Functional conservation of the *wingless–engrailed* interaction as shown by a widely applicable baculovirus misexpression system

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Background: The expression patterns of the segment polarity genes *wingless* and *engrailed* are conserved during segmentation in a variety of arthropods, suggesting that the regulatory interactions between these two genes are also evolutionarily conserved. Hypotheses derived from such comparisons of gene expression patterns are difficult to test experimentally as genetic manipulation is currently possible for only a few model organisms.

Results: We have developed a system, using recombinant baculoviruses, that can be applied to a wide variety of organisms to study the effects of ectopic expression of genes. As a first step, we studied the range and type of infection of several reporter viruses in the embryos of two arthropod and one vertebrate species. Using this system to express *wingless*, we were able to induce expression of *engrailed* in the anterior half of each parasegment in embryos of the fruit fly *Drosophila melanogaster*. Virus-mediated *wingless* expression also caused ectopic naked ventral cuticle formation in wild-type *Drosophila* larvae. In the flour beetle, *Tribolium castaneum*, ectopic *wingless* also induced *engrailed* expression. As in *Drosophila*, this expression was only detectable in the anterior half of the parasegment.

Conclusions: The functional interaction between *wingless* and *engrailed*, and the establishment of cells competent to express *engrailed*, appears to be conserved between *Drosophila* and *Tribolium*. The data on the establishment of an *engrailed*-competent domain also support the idea that prepatterning by pair-rule genes is conserved between these two insects. The recombinant baculovirus technology reported here may help answer other long-standing comparative evolutionary questions.

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Background

Comparisons of gene expression patterns have been a useful way to approach questions in evolution and development. The major drawback to this methodology has been the inability to test many of the hypotheses derived from these comparative studies in non-model systems [1]. For example, expression patterns of the segment polarity genes *wingless* (*wg*) and *engrailed* (*en*) are conserved in all arthropods studied to date, including the fruit fly *Drosophila melanogaster*, the beetle *Tribolium castaneum* and the grasshopper, *Schistocerca americana* [2–4] (D. DiPietro and N.H.P., unpublished observations). This conservation of expression suggests conservation of function, but there has been no way to test this idea directly. Mutational screens in *Tribolium* have identified both pair-rule and segment polarity mutants, but we do not yet know to which genes these mutants correspond [5,6]. In many model systems, misexpression assays have been a useful way to analyze gene function. For example, retrovirus-mediated gene misexpression has been a powerful way to analyze limb development during chick embryogenesis [7,8]. Although *P* elements are effective for studying misexpression of genes in *Drosophila*, there are no generally applicable methods for studying gene function in non-Drosophilid arthropods. Here, we describe the development of a system using recombinant baculoviruses for gene misexpression in a wide variety of organisms, including the arthropods *Drosophila* and *Tribolium*, and vertebrates such as *Xenopus*.

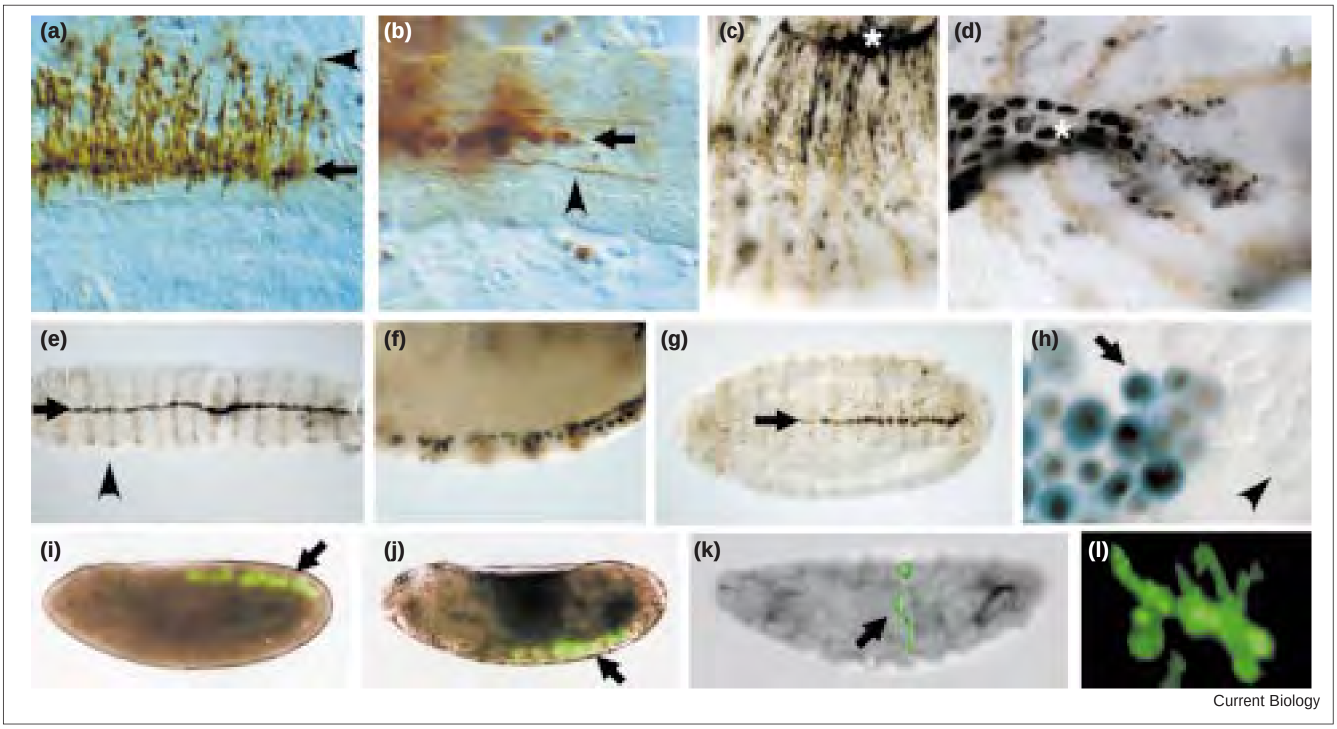
Baculoviruses are double-stranded DNA viruses best known for their use in recombinant protein expression [9,10]. We chose baculoviruses as the delivery vector to express genes ectopically for several reasons: recombinant baculoviruses are simple and inexpensive to engineer; they can enter many different cell types and species; they are relatively safe to use; and, finally, these viruses have a large capacity for DNA inserts [11–18]. Baculoviruses can infect post-mitotic cells, but are not capable of integrating into the genome of an infected cell and replicate only in host (Lepidopteran) cells. We constructed baculoviruses (*Autographa californica* nucleopolyhedrovirus) carrying the gene of interest under the control of the truncated promoter for the *D. melanogaster* heat shock protein 70 (Hsp70) [19], and injected these recombinant baculoviruses into *Drosophila*, *Tribolium* and *Xenopus laevis* embryos.

Results and discussion

Expression of reporter baculoviruses in embryos

We first used baculoviruses expressing β-galactosidase (v-h/lacZ), nuclear localized β-galactosidase (v-h/nuclacZ), or green fluorescent protein (v-h/GFP) to study general infection and expression dynamics in *D. melanogaster* (Figure 1). Expression was detectable within 2 hours after injection into *Drosophila* embryos, and embryos injected at the cellular blastoderm stage (stage 5) still showed detectable levels of expression as first instar larvae 24 hours later. As the DNA core of baculoviruses must separate from the viral envelope by endocytosis in a cell,

Figure 1



Baculovirus-mediated gene expression in *Drosophila* embryos and larvae, and Lepidopteran cells. Anterior is to the left in all panels. *Drosophila* (a–g,i,j) embryos and (k,l) larvae, and (h) Lepidopteran cells. (a) Dissected stage 14 embryo injected at stage 7 with v-h/lacZ. Ectodermal cells expressing β-galactosidase (brown) are prevalent along the midline (arrow) and up to two-thirds of the way around one side of the embryo (arrowhead). (b) Higher magnification view of (a) showing β-galactosidase expression in neurons on and near the midline (arrow); β-galactosidase can also be seen throughout a developing axon (arrowhead) from one of these neurons. (c) Lateral view of a whole mount of a stage 13 embryo injected laterally at stage 5 with v-h/lacZ and stained for β-galactosidase (black) and En (brown). Expression of β-galactosidase is restricted predominantly to dorsal and lateral ectoderm and amnioserosa (asterisk). The normal striped pattern of En expression shows that infection has not visibly affected segmentation of the embryo. (d) Whole-mount embryo at stage 11 injected dorsally with v-h/nuclacZ at stage 5 and stained for β-galactosidase (black) and En (brown); β-galactosidase expression is mostly in the amnioserosa nuclei (asterisk) and nuclei of the dorsal-most ectoderm. (e) Dissected stage 11 embryo injected on the ventral side with v-h/lacZ at stage 7 and stained for β-galactosidase (black) and En (brown); β-galactosidase expression is largely confined to the ventral midline of the embryo (arrow). Arrowhead, the En stripe of the first thoracic segment. (f) Side view of a stage 10 embryo injected on the ventral side with v-h/nuclacZ at stage 7; β-galactosidase accumulation (black) is seen in nuclei of midline mesectodermal and neural cells. En staining is in brown. (g) Ventral view of a stage 16 embryo injected and stained as in (f). Again, β-galactosidase expression is mostly restricted to ectodermal and neural cells along the ventral midline from the first abdominal segment and posterior (arrow). (h) *S. frugiperda* (Sf9) cells infected with a virus expressing both the *Ultrabithorax* (*Ubx*) gene and *lacZ* (v-h/Ubx + h/lacZ). The photograph was taken at the edge of a focus of infected cells formed by initial infection with a single viral particle (the virus is able to spread from cell to cell as the cell line is derived from its normal host). All infected cells (arrow) are both blue (β-galactosidase staining in the cytoplasm) and brown (antibody staining for Ubx) whereas uninfected cells show neither staining (arrowhead). (i) GFP expression in a lateral view of a living stage 10 embryo injected ventrally at stage 6 with v-h/GFP. The bright-field view of the embryo has been merged with a GFP fluorescence image. GFP expressing cells are visible along the ventral midline in the posterior abdominal region (arrow). (j) Same embryo as in (i), but allowed to develop for another 5 h and viewed at stage 14. The germ band has now shortened, and GFP expression is seen in the same set of cells in the midline of the abdomen (arrow). (k) Living first instar larvae that had been injected with v-h/GFP as described in (i). Several patches of GFP-expressing cells are seen on the ventral surface of the ectoderm (arrow). (l) Higher magnification view of GFP-expressing cells from the lateral side of the larvae in (k).

Table 1

Effects of injection of v-h/lacZ and v-h/nuclacZ on *Drosophila* and *Xenopus* development.

Stage of injection	Number of embryos injected	Injected material	Number of hatched larvae or normal embryos at time of fixation (%)	Number with *lacZ* expression (%)
Drosophila				
Stage 5/6	85	Buffer	36 hatched larvae (42%)	Not assayed
Stage 5/6	83	v-h/lacZ	24 hatched larvae (29%)	Not assayed
Stage 7/8	34	Buffer	14 hatched larvae (41%)	Not assayed
Stage 7/8	157	v-h/lacZ	55 hatched larvae (35%)	Not assayed
Stage 5	63	v-h/lacZ	59 normal at stage 11 (94%)	45 (71% of injected embryos)
Stage 5	68	v-h/lacZ	60 normal at stage 14 (88%)	48 (70% of injected embryos)
Xenopus				
Stage 20	45	v-h/nuclacZ	42 normal at stage 38 (93%)	41 (91% of injected embryos)

These are examples of typical results from injection experiments of these and other viruses. The first set of *Drosophila* data shows survivorship to hatching as first instar larvae when embryos at various stages are injected with v-h/lacZ. The second set of *Drosophila* data shows the percentage of embryos displaying β-galactosidase expression; all embryos were injected at the same stage but fixed at different stages.

efficient infection requires injection after cellularization of the embryo and into an extracellular space. Intriguingly, although β-galactosidase expression was controlled by the truncated *hsp70* promoter, no heat shock was necessary to induce expression. We checked whether virus infection itself induced a stress response that was in turn responsible for initiating expression. Using a monoclonal antibody to Hsp70, however, we were unable to detect expression of Hsp70 protein from the endogenous *hsp70* gene in virus-infected cells of injected wild-type *Drosophila* embryos. Thus, viral infection does not induce a stress response in infected cells. Enhancers on the viral genome near the *hsp70* promoter may be responsible for the observed constitutive expression [20].

Regions of infection and, to a certain degree, cell types infected, could be controlled by site and time of injection. For example, by injecting into the ventral side of the embryo just before gastrulation, we could largely restrict infection to mesodermal cells. Progressively more lateral injections biased infection towards the ventral ectoderm plus nervous system, lateral ectoderm, and dorsal ectoderm plus amnioserosa (Figure 1a–d). Strikingly, injections on the ventral side of the embryo just after gastrulation (stage 6) resulted in infection that was largely restricted to the ventral midline of the embryo (presumably because the virus solution is mechanically trapped in the narrow ventral indentation running the length of the embryo shortly after gastrulation; Figure 1e–g). Further, by injecting into the space between the gut and central nervous system at stage 12, internal tissues such as fat body and macrophages could be targeted (data not shown). Injections into the yolky center of the embryo before gastrulation (at the cellular or syncytial blastoderm stages) resulted in infection of the midgut presumably because the injected solution remains within the yolk and does not come into contact with cells until the midgut moves into this region. Not unexpectedly, β-galactosidase expression was mosaic, but we could frequently obtain embryos where up to 50% of the ectodermal cells within several adjacent segments were infected.

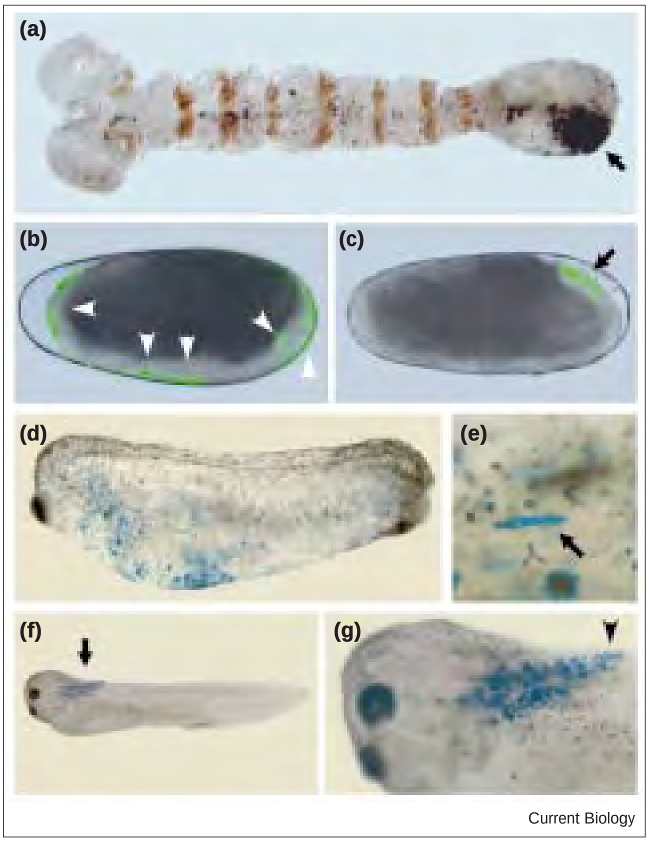
To determine the efficiency of infection, we compared infection rates of baculovirus in *Drosophila* tissue culture cells (Schneider line 2) with rates in Lepidopteran tissue culture cells (*Spodoptera frugiperda* line 9; Sf9 cells). We found that *Drosophila* cells required approximately twice the number of infectious units as Lepidopteran cells to infect the same number of cells (data not shown). In *Drosophila* embryos, we estimated that, at the highest concentration of virus, we injected approximately 500–1000 infectious units and that about half that number of embryonic cells were infected. Although *lacZ* expression was clearly achieved with baculovirus infection, it is worth noting that, as the viral genome does not integrate into the host cell genome and, thus, viral genomes may unevenly segregate into daughter cells, lineage tracing with baculoviruses is probably unreliable. Typically, 10–30% of mock-infected and virus-infected embryos displayed morphological defects as a result of injection trauma, but most of the remaining embryos went on to develop normally (Figure 1i–l). There was also some mortality associated with injection and infection (Table 1).

Embryos of *T. castaneum*, the red flour beetle, showed similar infection dynamics (Figure 2). For example, when embryos were injected in the posterior at the blastoderm stage or just after gastrulation, and examined 24–48 hours later, much of the infection, as assayed by β-galactosidase or GFP expression, was in the posterior end, but small clusters of cells throughout the ectoderm and mesoderm of the embryo were also infected (Figure 2a–c). Injections into later-stage embryos could result in infection that was limited to specific regions and structures of the embryo. We estimated the efficiency of infection in *Tribolium* embryos at about 25%, that is, if 500 infectious particles were injected, then approximately 125 cells became infected.

Baculoviruses have been shown to infect a broad range of vertebrate cell cultures [12,14,15] but it has not been established whether vertebrate embryos can also be infected. To determine whether baculovirus-mediated gene misexpression would also be useful for studying vertebrate development, v-h/lacZ baculoviruses were injected into the blastocoel of stage 8 (blastula) *X. laevis* embryos. Detectable levels of β-galactosidase were observed until at least stage 40, five days after injection (data not shown). The injected *Xenopus* embryos showed expression of β-galactosidase in many cell types including muscle, notochord, neural and ectodermal cells (Figure 2d,e). Infected regions became increasingly mosaic with time as cells continued to divide and the viral genomes were distributed to a smaller proportion of cells. Baculoviruses could also be injected at later stages of development. *Xenopus* embryos (stage 20) injected with v-h/nuclacZ virus into the neural tube exhibited substantial infection within a restricted region of the embryo (Figure 2f,g; Table 1). Thus, the baculovirus system offers potential for the manipulation of developmental processes in later stages of embryogenesis, which cannot be performed presently using standard mRNA or plasmid injections [21]. Furthermore, the technique can be refined to infect tissue explants for use in transplantation analyses.

An additional illustration of the versatility of the baculovirus system comes from the insertion of multiple, independent constructs into a single viral genome. For example, we constructed a single virus containing both the Hox gene *Ultrabithorax* (*Ubx*) and *lacZ* (v-h/Ubx + h/lacZ). Infection of host cells (Figure 1h) and *Drosophila* embryos (data not shown) revealed expression of both Ubx and β-galactosidase proteins from this virus. Using *lacZ* as a marker in this way will be useful when there are no antibodies to the gene of interest; this concept can be extended to using GFP in combination with a gene of interest to study the effects of gene misexpression in living embryos.

Figure 2

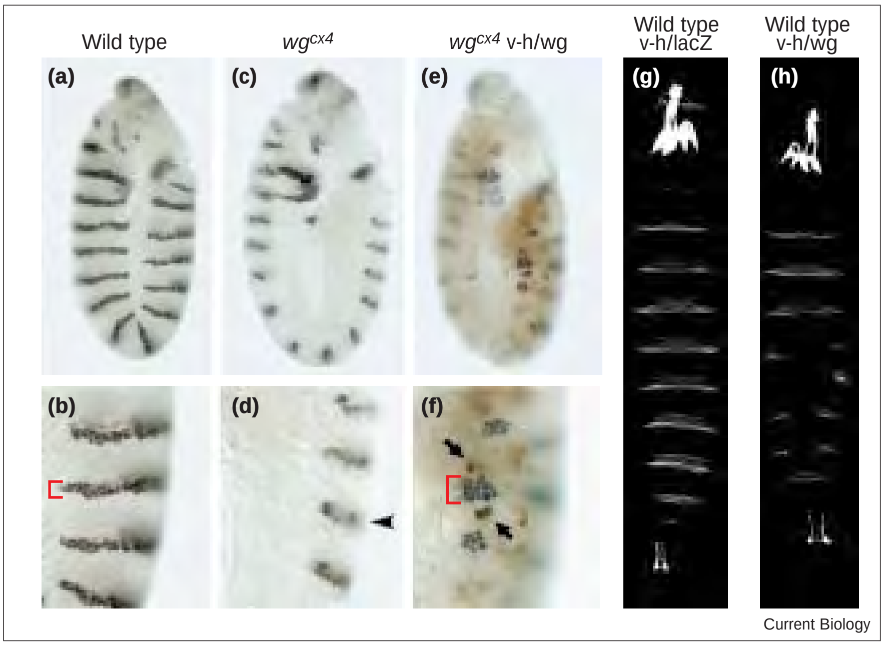


Baculovirus-mediated expression of cytoplasmic β-galactosidase, nuclear β-galactosidase and GFP in *Tribolium* and *Xenopus* embryos. Anterior is to the left in all panels. **(a)** *Tribolium* embryo injected shortly after gastrulation with v-h/nuclacZ, fixed during germ-band extension, and stained for β-galactosidase (black) and En (brown); β-galactosidase expression is prominent in a large posterior patch (arrow) and in scattered cells throughout the rest of the embryo. The normal striped pattern of En expression indicates that viral infection has no obvious effects on subsequent embryonic patterning. **(b,c)** Live *Tribolium* embryos injected with v-h/GFP shortly after gastrulation. GFP expression is seen in various patches (arrowheads) throughout the embryo in (b), but restricted to a single large patch (arrow) at the posterior end of the embryo in (c). **(d,e)** *Xenopus* embryo injected with v-h/lacZ into the blastocoel at stage 8 and fixed at stage 27. Scattered β-galactosidase staining is seen throughout the embryo in (d). The high magnification view in (e) shows β-galactosidase staining of an isolated muscle cell (arrow) within a somite. **(f,g)** *Xenopus* embryo injected with v-h/nuclacZ in the neural tube at stage 20 and fixed at stage 38. (f) Low magnification view showing β-galactosidase activity in a large patch within the neural tube (arrow). (g) Higher magnification view showing infected cells within the neural tube and also in the ectoderm at the needle entry point (arrowhead).

Functionality of virus-expressed *wg* in *Drosophila*

Having demonstrated that baculoviruses can express foreign genes in embryos, we began to test specific developmental and evolutionary questions. Specifically, we addressed the hypothesis that the role of *wg* in segmentation is evolutionarily conserved between *Drosophila* and *Tribolium*. In *Drosophila*, *wg*, expressed in the posterior of each parasegment, is required for the maintenance of *en*, another segment polarity gene expressed in stripes immediately posterior to the cells expressing *wg* [22] (Figure 3a,b). The *wg* gene is also required for the proper patterning of the larval cuticle [23]; denticle formation is repressed in areas where *wg* is expressed at high levels, leading to naked (denticle-less) regions on the ventral side of the embryo [22,23]. *P*-element-mediated transformation of *Drosophila* with constructs resulting in ectopic expression of *wg* has helped to elucidate these functions of *wg* [24] and we wanted first to demonstrate that these results could be reproduced in *Drosophila* using baculovirus-mediated *wg*

Figure 3



Effects of baculovirus-mediated *wg* expression in *Drosophila* embryos and larvae. Anterior is uppermost in all panels. **(a,b)** Uninjected wild-type *Drosophila* embryo, **(c,d)** uninjected *wg* mutant embryo (wg^{cx4}) and **(e,f)** wg^{cx4} embryo injected with v-h/wg, and stained for (a–d) En alone (black) or (e,f) both En (black) and Wg (brown). (a,c,e) Low magnification views; (b,d,f) corresponding higher magnification views. (b) In a wild-type germ-band-extended *Drosophila* embryo, En stripes are about two to three cells wide (red bracket). In a *wg* mutant embryo, (c) En expression in the ectoderm disappears, but (d) remains in the central nervous system (arrowhead). Virally-mediated Wg expression (brown staining; prominent expressing ectodermal cells indicated with arrows in panel f) in a *wg* mutant results in expression of En in patches that are wider (four to five cells wide; red bracket in panel f) than in wild-type embryos. **(g)** Virally-mediated expression of β-galactosidase along the ventral midline causes no cuticle defects in a *Drosophila* larva, but **(h)** virus-mediated expression of *wg* along the ventral midline causes the replacement of denticles with naked cuticle as is also seen when *wg* is expressed under the control of the heat shock promoter in transgenic *Drosophila* [24] (see Figure 1g for infection pattern of this type of injection).

expression, and thus establish a proof-of-principle for this methodology. For these experiments, we used a *Drosophila* mutant that does not express detectable Wg protein (wg^{cx4}). Without *wg* expression, *en* expression is initiated normally but is not maintained as *wg* is required for continued *en* expression [22] (Figure 3c,d). When *wg*-expressing baculovirus (v-h/wg) was injected into stage 5–6 embryos, virus-delivered *wg* was expressed by stage 8–9 (2–3 hours post-injection), the stage at which *en* is normally maintained by *wg* expression. In a wg^{cx4} mutant, either uninjected or injected with v-h/lacZ virus, *en* expression in the thorax and abdomen faded completely by stage 12, except for expression in the central nervous system which is not dependent on *wg* (Figure 3c,d). In wg^{cx4} embryos injected with v-h/wg virus, patches of ectodermal *en* expression were detectable in close proximity to the ectopic *wg* expression (Figure 3e,f; see Table 2 for data on survival and proportion of embryos with ectopic expression), showing that functional Wg protein capable of inducing *en* expression could be produced from the baculovirus-infected cells.

In a wild-type embryo at stage 10, the two to three rows of cells posterior to each Wg stripe, that is, the cells at the anterior of the next parasegment, express En. In wg^{cx4} embryos with virus-mediated ectopic *wg* expression, however, the En-expressing domain could be wider (four or five cells wide or about half the segment width), but did not cover the width of the entire segment (Figure 3f) even though infected cells were randomly distributed throughout the ectoderm. This observation is consistent with the results obtained from wild-type *Drosophila* embryos containing a transgene with a heat-shock inducible *wg* construct [24]. When *wg* is expressed in all ectodermal cells, only cells in the anterior half of each parasegment are competent to express *en*, which is thought to be due to pair-rule prepatterning dividing the segment into *wg*-competent and *en*-competent domains [25]. Thus, our baculovirus-mediated *wg* misexpression data confirm the existence of an *en*-competent domain in *Drosophila*.

We also analyzed the effect of baculovirus-expressed ectopic *wg* on denticle belt formation. When *wg* is overexpressed before stage 12, denticle formation is inhibited [24]. As described above, injections on the ventral side of the embryo just after gastrulation resulted in infection that was largely restricted to the ventral midline of the animal (see Figure 1e–g). We injected wild-type embryos with v-h/wg or v-h/lacZ virus in this way, and then examined the cuticles of larvae that hatched and emerged from the egg. Approximately 50% of the hatched v-h/wg-injected larvae showed gaps in the denticle belt pattern (Figure 3h). By contrast, in uninjected and v-h/lacZ-injected larvae, less than 1% had gaps in denticle belts (Figure 3g). Consistent with the position of the injection, these gaps

Table 2

Effects of v-h/nuclacZ and v-h/wg on ectopic En expression in *Drosophila* and *Tribolium*.

Stage of injection	Number of embryos injected	Injected material	Number with ectopic Wg expression	Number with ectopic En expression
*Drosophila**				
Stage 5	131	v-h/nuclacZ	0 (0%)	0 (0%)
Stage 5	132	v-h/wg	86 (65%)	18 (21%)
Tribolium†				
Cellular blastoderm	58	v-h/nuclacZ	0 (0%)	0 (0%)
Cellular blastoderm	137	v-h/wg	21 (14%)	10 (7%)

**Drosophila* embryos were fixed and assayed for Wg and En expression at stage 10–15. †*Tribolium* embryos were fixed and assayed for Wg and En at full germ-band extension.

were typically found along the ventral midline or immediately adjacent to the ventral midline. From these experiments, we conclude that the ectopically expressed *wg* delivered by baculovirus is functional and causes the expected phenotypes in *Drosophila*.

Functional interaction between *wg* and *en* in *Tribolium*

Embryonic pattern formation has been studied most extensively in *Drosophila* but most arthropods, such as *Tribolium*, have significant morphological differences in early embryogenesis compared with *Drosophila*. Whereas *Drosophila* embryos have long-germ development, in which all segments are simultaneously defined, *Tribolium* displays short-germ development, in which segments are progressively defined in an anterior-to-posterior direction. Furthermore, the *Drosophila* segmental pattern forms in a syncytium while much of the *Tribolium* pattern forms in a cellular environment. Nevertheless, the general expression patterns of developmental genes appear similar between these two insects [3,4,26,27].

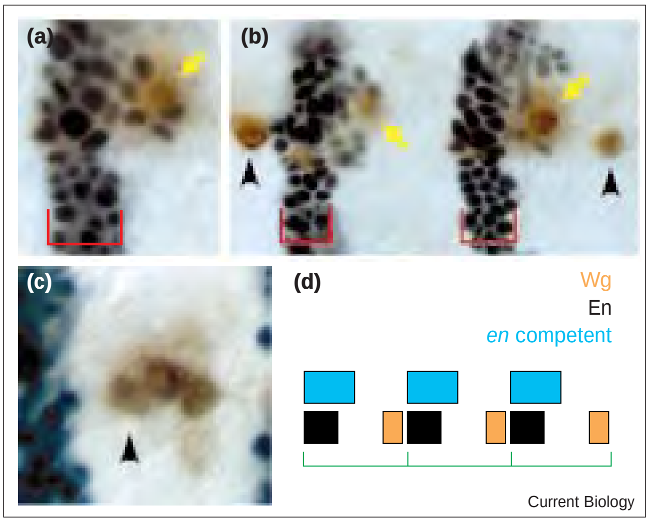
In both *Tribolium* and *Drosophila*, Wg is normally expressed in a stripe just anterior to En (at the posterior end of each parasegment) and, on the basis of this pattern of expression, it has been suggested that *wg* interacts with *en* in the same way in both insects [3]. To test this hypothesis functionally, we injected early *Tribolium* embryos, which were initiating segmentation, with v-h/wg virus. Extensive misexpression of *wg* led to morphologically aberrant embryos with widened En stripes (data not shown), similar to the effects seen in *Drosophila*. Even more informative, embryos containing only scattered cells misexpressing *wg* (Figure 4; Table 2) showed that *wg* misexpression just posterior to a normal En stripe could result in ectopic *en* expression and that ectopic *en* was expressed up to three cells away from a single *wg*-expressing cell (Figure 4a,b). Furthermore, whereas ectopic *en* expression extended approximately three cells more posterior from the normal En stripe, in segments where virally-mediated Wg protein was expressed just anterior to wild-type *en* expression (within the normal domain of *wg* expression or just anterior to it), no ectopic En was detected (Figure 4b). This result suggests that an *en*-competent domain exists in *Tribolium* as in *Drosophila*. Another possibility that might also explain this restricted expression of *en* is that the diffusion of ectopic Wg may be controlled such that Wg can only travel in one direction. In the context of our experiments, however, we detected Wg diffusion several cell lengths in all directions from the infected cell (Figure 4a). By analyzing infected embryos, we determined that this *en*-competent domain covered approximately half the segment (Figure 4d), which is comparable to the results from *Drosophila*. This also supports the concept that the ectoderm is already pre-patterned into competent domains earlier in development.

Finally, we noted a temporal aspect of *en* regulation by *wg* in *Tribolium*; *en* expression is not normally seen in the posterior growth zone, and ectopic *wg* expression in this region could not induce ectopic (and precocious) *en* expression (data not shown). In older embryos, as well, ectopic *wg* expression could not induce ectopic *en* expression (Figure 4c). As *Tribolium* segments develop in a progressive anterior to posterior pattern [4], scattered infection with v-h/wg in a single embryo can illustrate these temporal effects; the anteriormost segments can already be non-responsive, middle segments can show ectopic *en* expression, and the posterior region shows no engrailed expression. This suggests that, as in *Drosophila*, there is a specific developmental window in the segmentation process where *wg* signaling is able to maintain *en* expression.

Conclusions

These data provide further evidence that, although *Tribolium* development differs morphologically from *Drosophila* development, establishment of *en*-competent domains and the interaction between *wg* and *en* is conserved. Although the *wg–en* interaction might be predicted from previous comparisons of gene expression, the demonstration of an *en*-competent domain in *Tribolium* can only come from the type of functional/genetic

Figure 4



Effect of baculovirus-mediated *wg* expression in *Tribolium* embryos. Anterior is to the left in all panels. Histochemical staining for En (black) and virally-produced *Drosophila* Wg (brown). **(a)** Virus-mediated Wg expression in a single cell (yellow arrow) of a germ-band extending embryo located posterior to the normal domain of En expression (black) causes ectopic En expression in surrounding cells. The En stripes are normally three to four cells wide (red bracket) and ectopic En-expressing cells can be located two to three cells posterior to the normal domain. **(b)** Scattered infected cells expressing *Drosophila* Wg show that only certain domains of the *Tribolium* embryo are competent to respond to Wg signaling by expressing En. Infected cells just posterior to a normal En stripe (yellow arrows; red brackets indicate the normal width of engrailed expression) can lead to ectopic En-expressing cells, but infected cells just anterior to the En stripe (arrowheads) are not capable of causing ectopic En expression. **(c)** Ectopic expression of *wg* in older (fully germ-band extended) embryos is also not capable of causing ectopic En expression. Although the Wg-expressing cells (arrowhead) are just posterior to the En domain, ectopic expression at this time has no effect on En expression. **(d)** Schematic illustration of the relative position of normal En (black) and Wg (brown; from [3]) expression relative to the parasegment boundaries (vertical green lines at the bottom). The ectopic expression of Wg in *Tribolium* reveals that the domain of cells competent to express En stretches slightly posterior to the domain that normally does express En. This is consistent with the relative placement of *en*-expressing and *en*-competent domains in *Drosophila*.

analysis described here. Establishment of domains that are competent to express *en* or *wg* in *Drosophila* is thought to be defined by overlapping repression of *en* by the genes *sloppy-paired* and *naked*, and activation of *wg* by *sloppy-paired* [25,28]. We predict that the *Tribolium* orthologues of these genes may also define *en*-competent and *wg*-competent domains during *Tribolium* segmentation. As much of *Drosophila* pattern formation occurs in a syncytial environment, whereas *Tribolium* patterns are defined mostly in a cellular environment, we might expect that there are different ways of creating a segmental pattern. Our results suggest that, despite the morphological differences between early segmentation in *Drosophila* and *Tribolium*, several of the steps in the process of pattern formation at the level of segment polarity genes are conserved between these two insects. There are, however, several genes in *Tribolium* that are expressed somewhat differently from their homologues in *Drosophila* [29,30]. The functional roles of these genes are subjects of future study.

There are several transgenic systems presently available for ectopic misexpression in animal embryos including retroviruses, P elements, and plasmid-based systems [31–35]. The production of transgenic animals requires substantial resources and time, particularly for those organisms that have a long generation time. Baculoviruses are relatively cheap, simple and safe to produce, do not require intracellular injection, and as we have shown here, are capable of providing useful infection in animals as diverse as Drosophila, Tribolium, and Xenopus. In tandem with Sindbis virus [36] and interfering RNA [37,38], we believe this system will be useful for studying developmental processes in a range of species, and particularly important for investigating molecular mechanisms regulating pattern formation and the evolution of development.

Materials and methods

Recombinant baculovirus construction

The following recombinant viruses were used in this paper: v-h/lacZ, which expresses cytoplasmic b-galactosidase [39]; v-h/nuclacZ, which expresses b-galactosidase fused to a nuclear localization sequence (derived from pSP6nucbgal from R. Harland); v-h/wg, which expresses full-length Drosophila Wg (derived from pSP65wg from A. Bejsovec); v-h/GFP, which expresses full-length enhanced GFP (E-GFP; derived from pEGFP-1 from Clontech); v-h/Ubx + h/lacZ, which expresses full-length Drosophila UbxIIa (derived from pKSUbxIIa from M. Akam) and b-galactosidase. A cassette was constructed by inserting the gene of interest behind the hsp70 promoter derived from pAcDZ1 (described in [40]). Each cassette was then inserted into a transfer vector (named 3272 transfer vector) containing homologous viral sequences around the region of the polyhedrin gene. Baculoviruses were constructed by homologous recombination using BacPak (Clontech) as the parental virus and the transfer vector containing the cassette. The v-h/Ubx + h/lacZ virus was made by using v-h/Ubx virus as the parental virus and a transfer vector (derived from the PstI G fragment of the baculovirus genome) containing an hsp/lacZ cassette. Recombinant viruses were purified by three rounds of end-point dilution and confirmed by restriction analysis, sequencing and immunohistochemistry. More detail concerning construction and purification of baculoviruses can be found in the Supplementary material. Viral titers ranged from 5×10^7 to 2×10^8 PFU/ml. See [19] for detailed protocols. It should be noted that the cloning capacity of baculoviruses is at least 30 kb, but no upper limit has been established [11]. For example, baculoviruses containing double genomes have been described [18], suggesting that it may be possible to construct viruses with inserts over 100 kb in size.

Virus preparation and injection

Baculovirus was prepared for injection using filter-sterilized buffers and autoclave-sterilized centrifuge and microfuge tubes. Baculovirus was prepared for injection by prespinning the medium for 10 min at 800 × *g* to remove particulate matter, then pelleting 5–10 ml of virus through a 25% (w/v) sucrose cushion (sucrose dissolved in 0.1 × PBS, pH 6.2) for 30 min at 80,000 × *g*. The pelleted virus was rinsed once with

0.1 × PBS, repelleted in a microfuge tube and resuspended in a volume of 5 μl. Virus titer was in the range 10^{10}–10^{11} PFU/ml. Concentrated virus was injected within 6–8 h after concentration. *Drosophila* and *Tribolium* embryos were dechorionated, lined up on coverslips, briefly desiccated and covered in halocarbon oil. Insect embryos were injected using a standard *Drosophila* microinjection rig (Narishige 300 IM microinjector). *Drosophila* and *Tribolium* were injected in the perivitelline space between the vitelline membrane and ectodermal cells using a modification of [41] described here. Injection needles were pulled using standard microinjection parameters. Rather than the typical 5 μm diameter used for *P*-element injection, needles used for virus injection had diameters of 2 μm (see Supplemental material for a photograph). Embryos were lined up and placed on coverslips such that the desired location for injection was against the coverslip (for example, for ventral injections, *Drosophila* embryos were lined up ventral side down on the coverslip). Embryos were desiccated enough so that slight wrinkling was visible when focusing on the interface between coverslip and embryo (a flattened oval of vitelline membrane pressed against the coverslip). The needle was lowered until it just touched or was just above the coverslip and then just the tip was gently inserted into the embryo. When the needle is in the perivitelline space and virus injected, the embryo rocks slightly but there is no disturbance of individual tissues. *Xenopus* embryos were injected with a standard *Xenopus* oil-driven injection rig. Survival was typically 60–70% for stage 8 embryos and > 95% for stage 20 embryos (see Table 1). *Xenopus* embryos were staged according to [42]. Injected *Drosophila* and *Xenopus* embryos were incubated at 18°C and injected *Tribolium* embryos were incubated at 25°C.

Embryo fixation and immunohistochemistry

Drosophila and *Tribolium* embryos were fixed on the coverslip by rinsing off the halocarbon oil with heptane, letting the coverslips air dry briefly and then placing the entire coverslip in a 35 mm dish containing 3 ml 3.7% formaldehyde in PEM buffer for 18 min. To permit even penetration of fix into the embryo, eggs were gently poked with a very fine tungsten needle just after placement in the fixation buffer. Fixed embryos were rinsed with 100% methanol and then rinsed with PBS pH 7.4 containing 0.1% Triton X-100 and 0.1% bovine serum albumin. Embryos were dissected in this solution and then histochemically stained using the protocol described in [43]. Expression of β-galactosidase was assayed by immunohistochemistry with a rabbit antibody to β-galactosidase (Jackson Labs); GFP was assayed in living embryos by fluorescence microscopy; Wg protein was assayed using a mouse monoclonal to *Drosophila* Wg [44], and Ubx protein was assayed with a mouse monoclonal antibody specific for *Drosophila* Ubx (FP3.3) [45]. *Xenopus* embryos were fixed for 20–30 min in 3.7% formaldehyde in PEM buffer and assayed for β-galactosidase expression by X-gal staining [21]. For both v-h/Ubx and v-h/wg infections, we estimate that expression from viral infection is approximately 3–5 times higher than endogenous levels of expression. See Supplementary material for a photograph of v-h/Ubx infection illustrating this.

Supplementary material

Supplementary material including additional methodological detail and two figures showing ectopic Ubx expression in *Drosophila* embryos and the needles used for injection is available at http://current-biology.com/supmat/supmatin.htm.

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Because *Current Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub – for further information, see the explanation on the contents page.

Supplementary material

Functional conservation of the *wingless–engrailed* interaction as shown by a widely applicable baculovirus misexpression system

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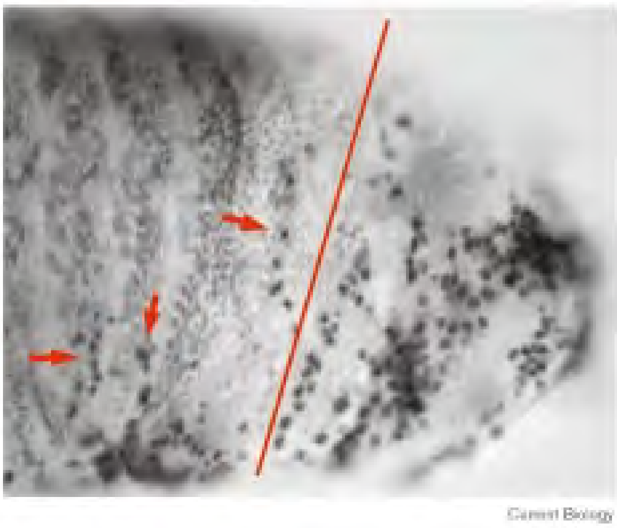
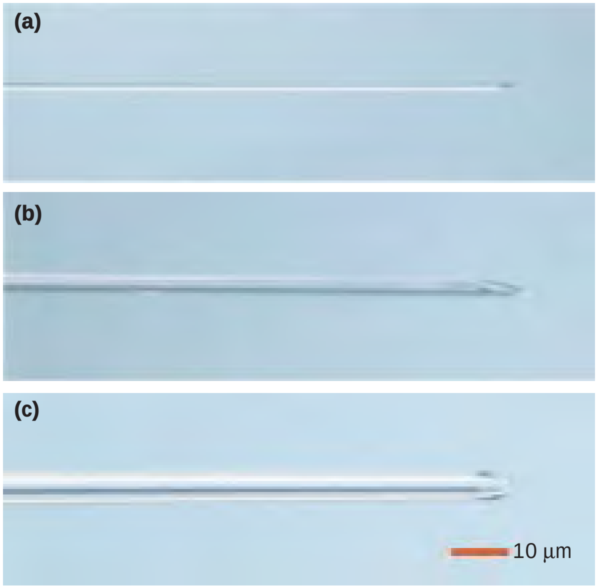
Figure S1



Current Biology

Expression and quantitation of ectopic Ubx in *Drosophila* embryos. Stage 15 embryo infected with *hsp–Ubx* and then stained with a *Drosophila*-specific Ubx antibody (FP3.3). The fainter expression to the left (toward the posterior end of the embryo) of the red line (which indicates the boundary between parasegments 4 and 5) is endogenous expression, whereas the more intense and mosaic expression to the right (toward the anterior end of the embryo) of the red line is ectopic expression. Further, the individual intensely expressing cells (red arrows) to the right of the red line are ectopic plus endogenous expression. By comparing signal intensity of endogenous and ectopic expression using Adobe Photoshop, we determined that virus-mediated Ubx expression is approximately threefold to fivefold greater than in the wild type.

Figure S2



Current Biology

Comparison of needle sizes used for injecting *Drosophila*, *Tribolium* and *Xenopus* embryos. **(a)** Needle used for *Drosophila* embryo injection. The needle diameter is approximately 2 µm and the point is beveled. **(b)** Needle used for *Tribolium* injection. The needle diameter is approximately 4 µm and the point is beveled. Although this needle size works quite well for *Tribolium*, a needle diameter of 2 µm is equally effective. **(c)** Needle used for *Xenopus* injection. The needle diameter is approximately 8 µm and the point does not need to be beveled.

Supplementary materials and methods

Information about baculovirus techniques as well as insect cell culture can be found in [S1]. All page numbers mentioned below refer to [S1]. Unless otherwise noted, protocols for baculovirus preparation and purification were followed exactly as indicated in [S1].

BacPak viral DNA can be purchased ready for use in homologous recombination from Clontech. BacPak virus contains a *lacZ* gene in place of the polyhedrin gene, and the transfer vector I used contains the polyhedrin gene. Thus, recombinant baculoviruses can be identified by their lack of the *lacZ* gene, which can be visualized by lack of X-gal metabolism, and gain of the polyhedrin gene, which can be visualized by the presence of occlusion bodies in infected cells.

Transfection information is described on page 145. Although many people have success with liposome-mediated transfection, we have found that calcium phosphate precipitation works quite nicely. The protocol for calcium phosphate precipitation is on pages 146–147.

The initial homologous recombination takes approximately 2 h to set up and then a 5 day incubation as virus is produced. Generally, the medium contains greater than 90% recombinant virus, so a scale-up of this mixture can be produced and used for injection to test efficacy of the virus. Each round of end-point dilution takes approximately 1 h to set up and then a 7 day incubation. Scale-up of virus requires 3–4 days of incubation. Slightly higher titers of virus can be achieved in monolayer cell culture, but we found it more efficient to produce virus in suspension culture, as we could produce larger quantities of virus in a smaller space.

Virus purification can be undertaken by either plaque purification or endpoint dilution. We found that end-point dilution is somewhat easier, faster and less prone to contamination than plaque purification. The concept in endpoint dilution is that a dilution series of virus is used to infect cells; at a particular dilution, there is only one (or no) virus particle in each well. Thus, a single virus can be isolated and expanded. The protocol for endpoint dilution can be found on pages 155–158. When looking for the loss or gain of β-galactosidase, we typically use X-gal at 240 µg/ml final concentration (stock concentration 20–40 mg/ml in DMSO). We recommend letting incubation proceed for a full 7 days, to ensure easily visible color reactions. After three rounds of endpoint dilution, the virus can be amplified according to the protocol described on pages 165–166. Be sure to confirm the identity of the recombinant virus by immunohistochemistry, restriction digests and/or Southern hybridization.

Example: v-h/wg virus

The full-length *wg* DNA fragment was cut out of pSP65wg using *Bam*HI and *Afl*II, blunted with Klenow enzyme and gel-purified. The plasmid KS/hsplacZ (Bluescript KS containing the *hsp70* promoter and *lacZ* gene) was cut with *Eco*RI and *Hind*III, blunted with Klenow enzyme and the larger fragment (containing the *hsp* promoter and Bluescript KS) was gel-purified. These two pieces were ligated together to produce KS/hspwg. After confirming that *wg* was inserted in the correct orientation behind the *hsp70* promoter, the entire cassette containing the *hsp70* promoter and *wg* open reading frame was cut out by a *Xba*I and *Bam*HI double digest. The transfer vector (called KS/3272bp transfer vector) contains baculovirus sequences, derived from the *Eco*RI fragment of the viral genome, which facilitate homologous recombination with the parental virus. This transfer vector has a unique *Eco*RV site just upstream of the polyhedrin gene. The hspwg cassette was inserted into the transfer vector at this *Eco*RV site. Orientation of the cassette in the transfer vector appears to have no effect. Purification of the transfer plasmid is important. Qiagen miniprep or midi/maxiprep purified DNA is suitable for transfection.

Although one can purchase predigested BacPak (parental virus) viral DNA from Clontech, one can maintain a stock of this virus and purify DNA when necessary. Viral DNA was purified as follows (see also page 141 in [S1]): pellet 10 ml of viral supernatant, resuspend the pellet in 10 mM Tris, 10 mM EDTA, 0.25% SDS, pH8.0 using a cut-off pipet tip to avoid shearing the viral DNA and add proteinase K to a final concentration of 50 μg/ml; incubate overnight at 37°C. The next day, phenol, phenol/chloroform, chloroform extract, then precipitate in ethanol and 100 mM sodium acetate pH 5.2. Be sure not to let the pellet dry out! Resuspend (again use a cut-off pipet tip) in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and let incubate several hours at 37°C to dissolve. Digest 10 μg viral DNA with 80 units *Bsu*36I overnight at 37°C. Add 20 more units and digest a further 2 h. Virus DNA is now ready for use in homologous recombination.

Transfection was carried out as described above, using 1 μg viral DNA and 1 μg transfer vector per dish. Use Sf9cells for the transfection, end-point dilution and scale-up of virus. We recommend at least two independent transfections because sometimes one of the transfection reactions will contain an incorrectly recombined virus. Let infection continue for 5 days. Often, it is difficult to see occlusion bodies in this initial transfection. One can either accept on faith that the homologous recombination has occurred (it has never failed for us) or try infecting a well or dish of cells with 0.25 ml of this undiluted virus. Be sure to purify at least one virus from each transfection reaction. For the hspwg virus, selection was straightforward, as the recombinant virus has occlusion bodies (visible as polyhedra in the nuclei of infected cells 48 h or more after infection) and do not have β-galactosidase, that is, both gain and loss of a phenotype as described in [S1]. After three rounds of end-point dilution, scale up production of the purified virus and store the supernatant at 4°C. To confirm identity of the virus, use whatever combination of PCR, restriction digests and/or Southern hybridization to confirm proper insertion. If possible, perform immunohistochemistry on Sf9 cells infected with desired recombinant virus to confirm protein synthesis.

For misexpression experiments, follow protocols described in the paper for concentration and injection of the virus.

Other possible species for misexpression

We would predict that baculoviruses will not be effective for misexpression in Lepidoptera, as injection of baculoviruses into many Lepidoteran species leads to a productive infection (see [S2,S3]). We have had limited success infecting amphipods (Crustacea: Amphipoda).

Supplementary references

S1. O'Reilly DR, Miller LK, Luckow VA: *Baculovirus Expression Vectors: a Laboratory Manual*. Oxford University Press; 1994.

S2. Lewis DL, DeCamillis MA, Brunetti CR, Halder G, Kassner VA, *et al.*: *Curr Biol*, 1999 9: 1279-1287.

S3. Kirkpatrick BA, Washburn JO, Volkman LE: AcMNPV pathogenesis and developmental resistance in fifth instar ***Heliothis virescens***. *J Invert Path* 1998, 72:63-72.