein sequences with proteins known to contain C4C4 RING domains, NOT4, a protein with ubiquitin ligase activity which is a component of the CCR-NOT complex, and the p44 subunit of the TFIIH transcription/repair complex showed a predicted RING motif located between residues 122 and 215 (Fig. 4A). HCF-1 contains cysteine residues in the correct context, however, the spacing of the cysteine CX2C motifs is different than in those of the other C4C4 RING-containing proteins. RING finger proteins have several prescribed functions including self-association and ubiquitin ligation. Mutational analysis of HCF-1 showed that all of the CxxC motifs present in the putative RING like domain are essential for HCF-1 self-association activity and suggest that self-association activity may be mediated by the putative RING finger structure. Confirmation that HCF-1 forms a RING finger remains to be determined by using structural analysis techniques.

The structure of RING finger domains is stabilized through the interaction between zinc ions and cysteine and histidine residues, however, the functions of the proteins are often dictated by the amino acids located in the so-called loop regions, the amino acids between the CxxC domains. Interestingly the region of HCF-1 that corresponds to one of the loop regions of the putative RING finger domain contained the only three other mutations, 11, 13, and 28, that had a significant effect on HCF-1 activity in the transient late-gene expression assay. Analysis of these mutations will be valuable to further define the activity of HCF-1.

In the context of a virus infection we have determined that several cysteine residues present in CxxC arrangements, specifically those located at amino acid numbers 52, 125, 154, 164, and 215, are essential for high levels of occlusion body formation in TN368 cells. The cysteine residue at position 47 had a less severe influence and thus appeared to have a supporting role in the activity of the protein. We have also shown that the same set of cysteine residues are required for protein self-association, specifically those located at positions 52, 125, 154, 164, and 215. Interestingly four of the cysteines in this group are also those that we predicted to form a RING finger structure. RING domains are known to mediate protein-protein thus the putative RING domain may function in HCF-1 self-interaction activity.

Interestingly, a previous report showed self-association activity by HCF-1 truncation mutants which would have lacked cysteine residues which we found to be essential for this activity (19). These data suggest the possibility that other regions of the HCF-1 protein may also be capable of self-association activity. In addition, it is possible that differences in the data between these two studies may stem from the different methods used to coexpress the wild-type and mutant HCF-1. In this study, we expressed tagged mutant HCF-1 using a transfected plasmid and expressed tagged wild-type HCF-1 by infecting cells with a recombinant virus. In the earlier report the HCF-1 species were both overexpressed from transfected plasmids. Perhaps differences in protein expression levels or the presence or absence of an active infection affected the results.

In addition to HCF-1 self-association activity, we also found that HCF-1 had gene repression activity on the hcf-1 promoter when overexpressed in TN368 cells. Gene repression activity was also abolished by mutation of an overlapping, but distinct set of cysteine residues in HCF-1 (summarized in Table 2). Gene repression activity by HCF-1 was observed in an overexpressed context so must be interpreted with caution, however, a mutation (C47A) that abolished HCF-1 gene repressor activity but did not disrupt HCF-1 self-association correlated with reduced activity of HCF-1 in transient viral gene expression assays and caused a delay in occlusion body production in
TN368 cells. Thus, it appears that the apparent gene repression activity is a necessary function for efficient occlusion body production in TN368 cells. In addition, a cysteine-to-alanine mutation (C154A) that abolished HCF-1 self-association but did not affect gene repressor activity led to impaired but greater than null levels of occlusion body production in infected TN368 cells. Thus, HCF-1 self-association activity is also required but not sufficient for efficient occlusion body production in TN368 cells. Only HCF-1 mutants in which both self-association and gene repression activity were abolished showed a viral phenotype which resembled that of the null mutant virus. Thus, both self-association and gene repression activities appear to be required for HCF-1 function. Since the sets of cysteine residues that are essential for gene repression and self-association differ, the two activities may require different structures within the protein.

By indirect immunofluorescent labeling, we have localized HCF-1 to punctate nuclear structures within infected cells. HCF-1 localization to nuclear foci is dependent on virus infection since HCF-1 expressed in the absence of an infection showed staining throughout the nucleus. The baculovirus proteins IE2, DBP, and LEF-3 have also been localized to punctate structures in infected cells which were identified as sites of viral DNA replication (31). Our data are also consistent with the possible localization of HCF-1 to within viral replication centers, however, coimmunoprecipitation experiments have failed to show HCF-1 association with components of the baculovirus replication machinery (19) (data not shown). Whether HCF-1 colocalizes with virus replication centers remains to be determined.

An alternative explanation for the punctate staining pattern of HCF-1 could be the association of HCF-1 with another virus-induced nuclear structure. The replication centers of many DNA viruses reside at the periphery of PML nuclear bodies, also known as PODs or ND1 (14). PML nuclear bodies are punctate structures found in the nucleus of mammalian cells and are believed to play a role in cell growth control and innate immunity. Many viruses express proteins that disrupt PML (14, 40) and this may be a general mechanism to counteract innate viral defense mechanisms. Whether HCF-1 associates with or alters PML nuclear body structures in insect cells is an open question since HCF-1 expressed in the absence of an infection appeared to self-associate differ, the two activities may require different cysteine residues that are essential for gene repression and self-association.

The precise role of the hcf-1 gene in the replication of baculovirus in TN368 cells is unknown. Several other viral RING containing proteins have E3 ubiquitin ligase activities that function to target host genes for degradation and benefit virus growth. For example, herpesvirus protein ICP0 resides within PML nuclear structures, is a RING finger E3 ubiquitin ligase, and alters host and viral transcription by targeting several host proteins, including p53 and IκB, for degradation and thus promotes virus replication (5, 11). In addition, paramyxovirus V proteins contain several cysteine residues capable of binding zinc, and several have been ascribed functions related to direct inactivation of STAT molecules through inactivation or degradation (22). Like hcf-1, paramyxovirus V proteins have no recognizable cellular homologues.

It will be interesting to determine if baculovirus HCF-1 has ubiquitin E3 ligase activity and if this activity targets components of the insect innate immune system in ways similar to virulence proteins of mammalian viruses. Many of the components of the cellular innate immune system, such as Toll-like proteins were first identified in insects, and we believe that insects will continue to be a powerful model system for the investigation of innate immune defense pathways.

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