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 engraveld, appears to be conserved between Drosophila and Tribolium. The data on the establishment of an engraveld-competent domain also support the idea that prepatternning 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.

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melanogaster, the beetle Tribolium castaneum and the grasshopper, Schistocerca americana [2–4] (D. DiPietro and N.H.P., unpublished observations).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,

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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 (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/lacZ and stained for β-galactosidase (black) and En (brown). Expression of β-galactosidase is mostly restricted to 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 15 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).
to Hsp70, however, we were unable to detect expression
ble for initiating expression. Using a monoclonal antibody
itself induced a stress response that was in turn responsi-
induce expression. We checked whether virus infection
within the yolk and does not come into contact with cells
midgut presumably because the injected solution remains
syncytial blastoderm stages) resulted in infection of the
center of the embryo before gastrulation (at the cellular or
stage or just after gastrulation, and examined 24–48 hours
embryos were injected in the posterior at the blastoderm
section running the length of the embryo shortly after
gastrulation; Figure 1e–g). Further, 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 in vivo; 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 yolk 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

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**Table 1**

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

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

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.
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, noto-chord, 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/nucZ 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.

**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 expression.
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<sup>cxd</sup>). 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<sup>cxd</sup> mutant, either un.injected 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<sup>cxd</sup> 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<sup>cxd</sup> 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 prepatternning 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

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<sup>cxd</sup>) and (e,f) Wg<sup>cxd</sup> 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).
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 [3,4,6,27].

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 anterior-most 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
As much of morphological differences between early segmentation may be caused 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 pSP65[wg] 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/UbxB + h/lacZ, which expresses full-length Drosophila UbxB (derived from pKSUbxB 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 3722 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/UbxB + h/lacZ virus was made by using v-h/UbxB virus as the parental virus and a transfer vector (derived from the PsI 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 $5 \times 10^7$ to $2 \times 10^8$ 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 resuspending the medium for 10 min at 800 x g to remove particulate matter, then pelleting 5–10 ml of virus through a 25% (w/v) sucrose cushion (sucrose dissolved in 0.1 x PBS; pH 6.2) for 30 min at 80,000 x 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 wrinking 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. Embryos were fixed for 20–30 min in 3.7% formaldehyde in PBS, repelleted in a microfuge tube and resuspended in a volume 3 ml 3.7% formaldehyde in PEm buffer for 18 min. To permit even penetration of fix into the embryo, eggs were gently rocked 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 histochromically 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 Supplemental material for a photograph of a v-h/Ubx infection illustrating this.

Supplemental material

Supplemental 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/supmat.htm.

Acknowledgements

We thank Loy Volkman and Jan W ashburn for the v-h/lacZ virus and baculovirus DNA fragments used for transfer plasmids; Stephen Cohen, Rob White and Susan Lindquist for antibodies; Richard Harland, Amy Bejoyec, and Michael Akam, respectively, for the nuclear lacZ, Drosophila Wg and Ubila cDNAs; Jeff Neul, Eva Decotto and John Hudson for advice on Drosophila microinjection; Cory Kending and Ramanuj Dasgupta for technical assistance; W Ilam B ronne for constructing the v-h/GFP virus; and Greg Davis, Sabbli Lal, Jon Walsh and Bridget Lear for comments. D.O.J. was a Howard Hughes Medical Institute Associate and is presently an NSF/Sloan Postdoctoral Fellow in Molecular Evolution, A.M.M. is supported by NIH grant CA 70846 and N.H.P. is an Assistant Investigator of HHMI.

References

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.

Figure S1

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

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.
Example: v-h/wg virus

The full-length wg DNA fragment was cut out of pSP65wg using BamHI and AflII, blunted with Klenow enzyme and gel-purified. The plasmid KS/hsplacZ (Bluescript KS containing the hsp70 promoter and lacZ gene) was cut with EcoRI and HindIII, 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 XbaI and BamHI double digest. The transfer vector (called KS/3272bp transfer vector) contains baculovirus sequences, derived from the EcoRI fragment of the viral genome, which facilitate homologous recombination with the parental virus. This transfer vector has a unique EcoRV site just upstream of the polyhedrin gene. The hspwg cassette was inserted into the transfer vector at this EcoRV 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, pH 8.0 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 Bsu36I 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 Sf9 cells for the transfection, endpoint dilution and scale-up of virus. We recommend at least two independent transfections because sometimes one of the transfection reactions will contain an incorrectly recombinant 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 Lepidopteran species leads to a productive infection (see [S2, S3]). We have had limited success infecting amphipods (Crustacea: Amphipoda).

Supplementary references

