Huckebein repressor activity in *Drosophila* terminal patterning is mediated by Groucho

Robert E. Goldstein¹, Gerardo Jiménez², Orna Cook¹, Devorah Gur¹ and Ze'ev Paroush¹,*

¹Department of Biochemistry, Hadassah Medical School, The Hebrew University, PO Box 12272, Jerusalem 91120, Israel
²Departament de Biologia Molecular i Cellular, CID-CSIC, Jordi Girona 18-26, 08034-Barcelona, Spain

*Author for correspondence (e-mail: zparoush@cc.huji.ac.il)

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**SUMMARY**

The Groucho corepressor mediates negative transcriptional regulation in association with various DNA-binding proteins in diverse developmental contexts. We have previously implicated Groucho in *Drosophila* embryonic terminal patterning, showing that it is required to confine *tailless* and *huckebein* terminal gap gene expression to the pole regions of the embryo. Here we reveal an additional requirement for Groucho in this developmental process by establishing that Groucho mediates repressor activity of the Huckebein protein. Putative Huckebein target genes are derepressed in embryos lacking maternal *groucho* activity and biochemical experiments demonstrate that Huckebein physically interacts with Groucho. Using an in vivo repression assay, we identify a functional repressor domain in Huckebein that contains an FRPW tetrapeptide, similar to the WRPW Groucho-recruitment domain found in Hairy-related repressor proteins. Mutations in Huckebein’s FRPW motif abolish Groucho binding and in vivo repression activity, indicating that binding of Groucho through the FRPW motif is required for the repressor function of Huckebein. Taken together with our earlier results, these findings show that Groucho-repression regulates sequential aspects of terminal patterning in *Drosophila*.

Key words: *Drosophila*, Transcriptional repression, *groucho*, Pattern formation, Terminal patterning, *huckebein*, *snail*, *brachyenteron*

**INTRODUCTION**

The developing *Drosophila* embryo consists of a segmented trunk flanked by presumptive head and tail regions. The maternal anterior and posterior coordinate systems regulate segmentation of the central portion of the embryo, whereas the termini are patterned in response to a signal relayed by the maternal terminal system (reviewed in St. Johnston and Nüsslein-Volhard, 1992). In all cases, maternally deposited gene products govern early zygotic gap gene expression. A key component in terminal body patterning is the gene *torso* (*tor*), which encodes a putative transmembrane receptor tyrosine kinase (RTK; Sprenger et al., 1989). Although the Torso (Tor) receptor is present uniformly along the plasma membrane of the early embryo, it becomes activated exclusively at the poles by its localised ligand (Casanova and Struhl, 1989, 1993; Sprenger and Nüsslein-Volhard, 1992). This activation triggers a signal transduction cascade resulting in restricted, partially overlapping expression of the terminal gap genes *tailless* (*tll*) and *huckebein* (*hkb*) at the embryonic termini (Pignoni et al., 1990; Brönner and Jäckle, 1991; reviewed in Lu et al., 1993). These two genes encode transcription factors, which regulate expression of downstream target genes that implement head and tail differentiation programs (Strecker et al., 1986, 1988; Casanova, 1990; Pignoni et al., 1990; Weigel et al., 1990; Brönner and Jäckle, 1991; Brönner et al., 1994). *tll* and *hkb* appear to be the only posterior targets of the Tor signal, as *hkb* and *tll* double mutant embryos display a posterior phenotype similar to that of embryos laid by females mutant in *tor* (Weigel et al., 1990).

Recent studies have shown that Tor signalling allows terminal gap gene expression indirectly, by locally counteracting a general repressor activity mediated by Groucho (Gro) (Liaw et al., 1995; Paroush et al., 1997). Thus, embryos deprived of maternal *groucho* (*gro*) function (thereafter referred to as *gro*mut*¹* embryos; see Materials and Methods) display ectopic *tll* and *hkb* transcription in their trunk region (Paroush et al., 1997), indicating that terminal gap gene expression is negatively regulated by a Gro-dependent repressor. The *tor* RTK-signalling pathway is required to relieve this Gro-mediated repression at the poles. Indeed, activation of *tll* and *hkb* is independent of *tor* signalling in *gro*mut*¹* embryos (Paroush et al., 1997).

Gro is a ubiquitously expressed nuclear protein that does not bind DNA, and which contains multiple WD-repeats that are believed to mediate protein-protein interactions (Hartley et al., 1988; Delidakis et al., 1991; Neer et al., 1994; reviewed in Parkhurst, 1998; Fisher and Caudy, 1998). It was initially shown to function as a corepressor for a subfamily of Hairy-related basic-helix-loop-helix (bHLH) repressor proteins...
encoded by hairy (h), the Enhancer-of-split (E(spl)) Complex and deadpan (Paroush et al., 1994 and references therein). All Hairy-related bHLH repressors terminate in a distinct WRPW tetrapeptide motif, which is necessary and sufficient for physical binding to Gro (Paroush et al., 1994; Fisher et al., 1996). Gro also binds to the Runt protein through a related C-terminal WRPY motif and mediates some of its functions (Aronson et al., 1997; Levanon et al., 1998).

More recently, Gro has been shown to act in conjunction with other repressors that are structurally unrelated to Hairy. In dorsoventral patterning of the embryo, Gro is necessary for the conversion of the Dorsal activator protein to a repressor (Dubnicoff et al., 1997). Also, repression by the Engrailed (En) homeodomain protein is Gro-dependent (Jiménez et al., 1997; Tolkunova et al., 1998). Finally, Gro associates with the dTCF/Pangolin (Pan)/LEF-1 transcription factor during Wingless signalling (Cavallo et al., 1998; Roose et al., 1998).

Thus, repression in diverse developmental settings depends on interactions between Gro and different classes of transcriptional repressors.

In this paper, we identify a novel function for Gro in terminal patterning. We show that, in addition to its role in regulating hkb and ill gene expression, Gro mediates the repressor activity of Hkb. We demonstrate that the two proteins physically interact with each other in vitro and that, in the absence of maternal Gro, putative Hkb target genes are derepressed. Mutations in an internal FRPW motif found in Hkb abolish its interactions with Gro in vitro and compromise its repressor activity in vivo. Our results indicate that Hkb is a Gro-dependent repressor and that gro fulfills multiple, sequential roles in Drosophila terminal patterning.

MATERIALS AND METHODS

Fly culture

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract-agar medium at 24°C.

Fly stocks and germ-line clones

Mutant alleles used were hkb1 and hkbXAMS (a strong hypomorph and a complete null, respectively; Weigel et al., 1990; Bröomer et al., 1994). hkb homozygous mutant embryos were distinguished from their blue-balanced siblings by lack of lacZ staining. Strong gain-of-function tord embryos were generated from tor\textsuperscript{w/hor\textsuperscript{D}} females (Klingler et al., 1988). gro\textsuperscript{E75} and gro\textsuperscript{E48} are strong gro alleles, and Df[3R]E(spl)Bx32 is a chromosomal deletion encompassing m5, m7, m8 and gro (Press et al., 1988; Delidakis et al., 1991).

gro\textsuperscript{mat+/-} embryos, lacking maternal gro activity, were derived from female females with either gro\textsuperscript{E75}, gro\textsuperscript{E48} or gro\textsuperscript{B32} mutant germlines, obtained using the FLP-DPS technique (Chou et al., 1993). Briefly, males carrying the FRT[82B]ovo\textsuperscript{D} chromosome and an X-linked hs-FLP1 chromosome were crossed to females carrying a FRT[82B]gro chromosome. Prenyge of this cross were heat-shocked (37°C/1.5 hours) on each of days 3, 4 and 5 following egg laying, and allowed to develop at 24°C. Non-heat-shocked control females were sterile (100% penetrance) and all eggs laid by fertile females displayed a severe neurogenic phenotype, as expected for gro\textsuperscript{mat+/-} embryos (Schroms et al., 1992; Paroush et al., 1994). All gro genotypes generated eggs with similar phenotypes.

Germline transformations

P-element-mediated transformations were performed as described (Rubin and Spradling, 1982). In general, two or more independent lines were analysed for each construct. Transgenes inserted on the X chromosome were maintained in males using an attached-X chromosome (C11M3); insertions on the autosomes were kept as unbalanced stocks, selecting each generation for transformant males and non-transformant females. To analyse the effect of hh-HairyHkb in gro embryos, mosaic gro\textsuperscript{E75} or gro\textsuperscript{E48} females were crossed to males carrying the hh-HairyHkb construct on the X chromosome, such that all female progeny were both gro\textsuperscript{mat+/-} and carried the transgene.

In situ hybridisation and antibody staining of Drosophila embryos

1-3.5 hour collections of wild-type or mutant Drosophila embryos were dechorionated in bleach and fixed in 4% formaldehyde/PBS/heptane for 15-20 minutes. Expression patterns were visualised by whole-mount in situ hybridisation using digoxigenin-UTP-labelled antisense RNA probes and antidigoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim; Tautz and Pfeifle, 1989). For SiX immunohistochemistry, embryos were incubated with a monoclonal antibody specific to the active form of SiX (Bopp et al., 1991), as previously described (Jiménez et al., 1997). Embryos were mounted in methacrylate (JB-4, Polyscience) and examined under Nomarski optics.

Plasmids

Molecular manipulations were conducted according to standard protocols (Ausubel et al., 1987-1997; Sambrook et al., 1989). Constructs containing Sna, and full-length and truncated versions of Hkb were prepared by inserting fragments generated by standard PCR amplification into pBluescript (Stratagene) and, after full sequencing of inserts, into appropriate vectors and sites. pGEX-Gro, pGEX-Hairy and pGEX-Hairy\textsuperscript{Anot}, pET-Gro, LexA-Gro, LexA-E(spl)-m7 and LexA-Dmcdc2 have been previously described (Paroush et al., 1994; Dubnicoff et al., 1997), hh-Hairy\textsubscript{Hkb} was constructed as described in Jiménez et al. (1997). The FEAW mutations were introduced by PCR (protocol adapted from Ho et al., 1989). Additional details on the construction of the plasmids are available on request.

Yeast two-hybrid interaction assays

Yeast two-hybrid interaction assays were performed as described (Gyuris et al., 1993; Paroush et al., 1994). Briefly, yeast strain EGY048 (MATa trpl ura3 his3 leu2:p3LexAop-LEU2), containing an integrated LEU2 reporter gene and upstream LexA operators, was transformed with pSH18-34 (2μ ura3 plasmid LexAop-lacZ). Next, this strain was transformed with the LexA(202+pl) plasmid, driving expression of LexA-fusion baits, and with the pG4-5 (2μ TRP1) vector that allows galactose-inducible expression of proteins fused to an activation domain. Reporter gene activation was analysed on ura3 trpl leu2-growing plates, including galactose and raffinose, to select for activation of the LexAop-LEU2 gene and on X-Gal indicator plates for lacZ expression. [35S]-labelled Gro or Hkb proteins, synthesised using the TNT-coupled rabbit reticulocyte lysate system (Promega), were added to each preincubation mix and the binding reactions were carried out overnight at 4°C. Beads were washed four
Deregulation of putative Huckebein target genes in embryos lacking maternal groucho function

We have previously pointed to similarities in gene expression patterns found in gro<sup>mat−</sup> embryos and in tor<sup>D</sup> gain-of-function mutant embryos (Paroush et al., 1997). In both cases, expression of the <i>tll</i> and <i>hkb</i> genes expands towards the centre of the embryo (though <i>hkb</i> broadens to a lesser extent than <i>tll</i> in gro<sup>mat−</sup> embryos; Paroush et al., 1997). Expanded <i>tll</i> consequently leads to repression of the central gap genes <i>Krüppel</i> (<i>Kr</i>) and <i>knirps</i> (<i>kni</i>) (Klingler et al., 1988; Pankratz et al., 1989; Brönner and Jäckle, 1991; Steingrímsson et al., 1991; Paroush et al., 1997). However, a more detailed comparison shows that differences between the two genotypes do exist; in particular, we note specific deregulation of putative Hkb repression targets only in gro<sup>mat−</sup> embryos.

One proposed Hkb target is the <i>snail</i> (<i>sna</i>) gene, which is transcribed in the ventral-most portion of the embryo. <i>sna</i> expression is thought to be excluded from the posterior pole by <i>hkb</i> activity. Accordingly, <i>sna</i> and <i>hkb</i> expression domains abut in cellularising wild-type embryos, whereas <i>sna</i> expression extends to, and includes, the posterior pole of <i>hkb</i> mutant embryos (Reuter and Leptin, 1994; Fig. 1A,D,G). In tor<sup>D</sup> embryos, <i>hkb</i> expression expands towards the centre of the embryo and the <i>sna</i> domain correspondingly retracts (Brönner and Jäckle, 1991; Reuter and Leptin, 1994; Fig. 1B,E). By contrast, in gro<sup>mat−</sup> embryos, the expression of <i>sna</i> does not respect its posterior border and spreads to the pole, overlapping extensively with the <i>hkb</i> expression domain (Fig. 1C,F).

The expression of the T-related gene <i>brachyenteron</i> (<i>byn</i>; also called <i>Trg</i>) also seems to be repressed by Hkb. <i>byn</i> is not expressed at the most posterior region of wild-type, or tor<sup>D</sup>, embryos, whereas it extends throughout the posterior cap of <i>hkb</i> mutant embryos, consistent with <i>hkb</i> setting the posterior limit of <i>byn</i> expression (Kispert et al., 1994; Singer et al., 1996; Fig. 1H,I,K). However, we find that <i>byn</i> is ectopically expressed at the posterior tip of gro<sup>mat−</sup> embryos (Fig. 1J). Together, these results suggest that gro is, directly or indirectly, necessary for <i>hkb</i> repressor functions.

Physical associations between Huckebein and Groucho in yeast and in vitro

The expression patterns of presumed Hkb target genes described above suggest that Hkb is a Gro-dependent repressor. We therefore tested whether Hkb can bind Gro. Full-length Hkb interacts specifically with Gro in the yeast two-hybrid system (Fields and Song, 1989; Gyuris et al., 1993), as detected by the activation of two reporter genes, lacZ (Table 1) and LEU2 (not shown). Specificity is confirmed by the binding of Gro to the E(spl)-m7 protein (Paroush et al., 1994), but not to negative controls such as Dmcdc2 and Snail. Likewise, Hkb does not complex with the Dmcdc2 control.

We also demonstrated Hkb:Gro associations in an in vitro pull-down assay (Fig. 2). Hkb and control proteins were expressed in bacteria as glutathione S-transferase (GST) fusions, immobilised on glutathione-agarose beads and assayed for their ability to retain in vitro translated Gro labelled with [35S]methionine (see Materials and Methods). Gro binds to GST-Hkb and GST-Hairy (Paroush et al., 1994; Jiménez et al., 1997), but not to control GST-HairyΔNot1 (a truncated form of...

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**Fig. 1.** <i>sna</i> and <i>byn</i> expression is deregulated in embryos lacking maternal <i>groucho</i> activity. Wild-type (A,D,H) or mutant tor<sup>D</sup> (B,E,I), gro<sup>mat−</sup> (C,F,J) and <i>hkb</i> (G,K) embryos were stained for <i>hkb</i> (A−C), <i>sna</i> (D−G) and <i>byn</i> (H−K). In wild-type and tor<sup>D</sup> embryos, posterior borders of <i>sna</i> and <i>byn</i> expression domains respect that of <i>hkb</i> (A,B,D,E,H,I). However, <i>sna</i> and <i>byn</i> transcription expands to include the posterior pole of gro<sup>mat−</sup> embryos (F,J), in a manner resembling their expression patterns in <i>hkb</i> mutants (G,K), despite the fact that gro<sup>mat−</sup>embryos express <i>hkb</i> (C). Ectopic <i>byn</i> transcription in the anterior of tor<sup>D</sup> and gro<sup>mat−</sup> embryos (I,J) probably reflects expanded <i>tll</i> expression in these genotypes (Steingrimsson et al., 1991; Singer et al., 1996; Paroush et al., 1997). All embryos in this figure, and in Fig. 4, are oriented with anterior to the left and dorsal up.
Fig. 2. Huckebein binds Groucho in vitro. (A) $^{35}$S-labelled Gro was incubated with GST-derivatives immobilised on glutathione-agarose beads and, after washing, retained Gro protein was examined by SDS-PAGE (not shown) and autoradiography. Gro binds to full-length Hkb (lane 3) and to Hkb$_{1-196}$ (lane 5), to the same extent that it binds Hairy (lane 8), but not to control Snail (lane 7), Hairy$\Delta$NorI (lacking its C-terminal Gro-binding domain; lane 9) or GST alone (lane 2). Mutating the FRPW motif (to FEAW) in Hkb (mHkb; lane 4) and Hkb$_{1-196}$ (mHkb$_{1-196}$; lane 6) dramatically attenuates the interactions with Gro. 10% of labelled Gro input was run in lane 1. Arrow indicates position of full-length Gro. (B) GST-Gro (lane 3), but not GST (lane 2), binds $^{35}$S-labelled Hkb. 10% of input Hkb was run in lane 1. Arrow indicates the position of full-length Hkb.

Hairy, lacking its 91 C-terminal amino acids, including the WRPW motif (Paroush et al., 1994), GST-Sna or GST alone (Fig. 2A). Reciprocal experiments show that GST-Gro, but not GST, binds $^{35}$S-methionine-labelled Hkb (Fig. 2B). Thus, Hkb and Gro can complex with each other both in yeast and in vitro.

**An internal FRPW motif in Huckebein is essential for its interactions with Groucho**

Hkb does not bear any apparent structural similarities to other Gro-interacting proteins that have been identified to date. It does not terminate with a WRPW/Y motif, shown to mediate both Hairy:Gro and Runt:Gro interactions (Wainwright and Ish-Horowicz, 1992; Paroush et al., 1994; Fisher et al., 1996; Aronson et al., 1997; Levanon et al., 1998), nor does it contain sequences resembling the Dorsal or En Gro-binding domains (Dubnicoff et al., 1997; Jiménez et al., 1997, 1999). To map Hkb protein motifs required for its association with Gro, we constructed a series of Hkb derivatives and tested their ability to interact with Gro in the yeast two-hybrid system. Deletion of Hkb’s carboxy-terminal 101 amino acids, which include its putative DNA-binding zinc-finger domain, or even the removal of a further 71 amino acids, does not abolish the binding to Gro (Fig. 3). In fact, a construct containing just the 52 N-terminal residues of Hkb interacts with Gro (Fig. 3). In contrast, truncation of Hkb’s N-terminal 23 amino acids completely abrogates its ability to complex with Gro (Fig. 3), implying that this portion of Hkb is indispensable for the association between the two proteins.

A closer examination revealed that Hkb’s N-terminal region contains, at amino acids 16-19, an FRPW motif reminiscent of the WRPW Gro-binding domain found at the C terminus of Hairy-related repressors. We therefore tested whether this motif in Hkb is of similar importance for the interaction with Gro. We find that mutating two amino acids in this motif (to FEAW) abolishes the binding of Hkb to Gro in the yeast two-hybrid system (Fig. 3). The requirement for the FRPW motif was also demonstrated in vitro: as shown in Fig. 2A, both full-length Hkb and Hkb$_{1-196}$ complex with Gro (lanes 3, 5), while neither binds Gro when carrying the FEAW mutation (lanes 4, 6). Thus, the FRPW motif behaves similarly to the WRPW tetrapeptide (Paroush et al., 1994), suggesting that it mediates the physical associations between Hkb and Gro.

**Huckebein acts as a transcriptional repressor in vivo**

$hkb$ encodes a postulated DNA-binding transcriptional regulator, yet it is unclear from its primary sequence whether it acts as an activator or as a repressor of gene expression (Brönner et al., 1994). On the one hand, Hkb contains a glutamine-rich region characteristic of some activation domains and is positively required for the transcription of several genes (Weigel et al., 1990; Rehorn et al., 1996). On the other hand, the similar derepression of Hkb target genes in $hkb$ and in $gro$ mutant embryos (Fig. 1), together with an ability to complex with Gro (Table 1; Figs 2, 3), argues that Hkb functions as a repressor. Consistent with this idea, LexA-Hkb does not activate transcription of a reporter gene when introduced into yeast cells (Table 1), suggesting that it lacks an intrinsic activation domain. To further test the role of Hkb as a transcriptional repressor, we investigated whether Hkb contains a functional repression domain, separable from its DNA-binding domain, that can inhibit gene expression.

To this end, we employed an in vivo assay that relies on the

### Table 1. Specific interactions between Huckebein and Groucho in the yeast two-hybrid assay

<table>
<thead>
<tr>
<th>LexA Fusions</th>
<th>pJG-Gro</th>
<th>pJG4-5</th>
<th>pJG-Hkb</th>
<th>pJG-Sna</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA-Hkb</td>
<td>2.7±0.8</td>
<td>3.4±0.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>LexA-Sna</td>
<td>7.1±2.0</td>
<td>7.7±2.1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>LexA-E(spl)-m7</td>
<td>25±11</td>
<td>25±11</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>LexA-Gro</td>
<td>nd</td>
<td>298±37</td>
<td>3.0±2.6</td>
<td>nd</td>
</tr>
<tr>
<td>LexA-Dmdc2</td>
<td>3.3±0.9</td>
<td>5.6±1.2</td>
<td>4.4±1.5</td>
<td>2.9±1.2</td>
</tr>
</tbody>
</table>

Full-length Hkb interacts specifically with Gro in yeast, regardless of which of the two proteins is expressed as a DNA-binding domain fusion and which as an activation-domain fusion. E(spl)-m7 is a positive control, whereas Snail and Dmdc2 serve as negative controls. pJG4-5 is the backbone vector. $\beta$-galactosidase activity is indicative of protein-protein interactions (see Materials and Methods). Analogous results were achieved by analysing many colonies on X-Gal indicator plates and for growth on leu$^{-}$ plates. nd, not determined.
Groucho mediates Huckebein repression

The interaction domain required for Huckebein’s association with Groucho maps to its N terminus. Fusion proteins, containing partial Hkb derivatives as shown, were used to delineate the Hkb Gro-recruitment domain in the yeast two-hybrid system. Results indicate that it resides in the N terminus of Hkb and encompasses the FRPW motif: First, Hkb’s 52 N-terminal aminoacids (Hkb1-52) are sufficient for mediating these interactions. Second, truncating the 23 N-terminal residues of Hkb (Hkb23-196) completely abrogates its ability to associate with Gro. Third, mutating the FRPW motif at aminoacids 16-19 (mHkb1-196) abolishes interactions with Gro. β-galactosidase liquid assay results are listed, and should be taken qualitatively rather than quantitatively. Hkb1-52 reproducibly interacts with Gro and activates both reporter genes (see Materials and Methods), whereas Hkb23-196 does not. When the Hkb derivatives are used as baits, Hkb1-52 interacts with Gro equally well as Hkb1-125, suggesting that the differences in their ability to interact with LexA-Gro should be attributed to variations inherent to the yeast two-hybrid system, rather than to a secondary interaction domain in other regions of Hkb. nd, not determined.

Groucho mediates Huckebein’s ability to repress gene expression

The results presented in Fig. 1 provide genetic evidence that Hkb requires Gro for the repression of downstream target genes such as sna and byn. To demonstrate this dependence more directly, we assessed whether repression by HairyHkb requires Gro activity. Males harbouring the hb-HairyHkb transgene were crossed to mosaic gro females (which only produce gro mutant embryos; see Materials and Methods), and progeny were stained for Sxl. As shown in Fig. 4B, HairyHkb does not repress Sxl in embryos lacking maternally supplied gro, suggesting that the Hkb repressor domain requires Gro for its function in vivo.

We also reasoned that disruption of the FRPW domain, required for the interactions with Gro in yeast and in vitro (Figs 2, 3), should result in a decline in Hkb repressor activity in the embryo. Therefore, a modified version of the hb-HairyHkb transgene, carrying the FRPW→FEAW mutations, was generated and introduced into wild-type embryos. As shown in Fig. 4C, Hkb contains a potent repression domain that depends on Gro in vivo. Effects of expressing the hb-HairyHkb transgene on Sxl expression in otherwise wild-type (A) or in gromut- embryos (B). Embryos were stained using a monoclonal antibody specific for Sxl. As shown in Fig. 4B, HairyHkb does not repress Sxl in embryos lacking maternally supplied gro, suggesting that the Hkb repressor domain requires Gro for its function in vivo.

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Fig. 4. Hkb contains a potent repression domain that depends on Gro in vivo. Effects of expressing the hb-HairyHkb transgene on Sxl expression in otherwise wild-type (A) or in gromut- embryos (B). Embryos were stained using a monoclonal antibody specific for Sxl. As shown in Fig. 4B, HairyHkb does not repress Sxl in embryos lacking maternally supplied gro, suggesting that the Hkb repressor domain requires Gro for its function in vivo.

Fig. 4. Hkb contains a potent repression domain that depends on Gro in vivo. Effects of expressing the hb-HairyHkb transgene on Sxl expression in otherwise wild-type (A) or in gromut- embryos (B). Embryos were stained using a monoclonal antibody specific for Sxl. As shown in Fig. 4B, HairyHkb does not repress Sxl in embryos lacking maternally supplied gro, suggesting that the Hkb repressor domain requires Gro for its function in vivo.
Fig. 5. Model for the role Groucho-mediated repression plays in establishing a transcriptional boundary between the central, segmental portion of the embryo and the termini. Initially, in stage 4 embryos (A), Gro and an as yet unknown partner protein ‘X’ repress terminal gap gene expression in the central region of the embryo. Local signalling by the Tor RTK pathway prevents Gro-complexes from repressing tll and hkb-expression at the poles. Later, in stage 5 embryos (B), Gro mediates repression by Hkb of downstream target genes like sna and byn, thus excluding genes normally transcribed in the trunk region and in the primordium of the hindgut from expanding to the posterior tip. In gro mat− embryos genes are found ectopically misexpressed across the boundary. For details, see text.

Fig. 4C, there is only very weak repression of Sxl in these embryos and the transgene does not cause female-specific lethality (not shown). These results provide further evidence that associations between the FRPW motif and Gro are essential for repression by Hkb in the Drosophila embryo.

DISCUSSION

Groucho potentiates Huckebein repression

Genetic studies have previously suggested that Hkb acts as a negative regulator during terminal patterning (Casanova, 1990; Weigel et al., 1990; Brömmer and Jäckle, 1991; Kispert et al., 1994; Reuter and Leptin, 1994; Margolis et al., 1995). In this paper, we present several genetic and biochemical lines of evidence indicating that Hkb is indeed a transcriptional repressor and that Gro mediates its activity. First, presumed Hkb target genes are derepressed in embryos lacking maternal Gro function. Second, Hkb and Gro physically interact with each other, both in yeast and in vitro. Finally, we have demonstrated that Hkb contains a repressor domain within its N-terminal region and that repression by this domain in vivo requires Gro. Therefore, Hkb fulfills all the criteria for being a Gro-dependent repressor.

Hkb also behaves genetically as a positive regulator of forkhead (fkh) and serpent (srp) expression. In hkb mutant embryos, the posterior/fkh domain is smaller than in wild-type embryos and srp expression at the poles is not initiated (Weigel et al., 1990; Rehorn et al., 1996). Perhaps Hkb functions as an activator of fkh and srp expression that, when associated with Gro, represses other target genes. Arguing against this possibility, there is no direct evidence that Hkb contains an activation domain. For example, it does not promote activation of reporter genes when introduced into yeast cells (Table 1). Additionally, the HairyHkb chimera containing the N-terminal 195 residues of Hkb does not cause activation of Sxl in male embryos (unpublished), whereas a Hairy fusion containing the viral VP16 activation domain does (Jiménez et al., 1996). These results suggest that Hkb regulates fkh and srp transcription indirectly, possibly by repressing a repressor of these genes.

Using in vitro and in vivo assays, we have established that the FRPW domain in Hkb couples its ability to associate with Gro with its ability to repress. In contrast to the extreme C-terminal position of Hairy’s WRPW Gro-binding motif, the FRPW sequence is situated within Hkb. Although this inner location appears unexpected for a WRPW-like repressor sequence, other Gro-binding domains are also positioned internally. Thus, the Dorsal rel domain and the conserved eh1 domains of En, Goosecoid and related proteins (Dubnicoff et al., 1997; Jiménez et al., 1997, 1999; Tolkunova et al., 1998) are located within the respective proteins. More recently, interactions between Gro and Pan/LEF-1 have been characterised in vitro (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998). Pan and LEF-1 contain pentapeptides (FRTPY and FRQPY, respectively) somewhat similar to the Gro-binding domains of Hairy and Hkb, within a highly conserved Pan/LEF-1 region; these may prove to be yet additional variants of the F/WRPW/Y motif.

Several works have suggested that the FRPW (and by implication, the FRPW) domain is sufficient on its own to recruit Gro (Fisher et al., 1996; Struhl and Adachi, 1998; Jiménez et al., 1999). That a repression domain consisting of only four aminoacids can recruit an ~80 kDa protein like Gro may at first seem surprising. However, the role of short polypeptide motifs as mediators of specific protein:protein interactions has been noted in numerous biological contexts. For example, a 5-aminoacid motif present in several transcriptional cofactors, such as RIP-140 and CBP, is necessary and sufficient for binding of these proteins to nuclear hormone receptors (Heery et al., 1997; Torchia et al., 1997) and the Drosophila phototransduction adaptor protein INAD binds to a C-terminal tripeptide in NORPA (Shieh et al., 1997). Perhaps these small motifs confer low-affinity interactions between partner proteins, facilitating the formation of transient, rather than lasting, protein complexes.

Groucho-dependent versus-independent repression

Gro-mediated repression plays a major role in several developmental and signalling pathways during early Drosophila embryogenesis, including sex determination, segmentation, neurogenesis and dorsoventral patterning. In these settings, Gro was shown to functionally associate with different classes of negative transcriptional regulators such as the Hairy-related bHLH proteins (Paroush et al., 1994), Runt
Hkb can now be added to this growing list of Gro-dependent repressors.

Previous studies have defined two different modes of transcriptional repression: (i) short-range, in which a repressor acts over short distances (less than \(-100\) bp) to inhibit nearby DNA-bound activators or the basal transcriptional machinery, and (ii) long-range, in which a repressor exerts its effects over longer distances (>1 kb) (reviewed in Gray and Levine, 1996). The distinction between the two classes of DNA-binding repressors suggests that different molecular mechanisms underly their mode of action. An analysis of Drosophila repressors suggests that those acting at short range operate independently of Gro, probably via the CBP corepressor (Jiménez et al., 1997; Nibu et al., 1998a,b; Zhang and Levine, 1999). In contrast, two long-range repressors, Hairy and Dorsal, are Gro-dependent (Ip et al., 1991; Huang et al., 1993; Paroush et al., 1994; Cai et al., 1996; Barolo and Levine, 1997; Dubnicoff et al., 1997). Thus, Gro may act as a long-range corepressor, perhaps interfering directly with the basal transcriptional machinery, or modulating chromatin organisation at promoters (e.g., via histone deacetylation; reviewed in Pazin and Kadonaga, 1997; Wolffe, 1997).

Interestingly, several Gro-dependent repressors also seem to direct Gro-independent repression. Thus, Runt (Aronson et al., 1997) and Hairy (Dawson et al., 1995) exhibit repressive potential even in the absence of their Gro-recruitment domains. Mutating the FRPW motif in Hkb still leads to weak repression of Sxl in the sex-determination assay (Fig. 4C), raising the possibility that Hkb also possesses some repressor activity that is independent of Gro. However, since this residual repression appears very weak, does not bring about female-specific lethality and is not observed when the \(hb\)-Hairy\_Hkb transgene is introduced into \(gro^{mat}\) embryos (Fig. 4B), the FEAW mutant is probably not a complete null. Indeed, \(h\) alleles with point mutations in their WRPW motif display hypomorphic phenotypes that are weaker than those seen with alleles encoding C-terminal truncations of Hairy (Wainwright and Ish-Horowicz, 1992).

**Transcriptional repression and posterior terminal patterning**

Transcriptional repression appears to play a pivotal role in embryonic terminal patterning, with both the \(hkb\) and \(tll\) gene products negatively regulating many downstream target genes. At the posterior pole, \(tor\) signalling is absolutely required for the relief of Gro-dependent repression and, consequently, for the expression of \(tll\) and \(hkb\) (Liaw et al., 1995; Paroush et al., 1997; anterior terminal patterning also requires input from additional factors.). Both \(tll\) and \(hkb\) encode for transcription factors that govern the expression of secondary transcriptional regulators and specify the pattern of the most posterior portions of the embryonic body (e.g., Casanova, 1990; Weigel et al., 1990; Brönnner and Jäckle, 1991; Kispert et al., 1994; Reuter and Leptin, 1994; Margolis et al., 1995; Singer et al., 1996).

Here we have shown that Hkb repression is mediated by Gro. However, several lines of evidence suggest that Tailless (Tll) functions independently of Gro. For example, although the giant (\(gt\)) posterior expression domain is normally repressed by both terminal gap genes, spreading to the posterior tip in \(tll\ hkb\) double mutant embryos (Brönnner and Jäckle, 1991), we find that this expansion does not occur in \(gro^{mat}\)-embryos (our unpublished results), indicating that Tll is still functional in the absence of Gro. Only in \(gro\ tll\) double mutant embryos does this expansion occur (our unpublished results). Also, we have previously shown that Tll represses the expression of the trunk gap genes \(Kr\) and \(kni\) independently of Gro (Paroush et al., 1997). Thus, Tll may associate with other corepressors, or act via a different molecular mechanism. Like Hkb, Tll also behaves as a positive regulator of certain genes (e.g., \(byn\) and \(hb\); Casanova, 1990; Kispert et al., 1994; Margolis et al., 1995; Singer et al., 1996), although it is unclear yet whether these effects are direct or indirect.

Gro is required repeatedly at different stages of terminal patterning (Fig. 5). First, Gro-repression acts to confine \(tll\) and \(hkb\) gene expression to the embryonic poles (Paroush et al., 1997). Subsequently, Gro mediates repression by Hkb, preventing genes normally expressed in the targeted portion of the embryo, and in the primordium of hindgut and anal pads, from being transcribed throughout the posterior cap. Thus, Gro-mediated repression establishes a border of gene expression between the centre and pole regions of the embryo (Fig. 5). In Gro’s absence, this boundary does not form and genes from either side are ectopically expressed across the border. Future experiments will determine whether repression is similarly involved in setting up transcriptional boundaries in other developmental contexts.

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