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Virology 367 (2007) 265–274
VIROLOGY
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Inter-subunit interactions of the *Autographa californica* M nucleopolyhedrovirus RNA polymerase

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Received 29 January 2007; returned to author for revision 2 February 2007; accepted 18 May 2007
Available online 2 July 2007

Abstract

Autographa californica M nucleopolyhedrovirus transcribes genes using two DNA-directed RNA polymerases; early genes are transcribed by the host RNA polymerase II, and late and very late genes are transcribed by a viral-encoded multisubunit RNA polymerase. The viral RNA polymerase is composed of four proteins: Late Expression Factor-4 (LEF-4), LEF-8, LEF-9, and P47. The predicted amino acid sequences of *lef-9* and *lef-8* contain motifs that are similar to those that participate at the catalytic center of known RNA polymerases. The requirement for the motif present in LEF-8 in late gene expression has been previously demonstrated. We have assessed the requirement of specific residues within the motif in LEF-9 for late gene expression. The conserved aspartic acid residues within the LEF-9 motif, corresponding to those essential for activity of the *Escherichia coli* RNA polymerase largest subunit, were required for late gene expression. Furthermore, we found that LEF-8 and LEF-9 interacted in coimmunoprecipitation experiments. We determined possible interactions of all the RNA polymerase subunits in pairwise combinations and found associations between LEF-9 and P47, LEF-4 and P47, and LEF-8 and P47. In contrast, LEF-4 and LEF-8 did not coimmunoprecipitate but coimmunoprecipitated in the presence of P47, suggesting that they do not associate directly. A weak association was observed between LEF-4 and LEF-9. Further analysis also suggested that LEF-8, LEF-9, and P47 have the ability to self-associate. Studies on protein-protein interactions may provide insight into the structural design of the complex and mechanistic aspects affecting late and very late gene expression.

Keywords: Baculovirus; RNA polymerase; Late gene expression

Introduction

Transcription of *Autographa californica* M nucleopolyhedrovirus (Ac*M*NPV) genes takes place in three temporal phases, early, late, and very late, during the infection cycle. Transcription of late and very late genes can be distinguished from that of early genes by their requirement for protein and viral DNA syntheses (Rice and Miller, 1986). Moreover, transcription of early and late or very late genes is carried out by distinct transcriptosomes, where early genes are transcribed by the cellular RNA polymerase II and the late and very late genes are transcribed by a viral DNA-directed RNA polymerase (Beniya et al., 1996; Grula et al., 1981; Grula and Weaver, 1981; Huh and Weaver, 1990; Yang et al., 1991).

The subunits of the DNA-directed RNA polymerase were initially identified by either characterizing viruses with temperature sensitive mutations (Carstens et al., 1994; Knebel-Mörsdorf et al., 2006; Partington et al., 1990) or by their requirement in transient late gene expression assays (Lu and Miller, 1994; Passarelli and Miller, 1993a; Passarelli et al., 1994; Todd et al., 1995). Subsequently, purification of factors necessary to transcribe a late promoter in vitro identified the polypeptides in the viral RNA polymerase as four previously identified late gene transcription factors: Late Expression Factor-8 (LEF-8), LEF-9, LEF-4, and P47 (Guarino et al., 1998b). This four-subunit complex was sufficient to recognize a late gene promoter, bind DNA, and synthesize a late message in vitro (Guarino et al., 1998b).

Two subunits, LEF-8 and LEF-9, encode motifs with homology to those present in other known RNA polymerases (Lu and Miller, 1994; Passarelli et al., 1994). In other RNA polymerases

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doi:10.1016/j.virol.2007.05.026

these motifs participate at the active center including the coordination of Mg^{+2} during transcription (Lu and Miller, 1994; Passarelli et al., 1994; Zaychikov et al., 1996). A 13-residue sequence required for activity of LEF-8 in transient late gene expression assays (Titterington et al., 2003) is also conserved in the *Escherichia coli* RNA polymerase β subunit region H (Passarelli et al., 1994). *lef-9* encodes a motif similar to the "invariable" RNA polymerase motif, NADFDGF, in the *E. coli* RNA polymerase β′ subunit and other RNA polymerase largest subunits (Lu and Miller, 1994). However, the role of this motif in late gene transcription has not been explored.

The other two subunits, LEF-4 and P47, have been characterized to different extents. LEF-4 has activities associated with 5′ capping, guanylyltransferase and RNA triphosphatase activities (Gross and Shuman, 1998; Guarino et al., 1998a; Ho et al., 1998; Jin et al., 1998). The specific function of *p47* in late gene transcription is not known.

DNA-directed RNA polymerases are the engines responsible for gene transcription and they play a major role in gene regulation. RNA polymerase II is a multisubunit complex of 8 to 14 subunits that interacts with promoter recognition and processivity factors. In contrast, the baculovirus RNA polymerase is a multisubunit and multifunctional four-subunit enzyme complex, and baculoviruses are the only nuclear-replicating viruses that encode a functional DNA-directed RNA polymerase. There is no significant overall sequence homology between the baculovirus RNA polymerase subunits and other RNA polymerases, except for the two motifs described above, implying that active site sequence conservation is essential for function.

In this study we were interested in defining the Ac*M*NPV RNA polymerase subunit interaction map to gather information on the overall architecture of this novel enzyme complex. In addition, we performed experiments to define the region(s) of LEF-8 that interfaced with two interactive partners. Finally, we altered the putative catalytic domain of LEF-9 to evaluate its contribution to late gene expression.

Results and discussion

Mutagenesis of the RNA polymerase motif within LEF-9

LEF-9 predicts a seven-amino acid region (NTDCDGD) that is also conserved in the β′ subunit of the *E. coli* RNA polymerase and largest subunit of the eukaryotic RNA polymerase II (NADFDGD) and the site coordinating the 3′-OH of the nascent RNA and the α-phosphate of the NTP being incorporated (Cramer et al., 2001). The aspartic residues bind Mg^{2+} at the catalytic center (Cramer et al., 2001). This region is also highly conserved in the predicted *lef-9* polypeptides of all sequenced baculovirus genomes (Fig. 1). Although there are slight differences in this sequence among different viruses, the three aspartic acids are invariable. We introduced alanines in place of aspartic acid residues at positions 282, 284, and 286 in the Ac*M*NPV LEF-9 NADFDGD motif to determine if the residues were necessary for late gene expression (Fig. 2A). The results indicated that mutation of the aspartic acid residues reduced late promoter activity to background levels (Fig. 2B, compare column 1 to columns 3 to 5). All of the LEF-9 mutants were

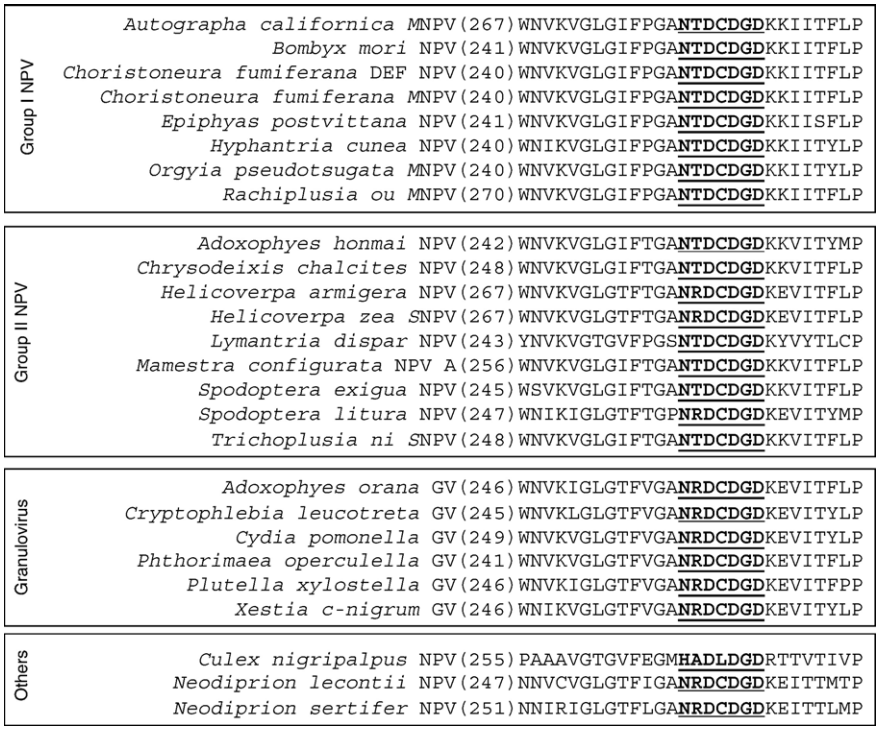

Fig. 1. Alignment of LEF-9 sequences. The region of Ac*M*NPV LEF-9 that contains a seven amino acid region also conserved in other RNA polymerases, NADFDGD, was aligned with selected baculovirus LEF-9 sequences from the baculoviruses indicated on the left. The position of the first amino acid in each region is listed in parentheses.

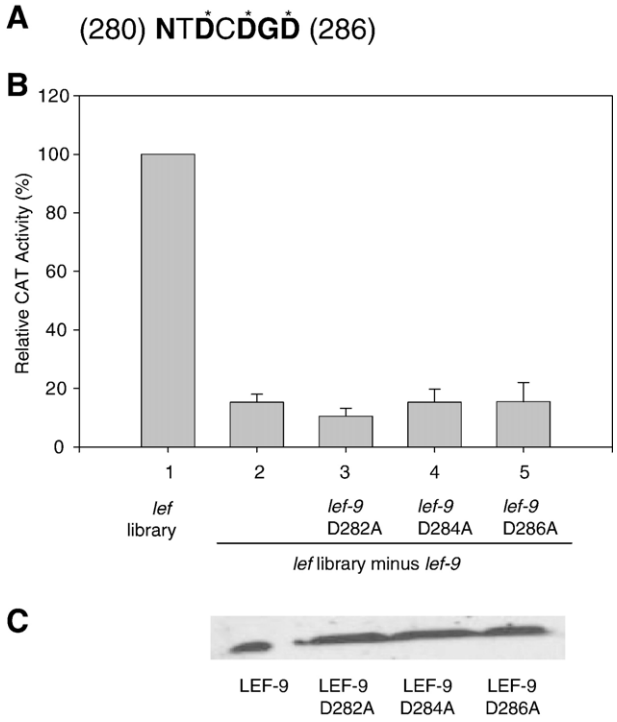

Fig. 2. Effects of mutagenesis of the LEF-9 RNA polymerase conserved motif on late gene expression. (A) Amino acid sequence of the LEF-9 RNA polymerase conserved motif. Amino acids in bold-type indicate residues conserved with those in the corresponding motif of the *E. coli* RNA polymerase largest subunit. The positions of the first and last amino acids in this LEF-9 sequence are shown in parentheses. The asterisks indicate aspartic acids that were altered and tested for their effect on *lef-9* activity. (B) A plasmid containing the late major capsid promoter controlling the chloramphenicol acetyltransferase (*cat*) gene, pCAPCAT, was transfected into SF-21 cells with the *lef* library (column 1) or the *lef* library lacking *lef-9* (columns 2 to 5) as indicated below the graph. Plasmids containing *lef-9* with an aspartic acid to alanine mutation were substituted for *lef-9* as indicated (columns 3 to 5). A relative value of 100% CAT activity was assigned to the activity obtained from the activation of the late promoter by the complete *lef* library (column 1). Bars indicate standard error from at least three independent experiments. (C) Immunoblots showing expression from plasmids expressing *lef-9* or the *lef-9* mutant constructs are shown. SF-21 cells were transfected with each plasmid and LEF-9 in each lysate was detected by immunoblotting using anti-HA.11 monoclonal antibody.

expressed as assessed by immunoblotting (Fig. 2C). Therefore, this region is essential for LEF-9 late gene expression, but its involvement at the catalytic center of the enzyme complex and in binding Mg^{2+} remains to be shown.

Coimmunoprecipitation strategy and setup

In order to monitor protein interactions among Ac*M*NPV RNA polymerase subunits (Figs. 3 and 4), we transfected two plasmids (unless otherwise noted) into SF-21 cells carrying either a FLAG- or an HA-tagged gene. Cells were lysed with 1% Triton X-100 lysis buffer. To monitor nuclear envelope lysis, we tested if the Ac*M*NPV nuclear protein, LEF-3 (Wu and Carstens, 1998; Berretta and Passarelli, unpublished results), was detected under the conditions used. LEF-3 was detected using 1% Triton X-100, 1% 3-[3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), or Laemmli loading buffer (results not shown). Thus, our lysis procedure disrupted the nuclear envelope. However, we do not know if each protein was able to localize to the nucleus in the absence of other viral factors. The FLAG-tagged protein was immunoprecipitated with anti-FLAG antibody and the interactive partner, if any, detected by immunoblotting with anti-HA antibody (column 1, top row in each panel). To asses what proportion of the interactive HA-tagged protein was pulled down with the FLAG-bound protein and FLAG antibody, the supernatant of the coimmunoprecipitation was immunoprecipitated with anti-HA antibody and immunoblotted with anti-HA antibody (column 1, middle row in each panel). Finally, to determine expression of the FLAG-tagged protein, we stripped the antibody off the membrane with the coimmunoprecipitation (membrane from the top row) and probed with anti-FLAG antibody to detect expression of the protein used to pull down an HA-tagged partner (column 1, lower row in each panel). We also controlled for non-specific binding of the interactive protein to FLAG antibody by attempting to immunoprecipitate the HA-tagged protein with FLAG antibody in the absence of a FLAG-tagged protein (column 2, top rows). Synthesis of this protein in the assay was verified by immunoblotting the supernatant of the immunoprecipitation with anti-HA (column 2, middle rows). In addition, we performed reciprocal coimmunoprecipitations, that is, we immunoprecipitated the HA-tagged protein and detected the FLAG-tagged partner (columns 3 and 4) and included all the controls described above.

Association of LEF-9 with LEF-8

The highly conserved RNA polymerase regions of LEF-8 and LEF-9 discussed above are thought to make up part of the catalytic region of other RNA polymerases (Markovtsov et al., 1996; Sosunov et al., 2003). Thus, LEF-8 and LEF-9 may interact with each other in a similar manner to that of the largest subunits of the *E. coli* RNA polymerase and the eukaryotic RNA polymerase II. Previous work has shown that the four subunits of the viral RNA polymerase can be isolated from virus-infected cells as a complex, and this complex is transcriptionally active in vitro (Guarino et al., 1998b), suggesting that the subunits interact in a protein complex. Moreover, preliminary results were reported suggesting that *Bombyx mori* NPV LEF-8 and LEF-9 interact (Acharya and Gopinathan, 2002).

We observed that FLAG-tagged LEF-9 and HA-tagged LEF-8 associated (Fig. 3A, columns 1 and 3, top row), but HA-tagged LEF-8 did not bind non-specifically to the anti-FLAG antibody during immunoprecipitation in the controls (Fig. 3A, columns 2 and 4, top row). Next, we immunoprecipitated proteins remaining in the supernatant of the anti-FLAG immunoprecipitation with anti-HA antibody to detect any HA-tagged protein that did not bind during anti-FLAG immunoprecipitation. The results indicated that not all of the HA-tagged LEF-8 or LEF-9 (Fig. 3A, columns 1 and 3, middle rows) bound to the FLAG-tagged LEF-9 or LEF-8, respectively. However, binding was roughly proportional to the

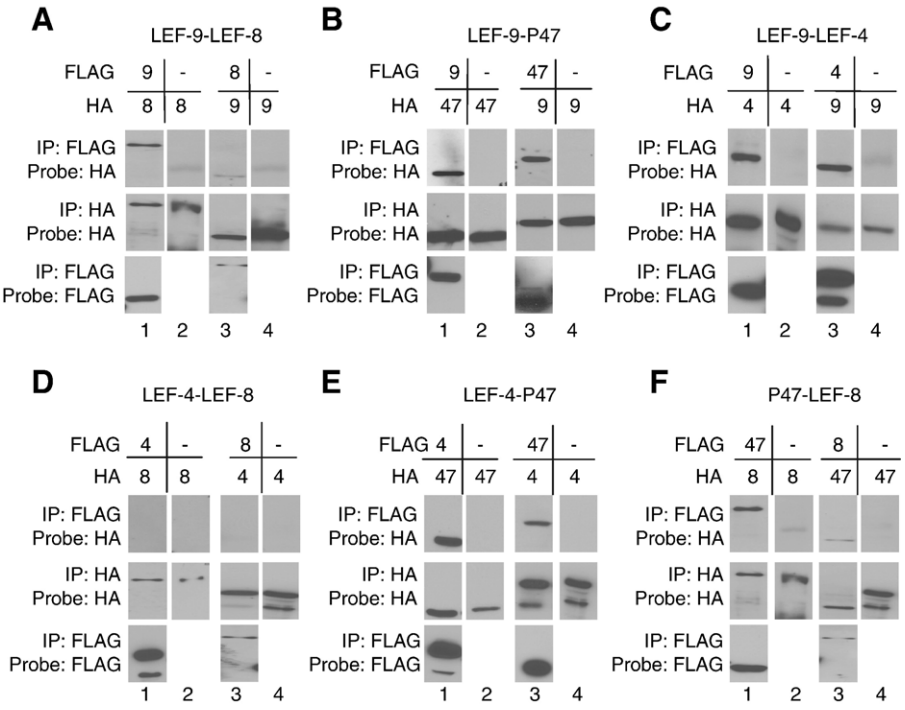

Fig. 3. Association of the RNA polymerase subunits in pairwise combinations. (A to F) SF-21 cells (4×10^6) were transfected with 8 μg of each plasmid as indicated and were immunoprecipitated with anti-FLAG and the immunoprecipitates were immunoblotted using anti-HA antibody (upper rows). The same membranes were stripped and treated with anti-FLAG antibody (lower rows), identifying the protein that was originally immunoprecipitated with the associated proteins, if any, in the upper rows. The supernatants of the immunoprecipitations containing unbound proteins were then immunoprecipitated with anti-HA antibody and immunoblotted with anti-HA (middle rows) to detect unbound HA-tagged proteins not present in the upper panels. The epitope tag on each protein is indicated on the upper left of each panel (FLAG or HA). Plasmids used to transfect SF-21 cells were as follows: pHSFLAGHislef9 (FLAG 9), pHSEpiHislef9 (HA 9), pHSFLAGHislef8 (FLAG 8), pHSEpiHislef8 (HA 8), pHSFLAGHisp47 (FLAG 47), pHSEpip47 (HA 47), pHSFLAGHislef4 (FLAG 4), and pHSEpiHislef4 (HA 4). In cases where only one plasmid was transfected into cells, balancing DNA was included to maintain a constant concentration of DNA per transfection. IP, antibody used for immunoprecipitation; Probe, antibody used in immunoblotting.

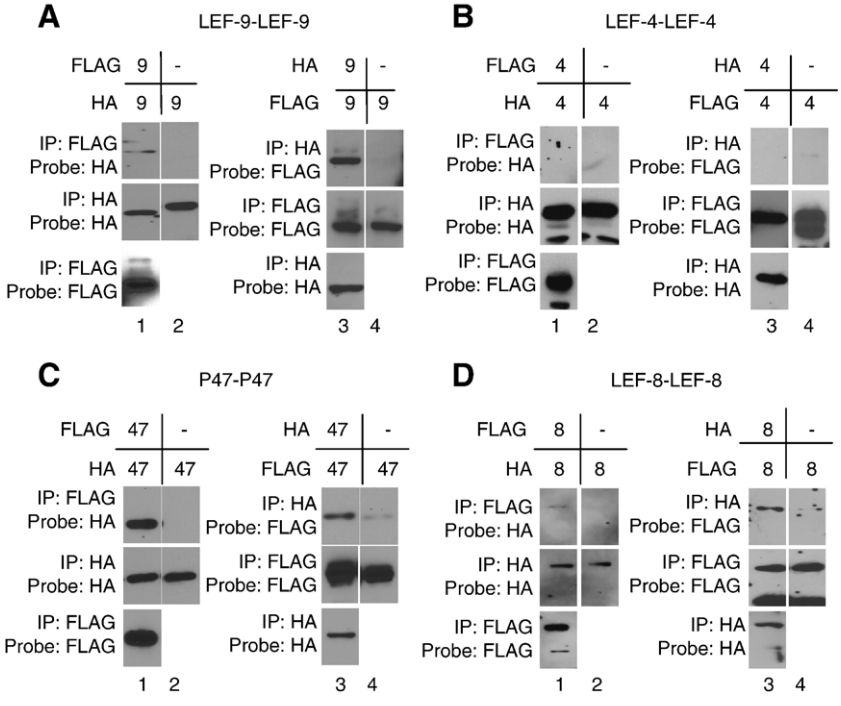

Fig. 4. Homodimer associations between the RNA polymerase subunits. SF-21 cells (4 to 8×10^6) were transfected with 8 to 16 μg of plasmid DNA and immunoprecipitated as described in the legend to Fig. 3.

amount of expressed protein used in the coimmunoprecipitations (Fig. 3A, columns 1 and 3, lower row).

Association of LEF-4, LEF-8, LEF-9, and P47 in heterodimer subcomplexes

To assemble a blueprint of subunit associations, we looked at possible interactions between LEF-4, LEF-8, LEF-9, and P47 in all pairwise combinations (Fig. 3). In addition to the association between LEF-8 and LEF-9 described above (Fig. 3A), we observed associations between LEF-9 and P47 (Fig. 3B, columns 1 and 3), LEF-9 and LEF-4 (Fig. 3C, columns 1 and 3), LEF-4 and P47 (Fig. 3E, columns 1 and 3), and between P47 and LEF-8 (Fig. 3F, columns 1 and 3). In contrast, LEF-4 and LEF-8 did not coimmunoprecipitate (Fig. 3D, columns 1 and 3, top row). Thus, P47 and LEF-9 immunoprecipitated with all three partners, while LEF-8 and LEF-4 only immunoprecipitated with two partners, LEF-9 and P47. In addition, it is possible that the interaction between LEF-9 and LEF-4 is weak, since we observed coimmunoprecipitation of these two molecules 60% of the time and no interaction about 40% of the time after multiple experiments. Furthermore, we were able to disrupt the association between LEF-4 and LEF-9, but not between other protein pairs, by including a non-specific protein, bovine serum albumin (BSA), in coimmunoprecipitations (results not shown). These results suggest that the interaction is not strong using our assay conditions but may not reflect what occurs during virus infection of cells. This interaction may be more stable in the presence of other viral factors during virus infection of cells.

During coimmunoprecipitation experiments, we sometimes observed some non-specific bands present in control experiments (e.g., Fig. 3A, columns 2 and 4 top row; Fig. 3C, column 4, top row; and Fig. 3F, column 2 top row). These were fairly faint and did not comigrate with the expected protein in the reactions. Also, during immunoprecipitation of LEF-4, we sometimes observed a lower band (e.g., Fig. 3C, column 3, lower panel) that reacted with the antibody. This may be a degradation product of LEF-4. In some experiments, we tested two HA-tagged proteins simultaneously, HA-tagged proteins LEF-4 and P47, for non-specificity to anti-FLAG antibody, thus, two proteins are apparent (Figs. 3D, E, and F, columns 4, middle rows).

The prevalence and number of interactions agree with the protein associations required for this enzyme complex to function adequately. The interaction between LEF-8 and LEF-9 may be related to their putative role during nucleotide incorporation. Since there are no clues to the specific function of P47 in transcription, it is difficult to speculate how its placement is important, but it may play a role in tethering the complex together.

Self-associations of LEF-4, LEF-9, LEF-8, and P47

The active and late gene-specific RNA polymerase was purified as a complex with molecular mass of 560,000 (Guarino et al., 1998b). Since all the subunits were purified in equimolar amounts, it was suggested that the active transcription complex was composed of two molecules of each subunit (Guarino et al., 1998b). Thus, we wanted to determine whether each of the four RNA polymerase subunits had the ability to dimerimize or whether they only heterodimerized with one or more different molecules.

Three of the four RNA polymerase subunits interacted with themselves. LEF-9 could immunoprecipitate with itself (Fig. 4A, columns 1 and 3, top row) but was not brought down non-specifically in the control immunoprecipitations (Fig. 4A, columns 2 and 4, top row). Similarly, P47 and LEF-8 appeared to self-associate (Figs. 4C and D); however, we observed weak non-specific pull down of FLAG-P47 and FLAG-LEF-8 during anti-HA immunoprecipitation in some of our experiments (Figs. 4C and D, column 4, top row). Since the extent of the non-specific immunoprecipitation was weak compared to the specific interaction (Figs. 4C and D, compare columns 3 to columns 4, top rows), we consider the interactions to be plausible. Interestingly, LEF-4 did not immunoprecipitate itself (Fig. 4B, compare columns 1 and 3, top and middle rows), suggesting that LEF-4 may not interact with itself in the RNA polymerase complex. LEF-4 dimerization has been reported at physiological salt concentrations; however, the association is weak (Guarino et al., 1998a). It is possible that the conditions did not allow a stable interaction in our reactions. Alternatively, LEF-4 may require other viral factors to localize to the nucleus of cells (Guarino et al., 1998b) or associate with itself.

Given the prediction that the RNA polymerase has 2 subunits of each molecule (Guarino et al., 1998b) and our results where LEF-4 does not coimmunoprecipitate with LEF-8, itself, and weakly with LEF-9, different scenarios may be envisioned. First, two molecules of LEF-4 may be part of the transcription complex but each binds a different subunit (*e.g.*, P47 and LEF-9). Second, LEF-4 interacts with itself during virus infection. It would be interesting to know if both LEF-4 subunits in the RNA polymerase complex participate in capping RNA or if LEF-4 has another function associated with transcription that positions it at another location within the complex. The entire complex containing two subunits of each molecule, as predicted, may be assembled as two juxtapositioned faces where each subunit interacts with itself or only one (or more) subunit(s) interacts with a different subunit(s) of the second complex. We confirmed our results using 1% CHAPS in our lysis procedure (results not shown).

We attempted to verify all coimmunoprecipitations by using an alternative method. The four RNA polymerase genes with either a FLAG or HA tag were cloned in a vector suitable for in vitro transcription and translation. Once the proteins were made in vitro, immunoprecipitations were carried out; however, these results were not consistent. It is possible that production or stability of these proteins required host factors. Expression of some of the RNA polymerase subunits is also difficult in *E. coli* (Detvisitsakun and Passarelli, unpublished results).

Mapping associations of LEF-9 and P47, and LEF-8 with LEF-8 domains

A previously constructed panel of deletions (Titterington et al., 2003), was used to map the LEF-8 domains required for interaction with the interactive subunits LEF-9 and P47. FLAG-

tagged *lef-9* or *p47* was cotransfected with different HA-tagged *lef-8* deletion constructs. We tested the HA-tagged *lef-8* N- and C- terminal deletions and approximately 50 amino acid internal deletions spanning the region not included in the terminal deletions (Fig. 5A). The complexes were immunoprecipitated with anti-FLAG antibody and immunoblotted using anti-HA antibody conjugated with peroxidase to detect HA-tagged LEF-8 protein immunoprecipitated with the FLAG-tagged bait proteins.

Full-length LEF-8 coimmunoprecipitated with LEF-9 as described above but did not immunoprecipitate non-specifically with the anti-FLAG antibody (Fig. 5B, lanes 1 and 12). Surprisingly, all of the LEF-8 N- and C- terminal deletions associated with LEF-9 (Fig. 5B, lanes 2 through 11). Several of

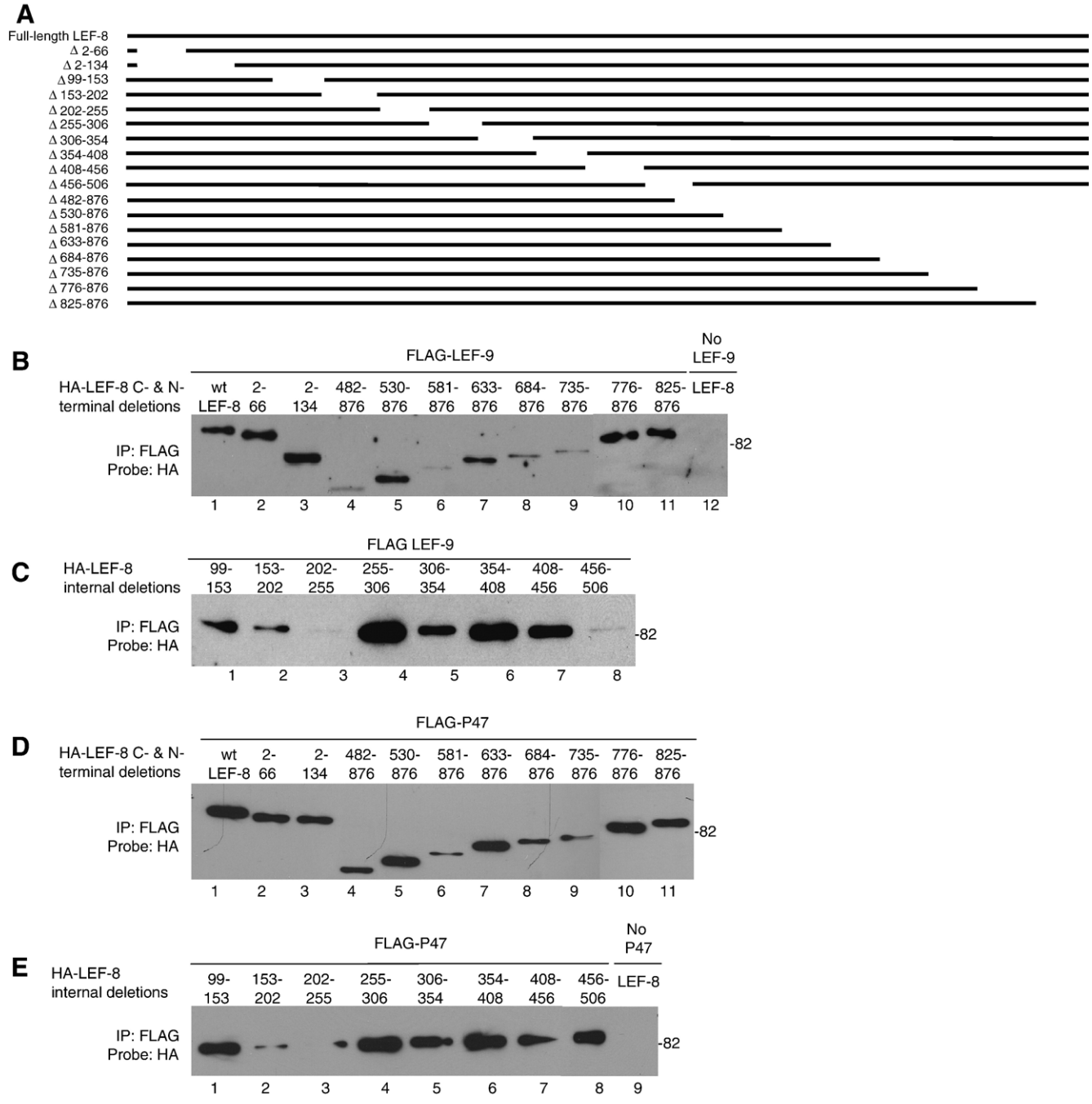

Fig. 5. Association of LEF-8 deletion mutants with LEF-9 and P47. (A) Schematic of LEF-8 deletions used to map LEF-8 interaction domains with LEF-9 and P47. The first line represents full-length LEF-8, the next two, next eight, and last eight lines, represent N-terminal, internal, and C-terminal deletions within LEF-8, respectively. The amino acids deleted in LEF-8 are indicated to the left. (B to E) SF-21 cells (2×10^6 in panels B and C and 4×10^6 in panels D and E were transfected with 11 μg, 16 μg, or 4 μg of each plasmid panels B, C, or D, respectively) or carrier DNA as indicated. The amino acids of LEF-8 deleted are indicated at the top. The lysates were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were immunoblotted using anti-HA antibody. Protein mass standards in kilodaltons are indicated to the right. IP, antibody used for immunoprecipitation; Probe, antibody used in immunoblotting.

the deletions seemed to associate weakly, but weak detection was consistent with weak expression (Fig. 5B, lanes 4, 6, 8, and 9 and results not shown).

We next tested the association of LEF-9 with LEF-8 internal deletions spanning the LEF-8 amino acids 134 to 482 to bridge the gap not covered by both sets of terminal deletions. All of the internal deletions of LEF-8 tested associated with LEF-9 (Fig. 5C, columns 1 to 8). Again, low levels of LEF-8 deletions 202–255 and 456–506 detected corresponded to weak expression. These data indicate that LEF-9 and LEF-8 interact at more than one site. In yeast, the RNA polymerase II forms a "V"-like structure and most interactions between the two largest subunits are at the base of this structure (Cramer et al., 2001). The large deletions within LEF-8 may disrupt the conformation of LEF-8 and allow association at sites not normally available. Studies with the Archeal RNA polymerase found enhanced binding of transcription factor B deletion mutants with the RNA polymerase D subunit (Goede et al., 2006).

Similarly, P47 associated with all the LEF-8 deletions tested (Figs. 5D and E). We thought that perhaps deletions within LEF-8 could have influenced LEF-8 non-specific aggregation to other proteins. To test for binding specificity, we coimmunoprecipitated selected LEF-8 deletions with P47 in the absence or presence of a non-specific protein, BSA. Disruption of the complexes was not altered by including BSA; in fact, it appeared to stabilize the interactions (results not shown). Inclusion of BSA had the ability to disrupt a weak interaction described above (e.g., between LEF-9 and LEF-4). Also, we tested if LEF-4, that does not bind LEF-8, would non-specifically coimmunoprecipitate with selected LEF-8 deletions. LEF-4 did not interact with either full-length or any of the LEF-8 deletions tested (results not shown).

Using smaller deletions within LEF-8 to map interaction domains may be able to rule out alterations in LEF-8 structure and provide more insight into mapping inter-protein domains. All the deletions used to map interactions disrupted LEF-8 function (Titterington et al., 2003), thus their lack of function was either due to alternative binding or disruption of a functional domain.

Tri- and quad-immunoprecipitations

Thus far, we have gathered a crude atlas of the RNA polymerase, giving us hints of the architecture behind the enzyme complex by looking at two-protein subcomplexes. To gain insight into the interactive nature of the four-protein complex, we transfected cells with either three or four plasmids and asked if immunoprecipitating one protein would bring the other two or three proteins down, respectively.

We first performed three-factor immunoprecipitations in which one factor was FLAG-tagged and used to immunoprecipitate two HA-tagged proteins (Fig. 6A). In two-factor immunoprecipitations, we found that LEF-4 did not associate with either LEF-8 or itself but associated with P47. Thus, we assumed that LEF-4 should be able to coimmunoprecipitate LEF-8 or itself if P47 was coexpressed. We observed that P47 served as a "bridge" between LEF-4 and either LEF-8 or LEF-4 (Fig. 6A, lanes 1 and 3). In contrast, LEF-4 was not able to immunoprecipitate LEF-8 or LEF-4 in the presence of LEF-9 (Fig. 6A, lanes 2 and 4). This is not surprising since the

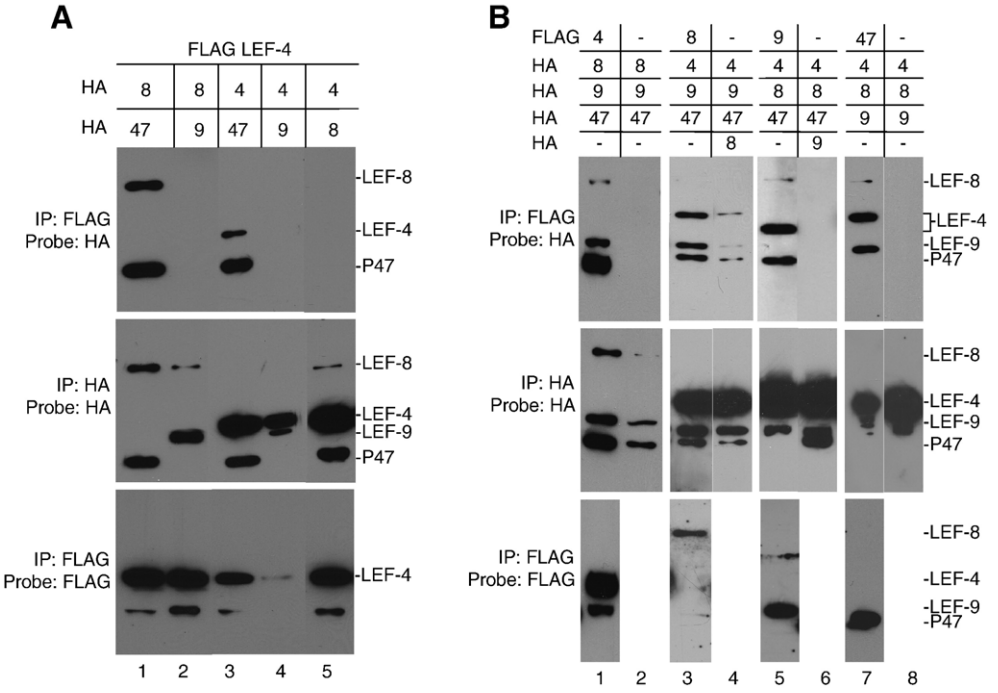

Fig. 6. Tri- and quad-immunoprecipitations. SF-21 cells (4×10^6) were transfected with 3 to 6 μg of plasmid DNA and immunoprecipitated as indicated in Fig. 3. (A) Three-factor immunoprecipitations and (B) four-factor immunoprecipitations with lysates containing the indicated proteins were performed as described in the legend to Fig. 3. The position where each subunit migrated is indicated to the right of each panel. IP, antibody used for immunoprecipitation; Probe, antibody used in immunoblotting.

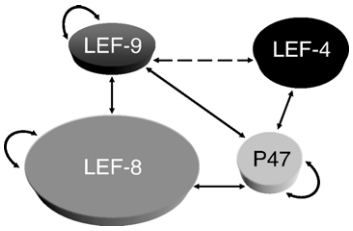

Fig. 7. RNA polymerase subunit interaction model. The four RNA polymerase subunits are represented by ovals and solid connecting arrows represent the interactions that were observed in coimmunoprecipitation experiments. The dashed arrow line represents a weak interaction and the arrowheads indicate the direction in which the association was tested. Curved arrows indicate self-interactions.

interaction between LEF-4 and LEF-9 was weak in two-factor immunoprecipitations. Alternatively, this interaction may be unstable in the absence of the fourth subunit. Although LEF-4 was not as well expressed in the experiment shown (Fig. 6A, lane 4, lower panel), it was well expressed in other experiments where similar results were obtained. Finally, we were not able to coimmunoprecipitate a three-factor subcomplex when FLAG-tagged LEF-4, HA-tagged LEF-8, and HA-tagged LEF-4 were assayed (Fig. 6A, lane 5), since any combination of these did not coimmunoprecipitate.

To complete the RNA polymerase interaction network, we inquired whether we could coimmunoprecipitate the four-subunit complex by using any of the factors to immunoprecipitate the other three. In a four-factor immunoprecipitation, we were able to immunoprecipitate three HA-tagged subunits by using the fourth FLAG-tagged subunit (Fig. 6B). Coimmunoprecipitation of the entire complex is consistent with fractionation of an active and specific complex in a single peak (Guarino et al., 1998b).

In summary, we found that P47 interacted with all four subunits including itself, LEF-8 bound to three (P47, LEF-9, and itself), LEF-9 coimmunoprecipitated with LEF-8, P47, itself, and weakly with LEF-4, and finally, LEF-4 only associated with P47 and weakly with LEF-9 (Fig. 7). From our experiments, we do not know if there are any intramolecular interactions, although some of the self-interactions may reflect these types of associations. We note that protein interactions may differ during virus infection of cells depending on the assembly order of the RNA polymerase complex. For example, interactions between the *E. coli* RNA polymerase β subunit region F and β subunit region I may lead to binding of α subunits (Naryshkina et al., 2000). Our studies portray a crude picture of the architecture of the Ac*M*NPV RNA polymerase; three-dimensional analyses will verify the anatomy of this enzyme complex and reveal additional structural parallels with other RNA polymerases.

Materials and methods

Cells

The lepidopteran cell line IPLB-SF-21 (SF-21) (Vaughn et al., 1977) was grown at 27 °C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 0.26% tryptone broth (O'Reilly et al., 1994).

lef library and reporter plasmid

The 19 plasmids encoding the HA-tagged Ac*M*NPV *lef* genes (*lef-1* to *lef-12*, *ie-1*, *ie-2*, *p143*, *p47*, *39K*, *p35*, and *dnapol*) necessary for optimal late promoter gene expression have been previously described (Rapp et al., 1998). The expression of each *lef* with an HA epitope and polyhistidine tags at the N terminus is controlled by the *Drosophila melanogaster* heat shock protein 70 gene promoter. Late gene expression was determined with the reporter plasmid pCAPCAT (Thiem and Miller, 1990), containing the late promoter of the capsid gene, *vp39*, in front of the bacterial reporter gene chloramphenicol acetyltransferase (*cat*).

Mutagenesis

The plasmid described above containing *lef-9*, pHSEpiHislef9 (Rapp et al., 1998), was used as a template to substitute each of three aspartic acid residues at the LEF-9 amino acid positions 282, 284, and 286 (D282, D284, D286) for alanines by site-directed mutagenesis using the Quik-Change Mutagenesis kit (Stratagene). Alanine was selected since it does not have a bulky side-chain, potentially minimizing structural changes. The following oligonucleotides and corresponding antisense oligonucleotides were used to generate the changes in the plasmids: pHSEpiHislef9D282A, 5′-GGCGCTAACACAGCCTGCGACGGTGAC'-3′; pHSEpiHislef9D284A, 5′-GCGCTAACACAGACTGCGCCGGTGACAAAAAAATTATTAC-3′, and pHSEpiHislef9D286A, 5′-GCGCTAACACAGACTGCGACGGTGCCAAAAAAATTATTAC-3′. Mutations were verified by nucleotide sequence analysis.

FLAG- and his-tagged lef constructs

Plasmids pHSEpiHislef4, pHSEpiHislef8, pHSEpiHislef9, and pHSEpiHisp47 were digested with *Bgl*II and *Not*I to isolate the open reading frame (ORF). The ORF was then inserted into the plasmid pHSFLAGHisVI+ (Prikhod'ko et al., 1999) digested with *Bgl*II and *Not*I to generate a FLAG epitope-tagged clone. The correct construction of pHSFLAGHislef4, pHSFLAGHislef9, pHSFLAGHislef8, and pHSFLAGHisp47 was verified by restriction endonuclease digestion. Each FLAG-tagged construct was able to activate late gene expression (results not shown).

Cotransfections and CAT assays

SF-21 cells (0.5×10^6) in 35-mm dishes were cotransfected for 4 h at 27 °C using 6 μl of a lipofection mixture (Crouch and Passarelli, 2002), 2 μg pCAPCAT, and 0.5 μg of each of 19 *lef*-containing plasmids or a plasmid with a mutation within *lef-9* substituting for pHSEpiHislef9. The plasmid pBluescript (0.5 μg) (Stratagene) was used to balance the DNA concentration in the reactions lacking *lef-9*. Cells were harvested 48 h

post-transfection, a protein lysate was obtained, and CAT activity was determined as previously described (Passarelli and Miller, 1993b).

Immunodetection

SF-21 cells (0.5×10^6) were transfected with 2 μg of pHSEpiHislef9 or pHSEpiHislef9-D282A, -D284A, or -D286A as described above. At 24 h post-transfection, cells were incubated at 42 °C for 30 min to induce expression from the heat shock protein 70 gene promoter. Cells were harvested 4 h after heat shock treatment by washing twice with phosphate-buffered saline pH 6.2 (Potter and Miller, 1980) in 100 μl of Laemmli loading buffer. Proteins were resolved by sodium dodecylsulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE), transferred to a PDVF membrane (Pierce) and immunodetected with a 1:1000 dilution of HA.11 monoclonal antibody (Covance), 1:3000 dilution of goat anti-mouse IgG-horseradish peroxidase (Bio-Rad), and SuperSignal West Pico Chemiluminescent substrate (Pierce).

Coimmunoprecipitations

SF-21 cells (2×10^6 or 4×10^6 plated on 60- or 100-mm cell culture dishes, respectively), were transfected with plasmids using 10 μl of lipofectin as described above. DNA concentration was maintained constant in each transfection experiment by adding pBluescript, herring sperm DNA, or pHSP70PLGFP-A (Crouch and Passarelli, 2002). Due to difficulties detecting some of the proteins (Berretta and Passarelli, 2006), cells were treated at 24 h post-transfection for 30 min at 27 °C with 50 μg/ml N-CBZ-LEU-LEU-LEU-AL (MG 132, Sigma), a proteasome inhibitor, to increase the levels of accumulated protein. After 2 to 4 h, cells were lysed with 500 μl of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and protease inhibitors (1 tablet of Complete Mini, EDTA-free protease inhibitor cocktail, Roche/10 ml lysis buffer). The lysate was incubated for 15 min at 4 °C with mixing and then centrifuged at 14,000×*g* to clarify the lysate. The supernatant was immunoprecipitated with 0.5 μl of anti-FLAG M2 antibody (Sigma) or 2.0 μl of anti-HA.11 monoclonal antibody (Covance) bound to 100 μl of a 10% solution of Protein G-Sepharose 4B Fast Flow beads (Sigma) at 4 °C overnight. The coimmunoprecipitated proteins were collected by centrifugation (14,000×*g*), washed with lysis buffer, and resolved by SDS-10% PAGE. The coimmunoprecipitated proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk/Tris-buffered saline-Tween-20 (20 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.1% Tween-20) at 4 °C overnight. The supernatants from the coimmunoprecipitated samples were immunoprecipitated with 2 μl of anti-HA.11 antibody or 0.5 μl of anti-FLAG M2 antibody with 100 μl of 10% Sepharose beads at 4 °C overnight to detect proteins that did not interact. Proteins in the supernatant were collected by centrifugation, washed, and separated by SDS-10% PAGE, transferred to a PVDF membrane, and the membrane was blocked as described above. Both the membranes with the coimmunoprecipitated sample and the proteins in the supernatant were incubated with either 1:4,000 anti-HA-peroxidase (Sigma) or 1:2000 anti-FLAG-peroxidase (Sigma) as indicated for 1 to 2 h at 25 °C to detect the immunoprecipitated proteins. Proteins were detected using SuperSignal West Pico Chemiluminescent substrate (Pierce) exposed to film. The membrane with coimmunoprecipitated proteins was then stripped of the bound anti-HA or anti-FLAG antibody using Restore Western Blot Stripping Buffer (Pierce) for 15 min at 25 °C. The blot was then probed with anti-FLAG or anti-HA for 1 to 2 h at 25 °C to detect the immunoprecipitated protein partners. Proteins were detected as described above. Independent experiments were repeated a minimum of two times but more often over 10 times to obtain accurate interpretation of weak associations or poorly expressed proteins.

Acknowledgments

We are grateful to Erin Harvey and Reinaldo Morales for constructing the FLAG-tagged plasmids and a *lef-9* mutation plasmid, respectively. We also thank Christophe Lehiy for help with sequence alignments, and Erica Cain for technical assistance.

This work was supported in part by the NRI Competitive Grants Program, USDA, under agreement number 2001-35302-09983 and a grant from the Plant Biotechnology Center at Kansas State University. This is contribution number 07-161-J from the Kansas Agricultural Experiment Station.

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