

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

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Accepted 10 June; published on WWW 5 August 1999

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 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*^{mat-} 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*^{mat-} 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 *tlx* 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 *hkb*¹ and *hkb*^{XM9} (a strong hypomorph and a complete null, respectively; Weigel et al., 1990; Brönnner et al., 1994). *hkb* homozygous mutant embryos were distinguished from their blue-balanced siblings by lack of *lacZ* staining. Strong gain-of-function *tor*^D embryos were generated from *tor*^{Y9/tor}⁴⁰²¹ females (Klingler et al., 1988). *gro*^{E75} and *gro*^{E48} are strong *gro* alleles, and *Df(3R)E(spl)*^{BX22} is a chromosomal deletion encompassing *m5*, *m7*, *m8* and *gro* (Preiss et al., 1988; Delidakis et al., 1991).

gro^{mat-} embryos, lacking maternal *gro* activity, were derived from mosaic females with either *gro*^{E75}, *gro*^{E48} or *gro*^{BX22} mutant germlines, obtained using the FLP-DFS technique (Chou et al., 1993). Briefly, males carrying the *FRT[82B]ovo*^{D1} chromosome and an X-linked *hs-FLP1* chromosome were crossed to females carrying a *FRT[82B]gro* chromosome. Progeny 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*^{mat-} embryos (Schrons 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 (*C(1)M3*); insertions on the autosomes were kept as unbalanced stocks, selecting each generation for transformant males and non-transformant females. To analyse the effect of *hb-Hairy*^{Hkb} in *gro* embryos, mosaic *gro*^{E75} or *gro*^{E48} females were crossed to males carrying the *hb-Hairy*^{Hkb} construct on the X chromosome, such that all female progeny were both *gro*^{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 anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim; Tautz and Pfeifle, 1989). For Sxl immunohistochemistry, embryos were incubated with a monoclonal antibody specific to the active form of Sxl (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-HairyΔNot, pET-Gro, LexA-Gro, LexA-E(spl)-m7 and LexA-Dmcdc2 have been previously described (Paroush et al., 1994; Dubnicoff et al., 1997). *hb-Hairy*^{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 trp1 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 *pJG4-5* (2μ *TRP1*) vector that allows galactose-inducible expression of proteins fused to an activation domain. Reporter gene activation was analysed on *ura*⁻*his*⁻*trp*⁻*leu*⁻ growth plates, including galactose and raffinose, to select for activation of the *LexAop-LEU2* gene and on X-Gal indicator plates for *lacZ* expression. β-galactosidase liquid assays were performed as described (Ausubel et al., 1987-1997). For each transformation, three colonies were assayed on two independent occasions.

In vitro GST pull-down assays

GST fusion proteins were expressed from the *pGEX2T* vector (Pharmacia) in the protease-deficient *Escherichia coli* strain AO5039 (gift of Amos Oppenheim, Hebrew University) and purified on glutathione-agarose beads (Sigma) essentially as described (Paroush et al., 1994). Pull-down assays were performed with equal amounts of fusion proteins (1-5 μg), bound to 50 μl beads and preincubated for one hour at 4°C in 1 ml binding buffer (PBS, 0.34 M NaCl, 1 mM EDTA, 0.1% NP40, 10% BSA). 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

times in 1 ml PBS/290 mM NaCl, boiled for 3 minutes in sample buffer and aliquots were examined by electrophoresis. Integrity and quantity of GST fusions was confirmed by Coomassie staining and autoradiography detected the amount of retained Gro or Hkb.

RESULTS

Deregulation of putative Hucklebein target genes in embryos lacking maternal *groucho* function

We have previously pointed to similarities in gene expression patterns found in *gro^{mat-}* embryos and in *tor* dominant (*tor^D*) gain-of-function mutant embryos (Paroush et al., 1997). In both cases, expression of the *tll* and *hkb* genes expands towards the centre of the embryo (though *hkb* broadens to a lesser extent than *tll* in *gro^{mat-}* embryos; Paroush et al., 1997). Expanded *tll* consequently leads to repression of the central gap genes *Krüppel* (*Kr*) and *knirps* (*kni*) (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^{mat-}* embryos.

One proposed Hkb target is the *snail* (*sna*) gene, which is transcribed in the ventral-most portion of the embryo. *sna* expression is thought to be excluded from the posterior pole by *hkb* activity. Accordingly, *sna* and *hkb* expression domains abut in cellularising wild-type embryos, whereas *sna* expression extends to, and includes, the posterior pole of *hkb* mutant embryos (Reuter and Leptin, 1994; Fig. 1A,D,G). In *tor^D* embryos, *hkb* expression expands towards the centre of the embryo and the *sna* domain correspondingly retracts (Brönner and Jäckle, 1991; Reuter and Leptin, 1994; Fig. 1B,E). By contrast, in *gro^{mat-}* embryos, the expression of *sna*

does not respect its posterior border and spreads to the pole, overlapping extensively with the *hkb* expression domain (Fig. 1C,F).

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

Physical associations between Hucklebein 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-HairyΔNotI (a truncated form of

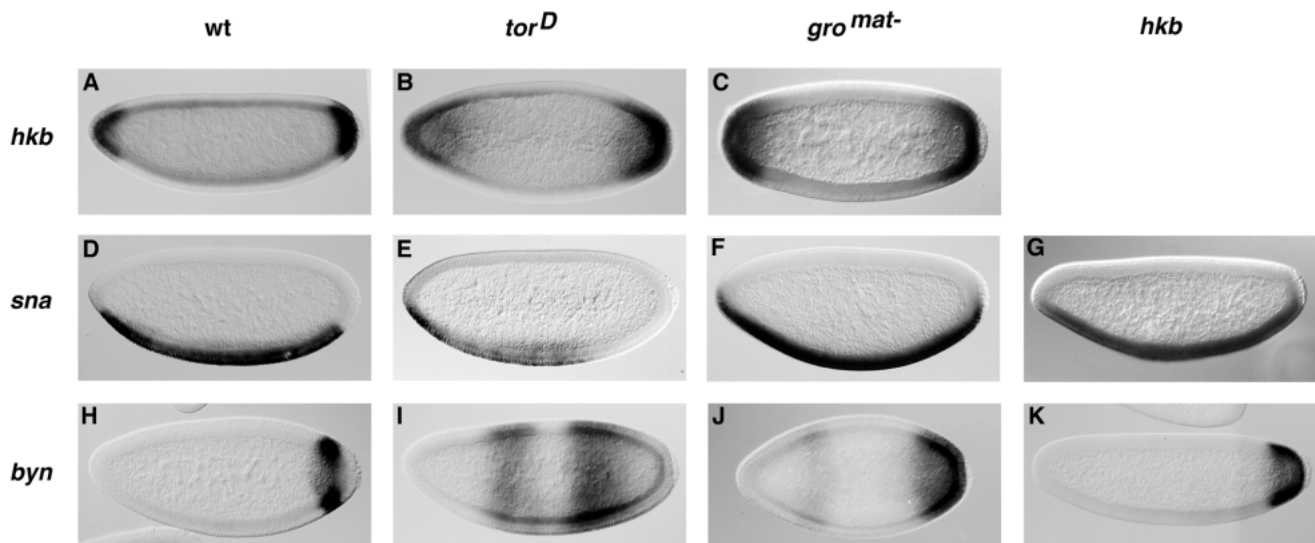
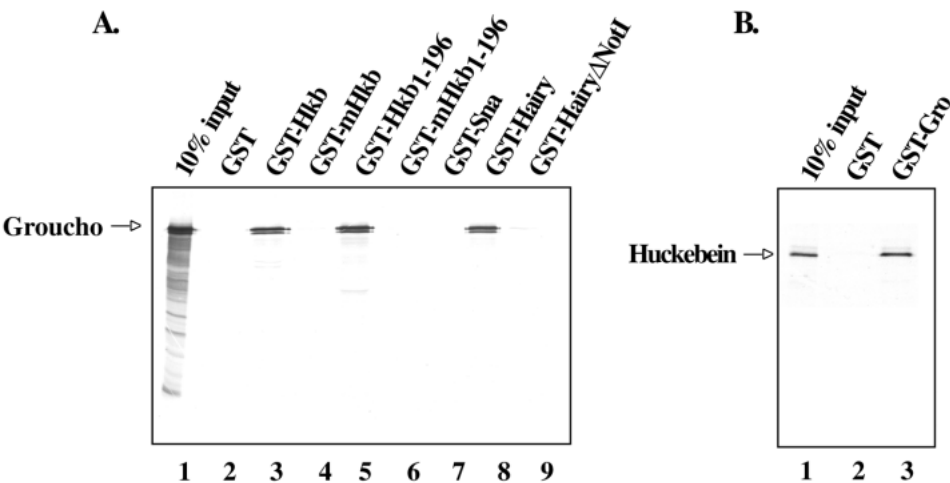


Fig. 1. *sna* and *byn* expression is deregulated in embryos lacking maternal *groucho* activity. Wild-type (A,D,H) or mutant *tor^D* (B,E,I), *gro^{mat-}* (C,F,J) and *hkb* (G,K) embryos were stained for *hkb* (A-C), *sna* (D-G) and *byn* (H-K). In wild-type and *tor^{Y9/4021}* embryos, posterior borders of *sna* and *byn* expression domains respect that of *hkb* (A,B,D,E,H,I). However, *sna* and *byn* transcription expands to include the posterior pole of *gro^{mat-}* embryos (F,J), in a manner resembling their expression patterns in *hkb* mutants (G,K), despite the fact that *gro^{mat-}* embryos express *hkb* (C). Ectopic *byn* transcription in the anterior of *tor^D* and *gro^{mat-}* embryos (I,J) probably reflects expanded *tll* expression in these genotypes (Steingrímsson 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. Hucklebein binds Groucho in vitro. (A) ³⁵S-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 Hkb₁₋₁₉₆ (lane 5), to the same extent that it binds Hairy (lane 8), but not to control Snail (lane 7), HairyΔ*NotI* (lacking its C-terminal Gro-binding domain; lane 9) or GST alone (lane 2). Mutating the FRPW motif (to FEAU) in Hkb (mHkb; lane 4) and Hkb₁₋₁₉₆ (mHkb₁₋₁₉₆; 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 ³⁵S-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 aminoacids, 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 [³⁵S]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 Hucklebein 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 aminoacids, which include its putative DNA-binding zinc-finger domain, or even the removal of a further 71 aminoacids, 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 aminoacids 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 aminoacids 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 aminoacids in this motif (to FEAU) 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 Hkb₁₋₁₉₆ complex with Gro (lanes 3, 5), while neither binds Gro when carrying the FEAU 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.

Hucklebein 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 Hucklebein and Groucho in the yeast two-hybrid assay				
LexA fusions	β-galactosidase activity			
	pJG-Gro	pJG4-5	pJG-Hkb	pJG-Sna
LexA-Hkb	23±15	2.8±0.8	nd	nd
LexA-Sna	7.1±2.0	7.7±2.1	nd	nd
LexA-E(spl)-m7	345±55	25±11	nd	nd
LexA-Gro	nd	nd	298±37	3.0±2.6
LexA-Dmcdc2	3.3±0.9	5.6±1.2	4.4±1.5	2.9±1.2

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 Dmcdc2 serve as negative controls. *pJG4-5* is the backbone vector. β-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.

Fig. 3. The interaction domain required for Hucklebein'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.

Huckebein derivatives		Interactions with Groucho	β-Galactosidase Activity			
			pJG-Hkb derivatives with:		LexA-Hkb derivatives with:	
			LexA-Gro	LexA-Dmcdc2	pJG-Gro	pJG4-5
Hkb ₁₋₂₉₇	<div><div>FRPW</div><div><div></div><div>Zn-finger</div></div></div>	+				
Hkb ₁₋₁₉₆	<div><div></div></div>	+	552±45	2.3±1.0	nd	nd
Hkb ₁₋₁₂₅	<div><div></div></div>	+	96±22	2.8±0.9	48±11	2.9±0.9
Hkb ₁₋₅₂	<div><div></div></div>	+	10±2.8	4.9±1.0	43±13	3.2±1.1
Hkb ₂₃₋₁₉₆	<div><div></div></div>	–	3.3±2.3	3.3±1.7	nd	nd
mHkb ₁₋₁₉₆	<div><div>FEAW (R17E, P18A)</div><div></div></div>	–	3.2±2.0	3.7±1.5	nd	nd

repression of the sex-determining gene *Sex-lethal* (*Sxl*) by ectopic Hairy (Parkhurst et al., 1990; Jiménez et al., 1997). *Sxl* is normally transcribed at the blastoderm stage in female embryos only (Keyes et al., 1992) while, in male embryos, it is silenced by autosomally encoded repressors that include the Hairy-related protein Deadpan (Dpn; Younger-Shepherd et al., 1992). Although Hairy is not involved in sex determination, its premature expression interferes with *Sxl* transcription (Parkhurst et al., 1990), probably because Hairy can mimic Dpn activity. Thus, early expression of Hairy from the *hunchback* (*hb*) promoter in the anterior halves of female blastoderm embryos causes repression of *Sxl* (Parkhurst et al., 1990). This repression requires *gro* activity, as it does not occur in *gro*^{mat} embryos (Jiménez et al., 1997).

A truncated form of Hairy, lacking its C-terminal 69 aminoacids (including the WRPW Gro-binding motif), is inactive in the above assay (Dawson et al., 1995; Jiménez et al., 1997). However, substituting this domain of Hairy with repressor domains taken from other proteins generates Hairy chimeras that do repress *Sxl* (Jiménez et al., 1997, 1999). Depending on the fused repressor domain, silencing of *Sxl* can be either dependent or independent of Gro (Jiménez et al., 1997; see below). To establish whether Hkb can function as a repressor, we constructed a Hairy^{Hkb} chimera by replacing the C terminus of Hairy with Hkb's N-terminal 195 aminoacids (lacking the Hkb Sp1-like zinc-finger DNA-binding domain). When expressed under the regulation of the *hb* promoter, the Hairy^{Hkb} chimera causes effective repression of *Sxl* in the anterior region of female embryos (Fig. 4A). Furthermore, this repression also causes female-specific lethality, probably due to the role of *Sxl* in dosage compensation (reviewed in Parkhurst and Meneely, 1994; unpublished data). These results indicate that Hkb contains a potent repression domain within its N-terminal 195 aminoacids.

Groucho mediates Hucklebein'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 Hairy^{Hkb} requires Gro activity. Males harbouring the *hb*-Hairy^{Hkb}

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, Hairy^{Hkb} 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*-Hairy^{Hkb} transgene, carrying the FRPW→FEAW mutations, was generated and introduced into wild-type embryos. As shown in

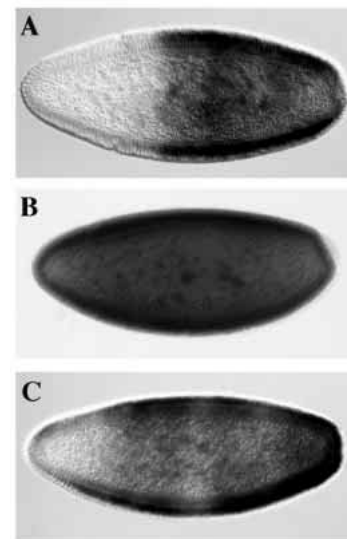


Fig. 4. Hkb contains a potent repression domain that depends on Gro in vivo. Effects of expressing the *hb*-Hairy^{Hkb} transgene on *Sxl* expression in otherwise wild-type (A) or in *gro*^{mat} embryos (B). Embryos were stained using a monoclonal antibody specific for full-length *Sxl*, which stains only female embryos (Bopp et al., 1991). Repression in the anterior halves of wild-type female embryos is evident (A) and is consistent with Hkb's repressor ability. In contrast, there is no *Sxl* repression by Hairy^{Hkb} in the absence of *gro* (B). The ability of Hairy^{Hkb} to repress *Sxl* in wild-type embryos is greatly compromised when the FRPW motif is mutated (C).

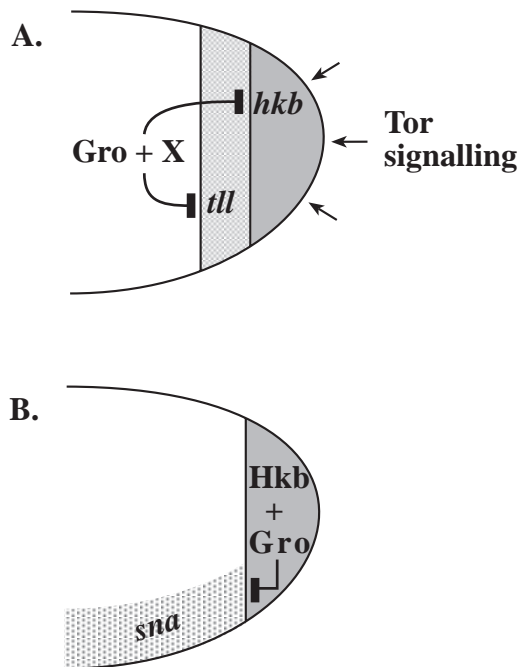


Fig. 5. Model for the role Groucho-mediated repression plays in establishing a transcriptional boundary between the central, segmental portion of the embryo and the termini. Initially, in stage 4 embryos (A), Gro and an as yet unknown partner protein 'X' repress terminal gap gene expression in the central 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 Hucklebein repression

Genetic studies have previously suggested that Hkb acts as a negative regulator during terminal patterning (Casanova, 1990; Weigel et al., 1990; Brönnert 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 *Hairy^{Hkb}* 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 WRPW (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

(Aronson et al., 1997), En (Jiménez et al., 1997; Tolkunova et al., 1998), Pan/LEF-1 (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998) and Dorsal (Dubnicoff et al., 1997). 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 CtBP 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; Brönner 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 (Brönner 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 segmented 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.

We should like to thank members of our laboratories for continued help and encouragement during this project. R. E. G. is grateful to Shosh Ravid, Chaya Miller and Naomi Zak for advice in pull-down experiments. G. J. thanks David Ish-Horowicz and Jordi Casanova for support. Z. P. is indebted to David Ish-Horowicz for hosting him in London. We would like to thank Jordi Casanova, David Ish-Horowicz, Adi Salzberg, Benny Shilo and Naomi Zak for their helpful comments on the manuscript, Sigal for fly food, and Günter Brönner, Al Courey, Michael Hoch, Judith Lengyel, Maria Leptin, Mike Levine, Jocelyn McDonald, Amos Oppenheim, Francisco Pelegri, Elisabeth Perkins, Sheena Pinchin and Mark Wainwright for probes, antibodies and fly stocks. We apologise for the incomplete referencing due to rigid space constraints. G. J. was supported by the Imperial Cancer Research Fund and the Spanish Ministry of Education and Culture. Work was supported by grants from the Israel Science Foundation (#359/96), the Israel Cancer Research Fund (RCDA) and from the United States-Israel Binational Science Foundation (#96-108). Z. P. is also grateful for support from the Jan M. and Eugenia Krol Charitable Foundation.

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