Conserved Regions of the *Drosophila* Erect Wing Protein Contribute Both Positively and Negatively to Transcriptional Activity*

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Genetic studies of the *Drosophila* erect wing (*ewg*) gene have revealed that *ewg* has an essential function in the embryonic nervous system and is required for the specification of certain muscle cells. We have found that EWG is a site-specific transcriptional activator, and we report here that evolutionarily conserved regions of EWG contribute both positively and negatively to transcriptional activity. Using gel mobility shift assays, we have shown that an EWG dimer binds specifically to DNA. In transfection assays, EWG activated expression of a reporter gene bearing specific binding sites. Analysis of deletion mutants and fusions of EWG to the Gal4 DNA binding domain has identified a transcriptional activation domain in the C terminus of EWG. Deletion analysis also revealed a novel inhibitory region in the N terminus of EWG. Strikingly, both the activation domain and the inhibitory region are conserved in EWG homologs including human nuclear respiratory factor 1 (NRF-1) and the sea urchin P3A2 protein. The strong conservation of elements that determine transcriptional activity suggests that the EWG, NRF-1, and P3A2 family of proteins shares common mechanisms of action and has maintained common functions across evolution.

The *erect wing* gene is essential for *Drosophila* embryogenesis, and analysis of mutants has revealed that *ewg* function is required for proper development of the embryonic nervous system as well as certain muscles (1). Viable alleles of *erect wing* give rise to flies with defects in indirect flight muscle development, leading to the erect wing phenotype. Immunostaining has shown that EWG is a nuclear protein expressed in neurons during the embryonic and larval stages (2). During pupal development, EWG is expressed in the myoblasts that give rise to the indirect flight muscles (3). The expression of EWG is regulated posttranscriptionally; the regulated efficiency of the splicing of the *ewg* transcript leads to an enrichment of the mRNA encoding the 116-kDa, 733-amino acid-long form of the EWG protein in the nervous system (4). The expression of a cDNA encoding the 116-kDa form of EWG in neurons rescues the lethality caused by loss-of-function mutations in *ewg*, and broad expression of this cDNA also restores muscle development, indicating that the 116-kDa form of the protein can provide all known *ewg* activities (2, 3).

EWG is a member of a family of related proteins that includes the human NRF-1 (also called α-Pal), chicken IBR/F, zebrafish Nrf, and sea urchin P3A2 proteins (2). Although these proteins share regions of significant homology, diverse activities have been reported for EWG homologs in different species. Similar to *ewg*, zebrafish *nrf* is an essential gene expressed in the developing nervous system (5). NRF-1, IBR/F, and P3A2 were identified as sequence-specific DNA-binding proteins, although the DNA binding domain in these proteins does not appear to correspond to a known structural motif (6–9). Human NRF-1 has been shown to activate the expression of several nuclear genes involved in mitochondrial function, including cytochrome c and mitochondrial transcription factor A, supporting the hypothesis that NRF-1 acts to coordinate nuclear and mitochondrial functions (8, 10–12). In sea urchins, P3A2 contributes to early pattern formation in the embryo by repressing expression of the CyIIIa cytoskeletal actin gene in oral ectoderm (6, 13, 14). In contrast to IBR/F, which has been found to repress transcription of the histone *h5* gene by binding to a site overlapping the start site of transcription (9, 15), P3A2 acts at a distance to repress transcription. The high degree of sequence similarity between EWG, NRF-1, and P3A2 raises the possibility that EWG may also function to regulate transcription either as an activator or a repressor.

In this study we show that EWG functions as a transcriptional activator and that evolutionarily conserved regions of EWG contribute both positively and negatively to transcriptional regulation. In gel mobility shift assays, an EWG dimer binds specifically to a consensus NRF-1 binding site. In *Drosophila* tissue culture cells, EWG activates transcription from a promoter bearing binding sites for EWG. Deletion analysis of EWG reveals a C-terminal activation domain and an N-terminal domain that inhibits transactivation. The core of the activation domain is highly conserved, and the mutation of evolutionarily conserved residues in the activation domain reduces activity. Our studies suggest that EWG participates in neurogenesis and indirect flight muscle development by directly binding to and regulating the expression of target genes important for differentiation or maintenance of these tissues. The finding that evolutionarily conserved regions of EWG contribute to transcriptional regulation suggests that EWG and its homologs in other species have maintained common mechanisms of action and regulation across evolution.

**MATERIALS AND METHODS**

Plasmids and Cloning—EWG deletion mutants were generated by polymerase chain reaction and cloned into pPac for expression from the *Drosophila* actin promoter or pTSTOP for use in *in vitro* transcription/translation reactions. EWG350 encodes amino acids 1–350, EWG564 encodes amino acids 1–564, EWG631 encodes amino acids 1–631, and EWG654 encodes amino acids 1–654. For analysis of N-terminal dele-
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FIG. 1. The Drosophila EWG protein is highly related to human NRF-1. A schematic of the EWG and NRF-1 proteins is shown with the extent of sequence similarity indicated. The NRF-1 nuclear localization sequence (NLS), DNA binding, and dimerization domains are all highly conserved in EWG (>70% homology). In addition, two small stretches in the C terminus, EWG residues 543–564 and 631–654, also share >70% homology with NRF-1. A region in the N terminus and one in the C terminus share >50% similarity.

RESULTS

EWG Is a Sequence-specific DNA-binding Protein—EWG is highly related to human NRF-1, chicken IBRF, and sea urchin P3A2 proteins in regions that correspond to the nuclear localization, DNA binding, and dimerization domains identified in these homologs (7, 9, 17, 18). In addition, an alignment of EWG with NRF-1 reveals patches of high homology in the C-terminal half of these proteins as well (Fig. 1). EWG residues 146–174 are 79% identical to the nuclear localization signal identified in NRF-1 (18). The predicted nuclear localization signal in EWG is likely to be functional because immunostaining has shown EWG to be nuclear in the neurons of Drosophila embryos (2). The high degree of sequence similarity between EWG residues 185–343 and the DNA binding and dimerization domain of NRF-1 predicts that EWG is also a sequence-specific DNA binding-protein. Using a gel mobility shift assay, we observed full-length EWG bound to a 24-base pair oligonucleotide containing a palindromic NRF-1 consensus site (Fig. 2A) (8, 9). As expected, in the absence of an unlabeled wild-type oligonucleotide efficiently competed the observed gel mobility shift. Much higher concentrations of an oligonucleotide with a single nucleotide substitution (5G) were needed to compete for binding, and a mutant oligonucleotide with substitutions in both halves of the palindromic site (5G8C) did not compete for binding by EWG. Consistent with these results, when labeled 5G and 5G8C oligonucleotides were used in gel mobility shift assays, EWG was found to bind very poorly to the 5G oligonucleotide and not at all to the 5G8C oligonucleotide (data not shown). Thus, EWG is a sequence-specific, DNA-binding protein with a sequence specificity similar to that of NRF-1.

We also used a gel mobility shift assay to investigate if EWG binds to this palindromic site as a dimer (19). A truncated form of EWG (residues 1–350) translated in vitro was found to bind to the NRF-1 consensus site, but similar to full-length EWG the truncated form bound very poorly to the 5G and not at all to the 5G8C mutant oligonucleotides (Fig. 2B and data not shown). When full-length EWG and EWG350 were cotranslated and used in the gel mobility shift assay, a single additional band of intermediate mobility was observed, indicating that EWG binds DNA as a dimer (Fig. 2B). When full-length EWG and EWG350 were translated individually and then mixed no intermediate band was observed, suggesting that EWG forms stable dimers in solution. Thus, EWG amino acids 1–350 are sufficient to bind specifically to DNA as a dimer, indicating
that these evolutionarily conserved sequences share a common function.

**EWG Is a Transcriptional Activator**—The observation that EWG is a sequence-specific, DNA-binding protein raised the possibility that EWG might regulate transcription through its cognate binding sites either as an activator like NRF-1 or as a repressor like P3A2. We therefore introduced three EWG binding sites upstream of a consensus TATA box driving luciferase expression to generate an EWG-responsive reporter gene, EWGBS3-luc. Cotransfection of a plasmid expressing full-length EWG stimulated luciferase expression from this reporter gene ~30-fold but did not stimulate a reporter gene lacking EWG binding sites (Fig. 3A). Consistent with our findings, EWG has recently been shown to activate expression of an NRF-1-responsive cytochrome c promoter in transfection assays (20). Unlike full-length EWG, the EWG350 deletion mutant did not stimulate expression from the EWGBS3-luc reporter (Fig. 3B). Immunoblot analysis indicated that EWG350 was expressed in the transfected cells at levels equal to or greater than full-length EWG (data not shown). Because EWG350 is able to bind DNA and form heterodimers with full-length EWG (Fig. 2B), we tested to see if this mutant could act as a dominant negative. Cotransfection of a plasmid expressing full-length EWG with increasing amounts of a plasmid expressing EWG350 resulted in a dose-dependent inhibition of luciferase expression (Fig. 3B). Thus, EWG is a transcriptional activator, and sequences in the C terminus are necessary for the activation function.

**The EWG Activation Domain Is Highly Conserved**—Having found that the C terminus of EWG was necessary for transcriptional activity, we tested to see whether the C terminus was in fact sufficient to function as an activation domain. EWG amino acids 351–733 were fused to the DNA binding domain of Gal4 (to generate fAD) and cotransfected into Drosophila tissue culture cells along with a reporter gene bearing five Gal4 binding sites upstream of a consensus TATA box driving luciferase expression (G5-luc). In this assay the C terminus of EWG was a very potent activation domain because the Gal4 + EWG fAD fusion stimulated transcription 1,200-fold more than Gal4 (1–147) alone (data not shown). We then generated and tested N- and C-terminal deletions to further define the regions of EWG that contribute to activation. The expression of all Gal4 + EWG fusions was confirmed by immunoblot analysis with anti-Gal4 antisera (data not shown). As shown in Fig. 4, the minimal EWG activation domain is contained in deletion M3, residues 631–654, that activates transcription to ~25–30% of the level of the entire C-terminal fragment. With the exception of the N2 deletion, which has only about 10% of the activity of the full-length Gal4 + EWG fusion, all of the active Gal4 + EWG fusions, N1, C3, M1, and M3 retain residues 564–654. The EWG minimal activation domain contains a highly conserved core, residues 631–654. However, both the highly conserved region and the adjacent region contribute to activation because deletion of this region significantly reduces the activation function (compare N1 versus N2, M1 versus M2, and M3 versus M4).

To confirm the importance of conserved residues in the activation domain, double alanine substitution mutants were analyzed in the context of the minimal activation domain, M3, amino acids 564–654. Alanine substitution of either Ser-642 and Tyr-644 or Val-639 and Val-641 in the highly conserved region significantly reduced activation (Fig. 5). Substitution of bulky hydrophobic residues in the adjacent region with alanine (Ile-592 and Val-595) also reduced activity, whereas the substitution of two hydrophilic residues in this region (Thr-591 and Thr-594) had no effect. Thus, particular conserved residues throughout the minimal activation domain contribute to activity.

**Deletions of EWG Confirm the Importance of the Conserved Activation Domain and Reveal an N-terminal Inhibitory Domain**—To confirm that the activation domain identified in Gal4 fusions contributes to activation by EWG, we tested additional deletion mutants in EWG for their ability to activate expression from the EWGBS3-luc reporter. The expression of all EWG deletions was confirmed by immunoblot analysis (data not shown). As shown in Fig. 6A, EWG654, which lacks the sequences C-terminal to residue 654, was able to activate expression of the reporter gene, although less well than the full-
length EWG. In contrast, EWG631, which lacks the evolutionarily conserved core of the activation domain identified in the Gal4 fusions, was essentially inactive. The deletion end point in EWG631 corresponds to the Gal4\(\text{N2}\) fusion indicating that, although this C-terminal deletion retains about 10% activity as a Gal4 fusion, it does not have significant activity in the context of EWG. A deletion mutant lacking the minimal activation domain identified in the Gal4 fusions, EWG564, was as inactive as EWG350, which lacks the entire C terminus. Thus, these data confirm that sequences in

Fig. 3. The C terminus of EWG is necessary for transcriptional activation. A, EWG activates transcription from a reporter gene with EWG binding sites. A plasmid expressing EWG or empty vector was cotransfected into Drosophila S2 cells along with a luciferase reporter gene bearing three EWG binding sites upstream of a consensus TATA box (EWGBS3-luc) or the control lacking EWG sites (E1B-luc). The luciferase activity obtained with empty expression vector and EWGBS3-luc was set at 1. The experiment was performed in triplicate; error bars indicate the standard deviation. B, EWG350 functions as a dominant negative. Plasmids expressing full-length EWG or the C-terminal deletion EWG350 were cotransfected with the EWGBS3-luc reporter as indicated. When increasing amounts of the EWG350 expressing plasmid (50, 100, and 200 ng) were cotransfected with 100 ng of a plasmid expressing full-length EWG, the resulting luciferase activity was reduced in a dose-dependent manner. The expression of full-length EWG and EWG350 in transfected cells was confirmed by immunoblot (data not shown). The luciferase activity obtained with empty expression vector and EWGBS3-luc was set at 1. The experiment was performed in triplicate; error bars indicate the standard deviation.

Fig. 4. A highly conserved region comprises the core of the EWG activation domain. A, fusions of C-terminal fragments of EWG to the DNA binding domain of Gal4 (residues 1–147) were cotransfected into Drosophila S2 cells with a reporter bearing five Gal4 sites upstream of a luciferase reporter gene (G5-luc). The regions of EWG contained in the Gal4 fusions are indicated schematically as described in Fig. 1. The activity of the Gal4 fAD fusion containing EWG residues 351–733, which activated expression 1,200-fold more than Gal4(1–147) alone (not shown), was set at 100%. The relative activity of N- and C-terminal deletions of the EWG activation domain is indicated. The experiment was performed in triplicate; error bars indicate the standard deviation. B, EWG amino acids 564–654 constitute the minimal activation domain. The activity of the indicated Gal4 + EWG fusions was determined as described in A.

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the C terminus of EWG are required for activation and support a critical role for the conserved core of the activation domain, residues 631–654.

We have found that evolutionarily conserved sequences in the N terminus of EWG down-regulate transcriptional activation. Although deletion of nonconserved residues prior to the first methionine at position 87 (EWG initiates with CTG) (2) did not reduce the activity of EWG, deletion of the first 144 residues was unexpectedly found to increase activation 35-fold (Fig. 6A). This was surprising because previous studies of NRF-1 have shown that deletion of the residues from the N terminus to the nuclear localization signal reduced DNA bind-
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We report here that evolutionarily conserved regions of the Drosophila erect wing gene product, EWG, function to regulate transcription. Using in vitro DNA binding assays and transfection assays in Drosophila tissue culture cells, we have shown that EWG is a sequence-specific, DNA-binding protein that activates transcription from its cognate binding sites. Our analysis of EWG deletion derivatives has revealed functional roles in transcriptional regulation for each of the regions of EWG that are conserved in the human NRF-1 and sea urchin P3A2 homologs with the exception of residues 543–564. In contrast, our studies failed to reveal a critical role for the nonconserved regions of EWG. Because of this strong correlation of sequence conservation with function, it seems likely that conserved residues 543–564 contribute to EWG activity in certain physiological contexts not reproduced in our assays. Consistent with the proposal that the highly conserved region of EWG from residues 146–343 participates in nuclear localization, DNA binding, and dimerization, we have found that deletion of the sequences C-terminal to amino acid 350 does not impair DNA binding or dimerization, and deletion of the sequences N-terminal to position 144 does not reduce, but rather stimulates, site-specific activation. We have mapped the minimal activation domain to residues 564–654, which includes a highly conserved core, residues 631–654, necessary for activation. Unexpectedly, a conserved region in the N terminus was found to inhibit activation by EWG. The identification of evolutionarily conserved regions that both positively and negatively influence transcriptional activation by EWG raises the possibility that the activity of EWG and its homologs may be regulated by common mechanisms dependent upon cell type or promoter context.

A wide variety of unrelated amino acid sequences has been shown to function as activation domains (22); thus it is particularly striking that the EWG activation domain is so highly conserved. An analysis of NRF-1 deletions has revealed a major role for NRF-1 residues 449–477 in activation that corresponds well to the highly conserved core that we have shown to be critical for the function of the EWG activation domain (18). In the case of NRF-1, the mutation of hydrophobic residues in the activation domain also significantly reduced activation, although mutation of multiple glutamines in the activation domain had no effect (18). NRF-1 has been shown to bind the coactivator PGC-1 through its DNA binding domain, but no targets of the NRF-1 activation domain have been identified (23). The high sequence conservation of the activation domain across evolution strongly suggests that EWG and NRF-1 activate transcription by a common mechanism. As shown in Fig. 5, the core activation domain is well conserved in the sea urchin P3A2 homolog, which has been shown to repress transcription (13, 14). It is interesting to note that in P3A2 there is alanine at the position equivalent to Val-641 in EWG because we have found that a double mutant in which alanine replaced both Val-639 and Val-641 was severely impaired for activation. Thus, it is possible that P3A2 does not activate transcription because of changes in the region homologous to the activation domain or, alternatively, that this function may be conserved and P3A2 may function both as an activator and as a repressor.

Analysis of EWG deletion derivatives revealed the presence of a novel conserved inhibitory domain in the amino terminus. Deletion of residues 87–144 resulted in a 55-fold increase in activation (Fig. 6B). Modulation of transcriptional activation by EWG may be critical because overexpression of EWG in Drosophila, particularly outside the nervous system, is lethal (4). Previous studies with NRF-1 found that deletion of the N terminus (Δ77) resulted in decreased DNA binding, which in the case of NRF-1 was shown to be due to a defect in dimerization (21). It was therefore surprising that deletion of the N terminus of EWG resulted in increased activation, particularly because deletion of EWG residues 1–144 was also found to reduce DNA binding activity in vitro (2). Our results suggest that the N terminus of NRF-1 may also function to inhibit activation. Interestingly, phosphorylation of the N terminus of NRF-1 in response to extracellular signals has been shown to increase transcriptional activation by NRF-1 because of an increase in DNA binding (although not dimerization) (12, 21). The N terminus of NRF-1 has also been reported to interact with dynein light chains, although the functional significance of this is unknown (20). The conserved inhibitory region is rich in acidic residues, but the sequence provides no clues as to whether inhibition involves interactions in cis with other regions of EWG or in trans with corepressor proteins. If the latter mechanism applies, it is possible that this amino-terminal domain contributes to transcriptional repression by P3A2. It will be interesting to determine whether the inhibitory activity of the EWG N terminus is modulated in response to extracellular signaling pathways or through interactions with other transcription factors at specific promoters.

Expression of the 116-kDa form of EWG is enriched in the Drosophila nervous system because of differential efficiency of splicing (4). Several other splice variants of ewg mRNA have been described with the potential of encoding additional EWG isoforms. Our identification of functional domains in EWG supports predictions of the functions of other forms of EWG protein that may be expressed. All of the observed splice variants encode the N terminus, DNA binding, and dimerization domains. Alternative splicing of exon D (amino acids 386–540), which is not conserved between EWG and NRF-1, has been observed in both neuron-rich heads and neuron-poor bodies. In our analysis of Gal4 + EWG fusions, residues encoded by exon D were not required for activity; therefore our data suggest that EWG forms both containing and lacking exon D should function as transcriptional activators. Interestingly, the highly conserved region that is critical for function of the EWG activation domain is present in a single small exon, exon H, which encodes amino acids 627–688. Thus, we predict that EWG proteins derived from the observed splice variants lacking exon H would fail to activate transcription. Although transgenic

2 I. K. Fazio and G. Gill, unpublished data.
studies have shown that the 116-kDa form of EWG rescues development of the indirect flight muscle, the form of EWG expressed in muscle has not been identified. It is of obvious interest to determine whether EWG activity in the nervous system and in myoblasts requires the same functional domains.

We have identified evolutionarily conserved regions of EWG that contribute to DNA binding and dimerization, transcriptional activation, and regulation of the activation function. Our studies suggest that the essential function of EWG in the developing Drosophila nervous system is to regulate expression of specific target genes. Two ewg mutants have been molecularly characterized; both introduce early stop codons before the DNA binding/dimerization domain (4). Characterization of additional ewg mutant alleles should help confirm that the functional domains we have identified contribute to EWG function in vivo. NRF-1 has been shown to regulate expression of nuclear encoded genes important for mitochondrial function, and NRF-1 binding sites have been implicated in the regulation of many genes involved in growth-responsive metabolic pathways as well as neuron-specific genes (8, 10, 11, 24, 25). Identification of target genes regulated by EWG will provide new insights into the role of this transcription factor in development of the nervous system and specification of the indirect flight muscles.

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