Multiple transcription factor codes activate epidermal wound–response genes in *Drosophila*

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Wounds in *Drosophila* and mouse embryos induce similar genetic pathways to repair epidermal barriers. However, the transcription factors that transduce wound signals to repair epidermal barriers are largely unknown. We characterize the transcriptional regulatory enhancers of 4 genes—*Ddc*, *ple*, *msn*, and *kkv*—that are rapidly activated in epidermal cells surrounding wounds in late *Drosophila* embryos and early larvae. These epidermal wound enhancers all contain evolutionarily conserved sequences matching binding sites for JUN/FOS and GRH transcription factors, but vary widely in *trans*- and *cis*-requirements for these inputs and their binding sites. We propose that the combination of GRH and FOS is part of an ancient wound–response pathway still used in vertebrates and invertebrates, but that other mechanisms have evolved that result in similar transcriptional output. A common, but largely untested assumption of bioinformatic analyses of gene regulatory networks is that transcription units activated in the same spatial and temporal patterns will require the same *cis*-regulatory codes. Our results indicate that this is an overly simplistic view.

cuticle | grainy head | fos | wound repair

Animals have evolved systems to sense and repair epidermal wounds and to fight microbial infections that follow wounding. Many wound responses are carried out by homologous genetic pathways in arthropods and vertebrates and thus apparently had evolved in a common ancestor of bilateral animals. These responses include pathways mediating reepithelialization (1–9), responses to microbial invasion (10), and regeneration of epidermal barrier layers (11, 12). The outermost epidermal barrier in mammals is the stratum corneum, a constantly regenerated layer of crosslinked keratinocytes, proteins, and lipids (13). The analogous protective barrier for arthropods is the cuticle, comprised of crosslinked chitin, proteins, and lipids (14). Although mammalian and arthropodal epidermal barriers are constructed in largely different ways, they share a homologous genetic pathway controlling barrier repair that involves transcription factors of the Grainy head (GRH) family (11, 12).

One central question in the control of epidermal wound repair is how signals are integrated at the level of transcription to activate the large battery of effector genes that mediate wound responses. Although many genes are known to be activated in epidermal cells after wounding (15–17), little is known about the *cis*-regulatory enhancers that mediate wound-induced gene activation. In *Drosophila*, there are only a few known genes activated at epidermal wound sites (11, 14, 17). Two of these genes encode the enzymes Dopa decarboxylase (*Ddc*) and Tyrosine hydroxylase (encoded by *ple*) (11). These 2 enzymes are limiting steps in the pathway to produce the highly reactive quinones that crosslink chitin and cuticle proteins during the construction and repair of cuticular barriers (18).

To better characterize the transcriptional wound response in *Drosophila* embryos, we have mutagenized minimal *Ddc* and *ple* epidermal wound enhancers, showing that both require AP-1-like and GRH consensus sites. We then searched for AP-1 and GRH consensus binding sites in the regulatory DNA for other epidermal wound–response genes, which led to the identification of epidermal wound enhancers for the genes *krotzkopf verkehrt* (*kkv*) and *misshapen* (*msn*). *Ddc*, *ple*, *kkv*, and *msn* are all transcriptionally activated within minutes after epidermal wounding. Three of the genes use overlapping transcriptional codes involving GRH and FOS to activate their epidermal wound enhancers, but *kkv* uses a fundamentally different code for wound activation. Whereas the common wound–response *cis*-regulatory codes we describe will be useful in the identification of new epidermal wound enhancers in both vertebrates and invertebrates, the evidence for alternative wound–response codes provides insight into how genetic control of wound healing has evolved in metazoans.

Results

Minimal *Ddc* Epidermal Wound Enhancers. By using reporter gene constructs in *Drosophila* embryos, we previously identified a 0.47-kb DNA fragment just upstream of the *Ddc* gene that functioned as an epidermal wound enhancer in late embryos (Fig. 1*A*) (11). This enhancer can activate GFP reporter expression in a zone around aseptic epidermal punctures in late embryos (Fig. 1*B*), in larvae just before molts (data not shown), and in very young adults (Fig. 1*C*). The *Ddc* .47 wound-enhancer DNA contains 2 regions upstream of the basal promoter that are highly conserved among all sequenced drosophilid species: CR1 (44 nt) and CR2 (13 nt) (Fig. 1*A* and *SI Appendix*). The CR2 sequence consists largely of a high-affinity GRH binding site (ACCGGTT) (12, 19, 20), which is required for wound-enhancer function (11). *Ddc* .47 CR1 contains a sequence matching the consensus binding site (TGANTCA) for AP-1, a transcription factor consisting of a JUN-FOS dimer (21).

To test for AP-1-like site function in the *Ddc* .47 wound enhancer, we mutated the conserved AP-1-like site in CR1 and a second AP-1-like site that lies between CR1 and CR2 (Fig. 1*A*). This construct is unable to activate reporter expression after wounding (Fig. 1*D*). We also deleted 355 bp between the conserved regions (*Ddc* Gap) (Fig. 1*A*), to test whether any required DNA elements are located within the region between CR1 and CR2. This deletion mutant functions as an epidermal wound enhancer, but the number of cells that activate reporter gene expression is reduced compared with WT *Ddc* .47 (Fig. 1*E*). In summary, both AP-1-like and GRH consensus sequences are absolutely required for *Ddc* .47 wound activation, and a minimal enhancer of 117 bp containing these sites is sufficient for modest activation in cells around wound

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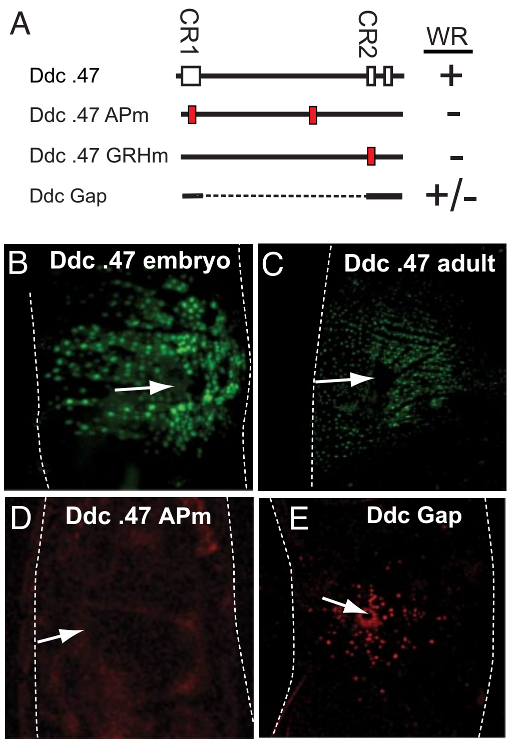

Fig. 1. Sequence requirements of the *Ddc* epidermal wound enhancer. (*A*) Diagram of WT and mutant *Ddc* .47 epidermal wound–response enhancers. White blocks indicate conserved regions with other drosophilids; red blocks denote mutant sequences. Functional wound enhancers are indicated by "+," nonfunctional enhancers by "−." (*B–E*) Dashed lines outline embryos, arrows indicate wound sites, and fluorescent nuclei surrounding wounds show GFP or DsRed reporter gene activation provided by wound enhancers. (*B*) *Ddc* .47 activated GFP-reporter expression around wounds in late-stage embryonic epidermis. (*C*) *Ddc* .47 activated GFP-reporter expression around wounds in early adult abdominal epidermis. (*D*) A mutation of AP-1-like consensus sites (APm) in *Ddc* .47 abolished wound-enhancer function. (*E*) *Ddc* Gap had reduced wound-enhancer function. Previous results showed that a GRH site mutant (GRHm) in *Ddc* .47 abolished wound-enhancer function (11).

sites. However, additional sequences within .47 *Ddc* contribute to the strength of this epidermal wound enhancer.

An Epidermal Wound Enhancer from the *ple* Gene Requires GRH and AP-1-Like Sequences. We previously identified 2 epidermal wound enhancers upstream of *ple* by searching for conserved clusters of AP-1-like and GRH consensus sites (11). We have refined the boundaries of the distal 3-kb *ple* fragment that contains a wound enhancer, delimiting the enhancer to a 687-bp DNA fragment that resides 3.45 kb upstream of *ple* (Wound Enhancer 1 or WE1) (Fig. 2 *A* and *B*). In addition to conserved AP-1-like and GRH consensus sites, analysis of this *ple* wound enhancer revealed conserved regions with consensus binding sites for other transcription factors. Of these sites, we noted putative binding sites for CREB homodimers (TGACGTMA) (22), EXD/PBX homodimers (WGATTGAW) (23–25), and Hox monomers (YMATTA) (26, 27). To test the importance of these sites, we mutated them in the context of *ple*-WE1 (Fig. 2*A*). Mutating the consensus AP-1-like sites abolished *ple* wound-enhancer function (Fig. 2*C*), whereas mutation of the GRH consensus site resulted in a consistent reduction of wound-enhancer function (Fig. 2*D*). In contrast, mutations in the CREB, EXD, or HOX consensus sites had no consistent effect on *ple* WE1 wound-dependent activation (Fig. 2*E–G*). Therefore, activation of *Ddc* and *ple* wound enhancers depends on AP-1-like and GRH consensus binding sites.

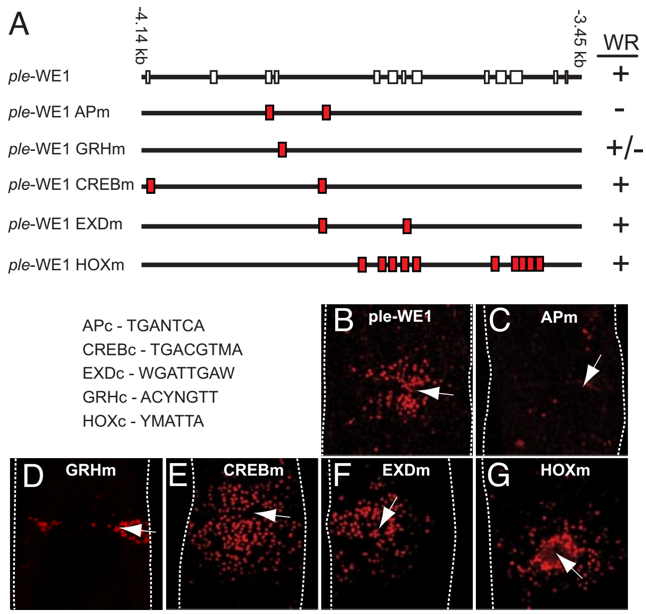

Fig. 2. *ple* epidermal wound enhancer binding site requirements. (*A*) Diagram of WT and mutant *ple*-WE1 enhancers. White boxes indicate regions of sequence conservation in drosophilids; red blocks denote mutant sequences. Wound responses of the elements are indicated at right. (*B–G*) Dashed lines outline the embryos; arrows indicate wound sites. (*B*) *ple*-WE1 was activated at epidermal wounds. (*C*) Mutation of two AP-1-like consensus sites abolished *ple*-WE1 wound-enhancer activity. (*D*) Mutation of a GRH consensus site strongly reduced *ple*-WE1 wound-enhancer activity. (*E–G*) Mutation of 2 CREB-like sites (*E*), or 2 EXD-like sites (*F*), or 14 HOX-like binding site had no effect on *ple*-WE1 enhancer function (*G*).

To test whether sequences matching the GRH and AP-1-like consensus sites in the *Ddc* and *ple* wound enhancers bound GRH and AP-1 family proteins in vitro, we used EMSAs (*SI Text*) (28). An oligonucleotide including a GRH consensus site (ACCGGTT) from the *Ddc* wound enhancer binds full-length GRH protein with high affinity and specificity (Fig. 3*A*) (19). The GRH-like site in the *ple* WE1 element (ACTCGTTT) is a weaker match to an optimal site (AACCGGTTT) (20), and oligonucleotides with this site bind GRH protein with low affinity and specificity (Fig. 3*A*).

EMSA experiments using full-length *Drosophila* JUN and FOS-B proteins (the fly *kay* gene produces 4 isoforms of FOS, A–D), indicate that they bind as heterodimers with high specificity and affinity to AP-1-like sites from CR1 of the *Ddc* wound enhancer, whereas JUN or FOS-B homodimers had lower affinities (Fig. 3*B*). Because the AP-1-like site in the *ple* enhancer has the same consensus sequence as the one in the *Ddc* enhancer, we did not test JUN/FOS binding to *ple* WE1 AP-1-like sites. In contrast to mammalian FOS, *Drosophila* FOS-B protein can bind as a homodimer to AP-1-like consensus sites, albeit with lower affinity than JUN/FOS heterodimers (Fig. 3*B*) (29). To explore the possibility that other *Drosophila* FOS isoforms might bind wound-enhancer AP-1-like sites, we also tested FOS-D binding to an oligonucleotide containing the *Ddc* AP-1-like site. As seen in Fig. 3*B*, FOS-D homodimers apparently have higher affinity with *Ddc* AP-1-like sites than FOS-B or JUN homodimers, albeit less than JUN/FOS-B or JUN/FOS-D heterodimers. The relevance of FOS-D homodimer binding will be seen later in this report. From the above, we concluded that the mutations we introduced into the GRH and AP-1-like consensus sites in the *Ddc* and *ple* wound enhancers would eliminate binding of GRH, JUN, or FOS transcription factors in vivo.

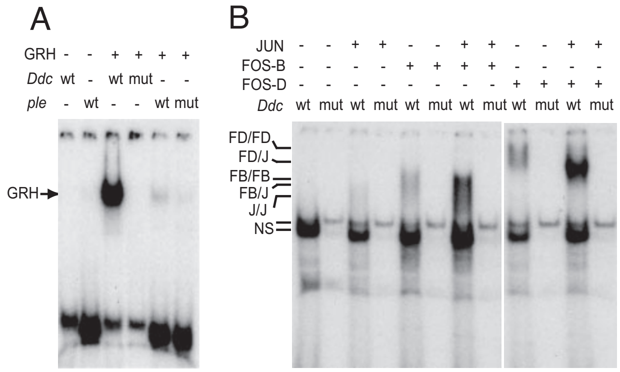

Fig. 3. GRH and FOS bind crucial sequences in the *Ddc* and *ple* wound enhancers. (*A*) Oligonucleotide DNA probes including the GRH consensus sites from *Ddc* and *ple* epidermal wound–response enhancers were tested in EMSA assays for binding to full-length *Drosophila* GRH protein (see *Materials and Methods*). Probes with mutant GRH sites were used to test binding-site specificity. Lanes 1 and 2 show *Ddc* or *ple* DNA probes with no GRH protein. Lanes 3 and 4 show GRH protein with WT and mutant *Ddc* probes, respectively. Lanes 5 and 6 show GRH protein with WT and mutant *ple* probe, respectively. Arrow indicates GRH–DNA complexes. The bands at the bottom of the frame are unbound probe. (*B*) Oligonucleotide probes including the AP-1-like site from the *Ddc* wound–response enhancer were tested in EMSA assays for binding to full-length *Drosophila* JUN, FOS-B, and FOS-D proteins (see *Materials and Methods*). Probes with mutant AP-1-like sites were used to test the specificity of binding. Lanes 1 and 2 show WT or mutant probes, no protein; NS denotes nonspecific shifted probe complexes that result from reticulocyte lysate alone; unbound probe is not shown. Lanes 3 and 4 show JUN protein with WT and mutant probes, respectively. Lanes 5 and 6 show FOS-B protein with WT and mutant probes, respectively. Lanes 7 and 8 show FOS-B and JUN proteins with WT and mutant probes, respectively. Lanes 9 and 10 show FOS-D protein with WT and mutant probes, respectively. Lanes 11 and12 show FOS-D and JUN with WT and mutant probes, respectively. The positions of the various shifted protein/DNA complexes are noted on the side.

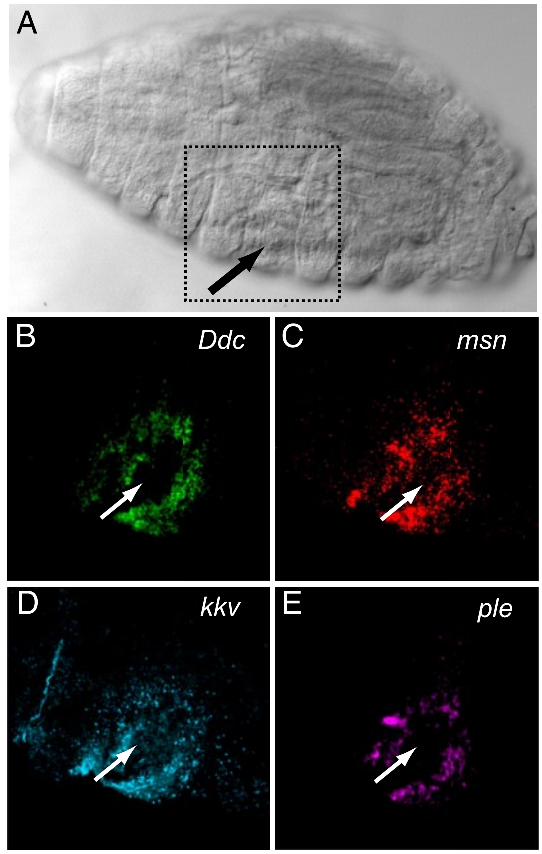

Fig. 4. *Ddc*, *ple*, *msn*, and *kkv* are transcriptionally activated in epidermal cells around wounds. (*A*) Image of wounded embryo, visualized with DIC optics, fixed 30 min after wounding. Arrow shows entry wound; dotted box shows the region imaged with fluorescence optics in frames *B–E*. (*B–E*) *Ddc* (*B*), *msn* (*C*), *kkv* (*D*), and *ple* (*E*) transcripts were simultaneously detected in the embryo around the aseptic wound using hapten-labeled probes (35). No signals were detected around wounds in embryos fixed immediately after wounding and hybridized with probes (data not shown).

Identification of Epidermal Wound–Response Enhancers. We next wished to determine whether clusters of AP-1-like and GRH consensus binding sites were a common feature of epidermal wound enhancers. Two other candidate genes for activation in embryonic epidermal cells after wounding were *kkv*, which encodes chitin synthase (30, 31), and *msn*, which encodes an upstream activating kinase in the JNK pathway (32). Chitin synthase is required for the final step in the production of chitin, a major component of *Drosophila* exo- and endocuticle (33), and we reasoned that *kkv* transcription was likely to be activated at epidermal wound sites to promote cuticle regeneration. *msn* transcription was likely to be activated around embryonic wounds, because previous results have shown a *lacZ* reporter gene inserted into the promoter region of *msn* is activated in larval and adult epidermal cells around wound sites (14, 34).

Using multiplex in situ hybridization (35), we tested for the transcriptional activation of *kkv*, *msn*, *Ddc*, and *ple* around epidermal wounds in late-stage embryos (Fig. 4). All 4 genes were transcriptionally induced within 30 min in a zone of cells surrounding wounds. Thus, it is possible that all 4 genes are regulated by the same wound-signaling pathways and combinatorial transcriptional codes. The accumulation of fluorescently labeled probes at wound sites was not an artifact of enhanced accessibility, as no localized probe signals were observed in embryos that were wounded, immediately fixed, and then hybridized (data not shown).

To identify candidate epidermal wound enhancers regulating *kkv* and *msn*, we surveyed these loci for evolutionarily conserved clusters of AP-1-like and GRH consensus binding sites. The 5′ region of the third intron of *msn* has a cluster of 2 conserved GRH sites and 1 conserved AP-1-like site. A 1.2-kb DNA fragment containing these sites functioned as an epidermal wound enhancer (*msn*-WE2) (Fig. 5 *A* and *D*).

The *kkv* first intron has 5 GRH consensus sites and 4 AP-1-like sites. We tested the wound enhancer function of 2 overlapping DNA fragments that each contained 4 GRH sites (2 conserved) and 3 AP-1-like sites (1 conserved). One of these fragments functioned as an epidermal wound enhancer (*kkv*-WE1) (Fig. 5 *B* and *E*), whereas the other did not (*kkv*2) (Fig. 5*B* and data not shown). To test whether AP-1-like and GRH consensus sites were required for the function of the *kkv* wound enhancer, we mutated the sites in the context of the 2.2-kb *kkv*-WE1 element (Fig. 5*C*). To our surprise, *kkv*-WE1 reporters with either AP-1-like (Fig. 5*F*) or GRH consensus sites (Fig. 5*G*) mutated were still induced in epidermal cells around wound sites. This evidence strongly suggests that the *kkv* wound enhancer is activated by a different transcription factor code than the *Ddc* and *ple* wound enhancers. A table of the quantitative responses of different mutant wound-enhancer lines is provided in Table S1.

Genetic Requirements for the Induction of *ple*, *kkv*, and *msn* Wound Enhancers. To test *trans* requirements for activation of the *ple*, *kkv*, and *msn* wound enhancers, we first tested their function in *grh* mutants (*Ddc* wound-enhancer function was previously shown to be dependent on *grh* genetic function) (11). In *grh* mutants, the *msn*-WE1 enhancer was not activated around embryonic wounds (Fig. 6 *A* and *B*), but the *ple* WE1 enhancer

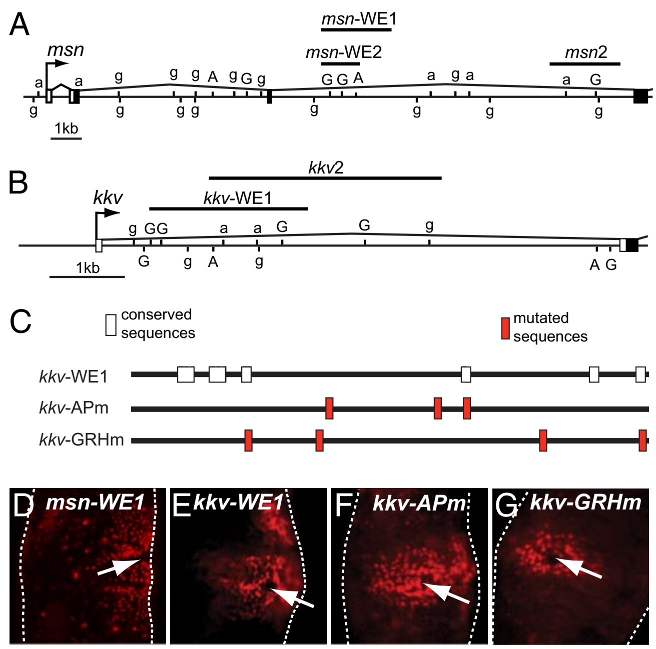

Fig. 5. Conserved AP-1-like and GRH consensus site clusters identify *kkv* and *msn* epidermal wound enhancers. (*A* and *B*) Diagrams of 5′ regions of the *msn* (*A*) and *kkv* (*B*) genes, with conserved AP-1-like and GRH consensus sites indicated by A and G, respectively, and nonconserved matches indicated in small case. Sites were found using GenePalette (60). The potential wound-enhancer regions that were tested overlie the diagrams. (*C*) Diagrams of the WT and mutant *kkv*-WE1 enhancers. In the 5′–3′ orientation, they were tested in reporter gene constructs. AP-1-like consensus binding site mutants (APm); GRH consensus binding site mutants (GRHm). White boxes indicate regions of conservation in drosophilids. Mutant sites are indicated by red boxes. (*D* and *E*) *msn*-WE1 (*D*) and *kkv*-WE1 (*E*) DNA epidermal wound enhancers driving DsRed fluorescent reporter protein expression around wound sites. Dashed lines indicate embryo boundaries; arrows indicate entry-wound sites. (*F* and *G*) *kkv*-WE1 enhancers with mutations in either AP-1-like or GRH consensus sites. See *SI Appendix* for all DNA sequences.

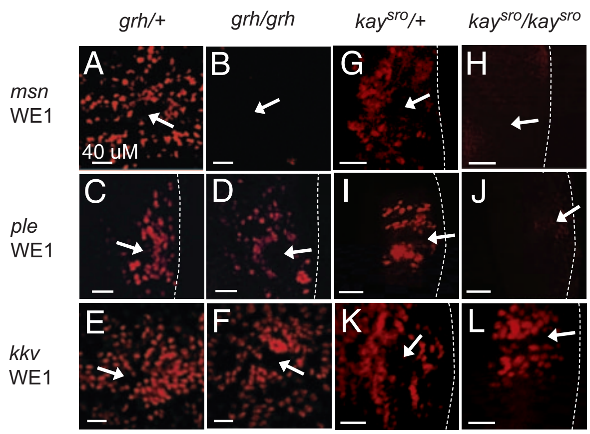

Fig. 6. Genetic requirements of epidermal wound–response enhancers. DsRed fluorescent protein reporter genes attached to the *ple*-WE1, *kkv*-WE1, and *msn*-WE1 epidermal wound enhancers were introduced into grh^{IM} and fos/kay^{sro1} mutant backgrounds balanced with *Kruppel*-GFP chromosomes carrying positive WT *grh* or *fos/kay* genes. DsRed signals around wound sites from *Kruppel*-GFP negative embryos (homozygous mutants) were compared with the signals observed in wounded *Kruppel*-GFP positive embryos (heterozygous for the mutant allele). Dashed lines show embryo boundaries; white arrows indicate puncture-wound sites. (*A* and *B*) *msn*-WE1 wound-enhancer function in a $grh^{IM}/+$ compared to a grh^{IM}/grh^{IM} embryo. (*C* and *D*) *ple*-WE1 wound-enhancer function in a $grh^{IM}/+$ compared to a grh^{IM}/grh^{IM} embryo. (*E* and *F*) *kkv*-WE1 wound-enhancer function in a $grh^{IM}/+$ compared to a grh^{IM}/grh^{IM} embryo. (*G* and *H*) *msn*-WE1 wound-enhancer function in a $kay^{sro1}/+$ compared to a kay^{sro1}/kay^{sro1} embryo. (*I* and *J*) *ple*-WE1 wound-enhancer function in a $kay^{sro1}/+$ compared to a kay^{sro1}/kay^{sro1} embryo. (*K* and *L*) *kkv*-WE1 wound-enhancer function in a $kay^{sro1}/+$ compared to a kay^{sro1}/kay^{sro1} embryo.

was activated to approximately normal levels (Fig. 6 *C* and *D*). This finding is seemingly at odds with the requirement of a GRH consensus binding site in *ple*-WE1 and suggests that another required factor acts through the low-affinity *ple*"GRH" consensus site in *ple*-WE1. The *kkv*-WE1 enhancer showed no significant reduction in function in *grh* homozygotes (Fig. 6 *E* and *F*), consistent with the *kkv*-WE1 enhancer's lack of dependence on GRH consensus binding sites.

We had previously found that a *Ddc* wound enhancer was activated normally in mutants for *jun* (Jra^{IA109}) and *fos* (kay^{1}) (11). At first glance, this finding seems paradoxical, because AP-1 consensus sites (binding sites for JUN/FOS dimers), are required for the activity of *Ddc* and *ple* epidermal wound enhancers. The *jun/Jra* mutation we tested is a null allele for the locus (36), and, thus, excludes JUN as an activator of *Ddc* wound enhancers. However, the fos/kay^{1} allele does not eliminate the function of all the *Drosophila* FOS isoforms (37–39).

We thus considered the possibility that other FOS isoforms might act through the required AP-1-like binding sites in the *Ddc* and *ple* wound enhancers. One intriguing candidate is the FOS-D protein isoform, encoded in transcripts that are abundantly expressed in late embryonic epidermis (39). In addition, a mutation in the *fos/kay* gene called $shroud^{P54}$ (or fos/kay^{sroP54}) is caused by a transposable element insertion in the promoter for FOS-D, and fos/kay^{sroP54} and other fos/kay^{sro} mutants have fragile, poorly differentiated cuticles that resemble those of *grh* mutants (39).

We therefore tested whether *Ddc*, *ple*, *kkv*, and *msn* wound enhancers could be induced in the epidermis of fos/kay^{sro1} mutants. The epidermal wound enhancers *msn*-WE1 (Fig. 6 *G* and *H*), *ple*-WE1 (Fig. 6 *I* and *J*), and *Ddc* .47 (data not shown) were not active in fos/kay^{sro1} mutants, whereas *kkv*-WE1 showed normal wound activation in fos/kay^{sro1} mutants (Fig. 6 *K* and *L*). The absence of wound-enhancer activation for *ple*, *Ddc*, and *msn* in fos/kay^{sro1} mutants was not due to a failure of epidermal development or function, as shown by the normal induction of *kkv*-WE1 in the mutants. The activation of *kkv*-WE1 in wounded fos/kay^{sro1} mutants is consistent with the finding that *kkv*-WE1 is also unaffected by mutation of AP-1-like consensus binding sites. Given that *Drosophila* FOS-D homodimers can bind with high affinity and specificity to wound-enhancer AP-1-like sites (Fig. 3*B*), it is possible that FOS-D does not require a heterodimeric binding partner on wound enhancers, but it is also possible that FOS-D heterodimerizes with a *Drosophila* basic-leucine zipper protein other than JUN on wound enhancers (40).

Discussion

One principal conclusion of our findings is that the activation of *Ddc* and *msn* epidermal wound enhancers requires the genetic function of both *grh* and fos/kay^{sro}. The current evidence indicates that fos/kay^{sro} mutants reduce or abolish the function of the *Drosophila* FOS-D isoform (39). The *grh* and fos/kay^{sro} gene functions are required not only for the activation of epidermal barrier-repair genes in *Drosophila* embryos but also for the generation of a normal epidermal barrier during embryonic development (11, 30, 39, 41, 42). The current evidence suggests that the combinatorial roles of *Drosophila grh* and fos/kay^{sro} in activating epidermal barrier-repair genes might be conserved by their mammalian homologs. In mice, one of the *grh*-like genes is required for embryonic wound repair and development of a normal epidermal barrier, and this is accomplished at least in part via activation of downstream target genes that are required for skin barrier formation (12, 43, 44). Some mammalian *fos* and *jun* genes have been implicated in epidermal barrier development and control of epidermal wound repair, although their genetic roles in the epidermis are still being explored. It is known that mouse *fos* and *jun* paralogs are expressed in differentiating epidermis and up-regulated in wounded epidermal cells (45). Also, in the cells surrounding epidermal wounds, there is some genetic evidence that certain *jun* and *fos* paralogs regulate wound healing, although whether they act to accelerate or retard (or both) the process of wound healing is still unclear (46–49).

On the basis of the evidence just described, we propose that GRH and FOS family proteins are part of an ancient, evolutionarily conserved code that serves to activate an immediate transcriptional response in epidermal cells near wound sites. A recent report found that the *Drosophila* GADD45 gene is strongly activated around epidermal wounds (17) and that it may be controlled by the same activation code because a cluster of GRH and FOS (AP-1) consensus binding sites exist $\approx$2 kb upstream of the GADD45 transcription start site. However, there must be other epidermal wound transcriptional codes, because the *ple* wound enhancer required *fos*/*kay*^{sro} function (but little or no input from *grh*), and the *kkv* wound enhancer required neither of these inputs. A common assumption is that similar combinations of transcription factors will control transcription units that are activated in the same temporal and spatial patterns. Our results indicate that, at least for *Drosophila* wound enhancers, this assumption is incorrect.

The signals that are sensed by *Drosophila* cells surrounding wounds are as yet unknown. The functional activation of either or both of the GRH or FOS-D proteins may depend on a receptor tyrosine kinase pathway, because both phospho-tyrosine and diphospho-ERK levels increase rapidly around wound sites and because ERK inhibition reduces the function of a *Ddc* epidermal wound enhancer (11). It is known that *Drosophila* GRH and FOS proteins can be phosphorylated by ERK in vitro (50–52), but at present it is unknown whether GRH and FOS are phosphorylated in a wound-dependent manner in the epidermis.

Although JUN kinase function is apparently not required for activation of the *Ddc* wound enhancer (11), the immediate transcriptional activation of *msn*, which encodes a JUN kinase kinase kinase kinase (32), is consistent with previous findings that the JNK pathway is required for wound-dependent reepithelialization (14, 34, 52, 53). The immediate activation of *msn* transcription after wounding (Fig. 4) suggests that robust JNK activity after epidermal wounding might be dependent on an immediate response involving GRH, FOS-D, and ERK (11). Genetic linkages between the reepithelialization response and the epidermal barrier-repair response are still poorly understood, but in a biological sense the two responses must be coordinated, with reepithelialization occurring in concert with barrier regeneration. The activation of the *msn* epidermal wound enhancer in a manner that depends on *grh* and *fos*/*kay*^{sro} suggests that *msn* may mediate crosstalk between the reepithelialization and cuticle barrier-repair pathways during the complex process of wound repair. Ongoing genetic screens in *Drosophila* are likely to clarify the relationships between the reepithelialization and epidermal barrier–repair pathways, as well as identify diffusible signals and receptors that mediate the immediate response to epidermal wounding.

Materials and Methods

***Drosophila* Stocks and Genomic DNA.** *D. melanogaster* strain *w*^{1118} was used for germline transformation (54, 55), for in situ hybridizations, and as a source for genomic DNA. Fly stocks for *Drosophila pseudoobscura*, *Drosophila virilis*, *Drosophila immigrans*, and *Drosophila hydei* were supplied by the Tucson *Drosophila* Stock Center. Genomic DNA was prepared by using standard procedures. *kay*^{sro1} mutants were obtained from the Bloomington Stock Center. Transgenic lines containing epidermal wound-enhancer test constructs were obtained by using injected *w*^{1118} embryos. The reporter constructs were tested in pH-Stinger vectors that contain GFP (56) or DsRed (57) reporter genes.

Wounding Procedure. Embryos were collected on apple juice agar plates and aged to 15–17 h at 25 °C. Embryos were washed into mesh baskets, dechorionated in bleach for 1 min, then washed copiously with water. Embryos were then transferred to a clean slab of apple juice agar and aligned for 30–60 min at 18 °C, transferred to slides with double-sided tape, then covered in a 1:1 ratio of 700:27 weight halocarbon oil or PBS. Embryos were then wounded laterally with fresh microinjection needles made from an automated puller mounted on a micromanipulator, allowed to recover for 3–8 h at room temperature, and visualized under fluorescent light in either a compound microscope or a Leica SP2 confocal microscope to determine wound response. At least 2 independent experiments with at least 20 successfully wounded embryos were performed, testing at least 2 independent transformant lines. Assays involving altered enhancers were performed in parallel to unaltered enhancers, impaling all embryos using a micromanipulator so that the needle protruded 1 embryo-width from the exit wound, and responses rated on a scale of ''no response, weak, moderate, strong.'' Images were obtained by wounding embryos with microinjection needles by hand and imaged on a Leica SP2 confocal microscope, selecting representative embryos to image. Pixel-intensity levels of images were adjusted for clarity; Adobe Photoshop despeckle, blur, and sharpen functions were used occasionally to enhance clarity. Original images are available on request.

In Vitro Synthesis of Proteins. The following plasmid clones were used to produce *Drosophila* GRH, JUN, FOS-D, and FOS-B proteins in reticulocyte lysates; pcDNAMyc/His-GRH, pcDNAMyc/His-DJUN, pcDNAMyc/His-DFOS-B, and pcDNAMyc/His-DFOS-D. PCR synthesized ORFs for these proteins were inserted into pcDNA3.1(−)/Myc-His A plasmids (pcDNAmyc; Invitrogen). The primers used to synthesize the ORFs are reported in *SI Text*.

Synthesis of proteins was performed in a quick-coupled transcription/translation system (TNT kit, Promega) in vitro, programmed with 0.05–0.3 μg of pcDNAMyc/His-GRH, pcDNAMyc/His-DJUN, pcDNAMyc/His-DFOS-B, or pcDNAMyc/His-DFOS-D plasmids. For assaying expression level of TNT products, proteins were subjected to 10% SDS/PAGE and transferred to PVDF membranes. The blots were incubated for 1 h with a 1/1,000 dilution of anti-myc antibody 9E10 (Developmental Studies Hybridoma Bank). The blots were washed, incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch), and visualized by chemiluminescence as described by the supplier (SuperSignal West Pico chemiluminescent substrate; Pierce). EMSAs were performed as described in the *SI Text*.

Multiplex Fluorescent in Situ Hybridization. Probes were generated from partial or full cDNA clones from the *Drosophila* Gene Collection (58, 59). Probe labeling and hybridization protocol was as described in Dave Kosman's multiplex FISH protocol (35).

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