Differential Regulation of Target Genes by Different Alleles of the Segmentation Gene hunchback in Drosophila

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ABSTRACT

hunchback (hb) is a key regulatory gene in the early segmentation gene hierarchy of Drosophila. It codes for a transcription factor of the Cys2-His2 zinc finger type and shows two separate zinc finger domains in its coding region. hb forms a morphogenetic gradient in the middle of the embryo that is required for setting the spatial boundaries of several target genes. We have analyzed the molecular lesions found in the different hb alleles and have studied the differential effects of these alleles on a number of such target genes. We find that in mutants in which the HB protein lacks a functional second finger domain, the regulation of the target genes Krüppel (Kr) and knirps (kni) is differentially affected. While this domain is required for the correct regulation of Kr, it is not necessary for the repression of kni. Furthermore, mutations affecting this domain lead to a decreased protein stability. The integration of the expression pattern of target genes was found to be distorted in a second class of mutants between the two finger domains which lead to gain of function or neomorphic phenotypes. The effects of these mutations were studied in detail and it was found that they fall into two classes, the first one interfering with the function of the maternal hb product, the second leading to a delayed segmentation. The function of the latter class appears to be linked to the secondary expression of hb in the parasegment 4 (PS4) stripe at blastoderm stage.

The early segmentation process of the Drosophila embryo is governed by a cascade of gene activities which divide the embryo into more and more refined subdomains. Most of the early gene products in this cascade code for transcription factors which regulate the next steps in the cascade via morphogenetic gradients. The maternal morphogen organizing the anterior region of the embryo is bicoid, while the abdominal region of the embryo is organized by nanos (nos) and hunchback (hb) (reviewed in St. Johnston and Nüsslein-Volhard 1992). Maternal hb RNA is homogeneously distributed in the early embryo. The translation of this RNA is differentially controlled by nos, which forms itself a posterior-anterior gradient in the embryo (Wang and Lehmann 1991). This results in a complementary hb protein gradient in the posterior region of the embryo (Tautz 1988) which is responsible for the organization of the abdominal pattern (Hülskamp et al. 1990; Struhl et al. 1992). Interestingly, this gradient can also be provided by the bcd dependent zygotic expression of hb, independent of the nos system. It is therefore possible to remove the maternal component of hb, as well as nos without affecting the normal development of the embryos (Hülskamp et al. 1989; Irish et al. 1989a; Struhl 1989).

The genetic analysis of the differential and combined function of the maternal and the zygotic expression of hb has revealed the following direct or indirect target genes of hb at the gap gene level (Hülskamp et al. 1990; Struhl et al. 1992). Low levels of hb are required to activate the gap gene Kr, while high levels of hb repress Kr. Two further gap genes, namely kni and giant (gt) are also repressed by hb, though at different concentrations of the HB protein. All of these gap genes form then themselves morphogenetic gradients, which are involved in defining the spatial limits of the individual stripes of the pair-rule genes (Howard et al. 1988; Pankratz et al. 1990). The setting of the borders of the gap gene expression domains in the right order and polarity is therefore crucial for the further segmentation process (reviewed in Hülskamp and Tautz 1991).

hb controls also the expression of homeotic genes such as Ubx (Lehmann and Nüsslein-Volhard 1987; White and Lehmann 1986; Qian et al. 1991, 1993; Zhang et al. 1991) and Antp (Harding and Levine 1988; Irish et al. 1989b). It was shown that hb binds directly to the early Ubx enhancer elements BRE and PBX (Qian et al. 1991; Zhang et al. 1991) and that it is capable to compete against the binding of fushi tatrazu (ftz) in the BRE enhancer (Qian et al. 1993). hb integrates in this way the segmental subdivision of the embryo and the specification of the identity of the segments.

hb codes for a transcription factor with zinc fingers of the Cys2-His2 type (Tautz et al. 1987). Two such zinc finger domains are found in the protein, one with four
fingers in the middle and one with two fingers at the very C terminus. Evolutionary comparisons have shown that these two domains are conserved over large evolutionary distances (Treier et al. 1989; Sommer and Tautz 1991; Sommer 1992). In addition, these comparisons have revealed four further conserved domains in the protein, the A and the B box (Tautz et al. 1987), as well as a region after the first finger domain (the C box) and a region between the two finger domains (the D box) (Sommer 1992). However, nothing is known about the function of these boxes so far. In order to elucidate potential differential functions of separate HB protein domains, we have analyzed the various hb alleles at the sequence level and with respect to their effects on various target genes as well as on the segmentation process. These experiments were complemented by several specifically designed P element constructs. We show that the functions of the first and the second finger domain of hb are separable and that the D box is specifically involved in the regulation of a subset of genes. Our results shed also some light on the nature of the gain of function or neomorphic hb alleles.

MATERIALS AND METHODS

Sequencing of the alleles: DNA from homozygous mutant embryos was isolated by crossing the balancer out and collecting the non-hatching embryos from the respective backcross. The hb locus was then amplified by polymerase chain reaction using primers that flank the coding region. The fragment was cloned and sequenced using sets of nested sequencing primers. Any mutation found was verified or excluded by at least one additional replication of the experiment.

Expression analysis: Whole mount in situ hybridizations were essentially done according to Tautz and Freier (1989). For antibody staining we used the universal en antibody described in Patel et al. (1989). The mutant expression patterns were identified on a statistical basis. The locations of the borders of domains or of stripes were measured in percent egg length with the help of a wedge shaped grid and a camera lucida equipped microscope. When care is taken that only equivalent stages are compared, this method is very reliable and shifts as little as 2-4% egg length can reliably be detected.

Construction of P elements and artificial alleles: All P element constructs described here are derived from a rescue construct that carries a 4.3-kb genomic fragment from the hb locus cloned into the P element vector Carnegie20 (Rubin and Spradling 1983). This fragment extends from the HindIII site at position 3445 to a BamHI site outside of the sequenced region (listing of the nucleotides according to Tautz et al. 1987; note that this is an artificial BamHI site generated from a Sau3A site of the genomic fragment cloned into λ E9)). It is expressed only at blastoderm stage in the anterior half of the embryo and hence mediates normally only a rescue of the head structures and the thoracic segments T1 and T3 though an occasional rescue of T2 is also seen [see Lukowicz et al. (1994) for further details]. Within this construct the hb coding region is included in both a single SalI and a single XbaI fragment. This enabled us to use different subclones of the 4.3-kb genomic fragment for manipulating the coding region in vitro and eventually to exchange the manipulated sequences (either as SalI or as XbaI fragments) against the wild-type one. The result of all manipulations was controlled by sequencing.

p9K49 and p9K57 constructs: The alleles were cloned as XbaI fragments into the above P element construct.

pSpuV construct: A premature stop-codon was introduced before the D box. This was done by deleting the 56 bp DNA fragment between the two PvuII sites around codon 400 and 418 and replacing it with a 6-mer XbaI linker. The translated protein sequence reads: (399)Ala-Ala-Val-TER.

pAPST and pipPST constructs: These carry an in frame deletion (Δ) and in-frame inversion (i) of the central 39 codons of the D box. In pAPST the 117-bp DNA fragment between the two PstI sites around codon 475 and 514 was deleted. The translated protein sequence reads: (475)Ser-Leu-Leu-Gln-Gln-Gln-Asn(518) (site of deletion underlined). In pipPST the respective DNA fragment is inverted, the translated protein sequence reads: (473)Ser-Leu-Leu-Gln-Leu-Ser-His-Ser-Trp-Arg-Gln-Leu-Glu-Gln-Asp-Ser-Gly-Leu-Leu-Gly-Gly-Glu-Gln-His-Leu-Glu-Ile-Glu-Ala-Pro-Val-Gly-Glu-Glu-Gly-His-Val-Ala-Val-Leu-Leu-Gln-Asn(518) (inversion underlined).

pSxo construct: This construct carries a premature stop-codon between the D box and the domain 2. This stop-codon is created by a 14-mer XbaI linker (CTAGCTCAGCAGTAG) introduced into the blunted XbaI site around codon 583. The translated protein sequence reads: (380)Ser-Asn-Ala-Ser-Thr-Ser-Leu-Asp-TER  Note that the last four positions (underlined in the above listing) differ from the wild-type sequence.

P element transformation: P element transformations were done as described by Rubin and Spradling (1982) using the Δ2,3 P element helper plasmid (Laski et al. 1986). The recipient fly strain for the constructs was ry108. Multiple independent lines were established for each construct and backcrossed to hb16,ry/TM3,ry (kindly supplied by R. Lehmann). Embryos that were homozygous mutant for hb could be unequivocally recognized by the fusion of abdominal segments A7/A8, since the phenotype of this defect was not expected to be affected by the constructs.

RESULTS

Molecular characterization of the hb alleles: We have sequenced eleven different hb alleles to define the molecular nature of the respective mutations. All but one were induced by EMS and we found the expected G to A transitions for most of them (Table 1). The X-ray-induced allele hb16 showed a small deletion of 10 bp, resulting in a frameshift and a premature stop codon.

The different molecular lesions found can be grouped into several classes which roughly coincide with the previously defined phenotypic classes of the hb alleles (Lehmann and Nüsslein-Volhard 1987). The amorphic alleles (hb16, hb9Q and hb16) are characterized by stop codons before the first finger domain, thus abolishing all DNA-binding capacities of the protein (Figure 1). The hypomorphic alleles can be grouped into two classes, one with amino acid exchanges in the first finger domain (hb7L and hb19P) and one with a functional loss of the second finger domain (hb10L, hb7A and hb11C) (Figure 1). The two neomorphic alleles (hb14C and hb9K57) have stop codons within the D box, the third gain of function allele (hb9K49) shows an amino acid replacement behind the first finger domain in the C box (Figure 1).

To understand the function of the HB protein in more detail, we have created a number of artificial al-
Differential Regulation by hb Alleles

TABLE I

Mutations in the hb alleles analyzed

<table>
<thead>
<tr>
<th>hb allele</th>
<th>Wild-type sequence</th>
<th>Mutant sequence</th>
<th>Amino acid position in protein</th>
<th>Mutagen</th>
<th>Phenotypic class</th>
</tr>
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<tbody>
<tr>
<td>hb1B</td>
<td></td>
<td>Del (10n)</td>
<td>150</td>
<td>X-ray</td>
<td>Amorph</td>
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<td>Stop (TAA)</td>
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<td>EMS</td>
<td>Amorph</td>
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<tr>
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<td>Stop (TGA)</td>
<td>256</td>
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<td>Amorph</td>
</tr>
<tr>
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<td></td>
<td>Asn (AAG)</td>
<td>296</td>
<td>EMS</td>
<td>Hypomorph</td>
</tr>
<tr>
<td>hb1U</td>
<td></td>
<td>Ile (ATA)</td>
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<td>EMS</td>
<td>Hypomorph</td>
</tr>
<tr>
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<tr>
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<td>710</td>
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<tr>
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<td>EMS</td>
<td>Gain of function</td>
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<tr>
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<td>Neomorph</td>
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<tr>
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<td>477</td>
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<tr>
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<td>Neomorph</td>
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<tr>
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<td>Hypomorph</td>
</tr>
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<td>Inv (108n)</td>
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<td>IVM</td>
<td>Hypomorph</td>
</tr>
<tr>
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<td>Hypomorph</td>
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<tr>
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The nature and position of the mutations is given for each of the described hb alleles. The X-ray-induced allele hb1B exhibits a small deletion of 10 bp which causes a frameshift and a premature stop codon. Mutations in the artificial hb alleles were generated in vitro as described in MATERIALS AND METHODS. The phenotypic classification is based on Lehnmann and Nüsslein-Volhard (1987). EMS, ethylmethanesulfonate; IVM, in vitro mutagenesis; Inv, inversion; Del, deletion.

![Figure 1](image)

**Figure 1.**—Schematic representation of the HB protein and the positions of mutations in the hb alleles. The protein coding region of hb is depicted as a black bar and the relative position of the A, B, C and D box as well as the two finger domains are shown as white boxes. The A and B box are short regions of similarity between hb and Krr in similar positions of the proteins (Tautz et al. 1987), the C and D box were defined on the basis of their evolutionary conservation between the Drosophila and Musca hb proteins (Sommer 1992). The C box is located between amino acid position 350-380 and shows 87% similarity to the Musca sequence, the D box is located between amino acid position 466-522 and shows 98% similarity (amino acid positions according to Tautz et al. 1987).

alleles. For this purpose, we have mutagenized the hb coding sequence in vitro and placed it into a P element vector under the bcd dependent promotor of hb. Such a construct can rescue most or all of the anterior cuticle pattern of amorphic hb alleles when it carries a wild-type hb gene. However, it will show no rescue of the posterior defects or of maternally induced defects, since the respective promotor elements are missing (Luikottz et al. 1994). This allows to differentially analyze the effects of hb caused by the bcd dependent expression. The artificial alleles created in this way are included in Table 1 and Figure 1 and their specific effects are discussed below.

**Phenotypic analysis:** The hb alleles can be classified according to the strength of the mutant phenotype (Lehnmann and Nüsslein-Volhard 1987). However, for the following description we should like to group the known alleles, as well as the artificial alleles according to the characteristics of their molecular lesions. The residual function of the alleles was analyzed at different “phenotypic” levels, namely at the level of the expression of the two pair-rule genes hairy (h) and ftz) as well as at the cuticle pattern of the first instar larvae (Figure 2).

In amorphic hb alleles, all thoracic segments and the most posterior gnathal segment, the labium, are missing (Figure 2G). The corresponding parasegments, as represented by the first three h and ftz stripes, are strongly affected. h stripes two and three are missing and the first three ftz stripes appear to be fused (Figure 2, H and I). Mutations in either the first (hb1B, hb2) or the second (hb1U, hb70, hb14C, pSXHO) finger domain result in a hypomorphic phenotype (Figure 2, J and S). These mutants lack the second and third thoracic segments. At the
level of the pair-rule genes, h and ftz stripes three are missing and stripes two are reduced (h) or expanded (ftz) (Figure 2, K and L, and T and U). Artificial alleles in which the D box is deleted or mutated (pΔPST, pΔPST) show essentially the same phenotype (Figure 2, P, Q and R). On the other hand, the reference construct with the wild-type coding sequence of hb leads to a rescue of all anterior structures apart of the second thoracic segment (Figure 2D). All anterior h and ftz stripes are formed in this “allele,” though stripes two and three are slightly disturbed (Fig. 2, E and F).

The only known mutation mapping to the C box, the allele hb9K49, leads to a gain of function phenotype, represented by a duplication of A1 at the cuticle level (Figure 2M). However, this effect is not evident at the level of the expression pattern of h and ftz which are only

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**Figure 2.**—Comparison of the cuticle pattern (left panel), the expression pattern of hairy (h) (middle panel) and the expression pattern of fushi tarazu (ftz) (right panel) in different hb alleles. (A) Cuticle pattern of wild-type larva showing three thoracic (T1–T3) and eight abdominal denticle belts (only A1 and A2 are labeled; segment A2 is labeled with an arrowhead in this and in the pictures below for better orientation); (B and C) wild-type embryos stained with h and ftz which serve as early markers for the odd-numbered and even-numbered parasegments at blastoderm stage. (D–F) P element rescue with the hb coding sequence under the bcd dependent promotor of hb. All head and thoracic segments apart of the second thoracic segment are rescued. Note that the posterior segment A8 is still fused with A7, thus allowing to identify the homozygous mutant hb embryos. The expression patterns of h and ftz show only minor deviations from normal. The second and third stripes appear somewhat weaker or dorsally fused. (G–I) Amorphic hb phenotype (e.g., hbl6, hb186, hb9C). The labial and all thoracic segments are lacking. A8 is fused with A7 and the filzkörper are rudimentary. In such embryos the h stripe 2 is absent, while the first three ftz stripes appear to be fused. (J–L) Phenotype typical for hb alleles classified as finger domain 1 mutants (e.g., hb2L, hb196). The meso- and metathoracic segments are missing which corresponds to the lack of h stripe three and a fusion of the second and third ftz stripes. (M–O) hb9K49 phenotype, A1 is duplicated. The third h stripe is weak or missing. The second and third ftz stripes are either fused or very close together. (P–R) Phenotypic rescue with P element constructs carrying mutations affecting the D box (e.g., pΔPST and pPST). Both, the cuticle pattern and the expression pattern of pair-rule genes are very similar to hypomorphic hb alleles. (S–U) Finger domain 2 mutants (e.g., hb12L, hb129, hb11C, pxho). The cuticle phenotype and the expression pattern of h and ftz are indistinguishable from finger domain 1 and D box mutants. (V–X) D box/finger domain 2 mutants (e.g., hb9K49, hb11C, pxPvu, p9K37). Gnathal and thoracic segments are missing. Up to three abdominal denticle belts of irregular shape but with normal polarity are formed anterior to the abdominal segments A2–A7. h stripes h1–h4 appear to be affected. Although the pattern is very variable, up to six h stripes may be found. In ftz staining, the second and third ftz stripes are missing.
The embryo at late blastoderm stage hybridized with weakly affected (Figure 2, N and O). This suggests that the main effect of this mutation occurs only after the segmentation process has been initiated (see below).

Lesions that remove the second finger domain and the D box (hb857, hb14c, psPVU, p9K57) result in a neormorphic phenotype. The head is even more reduced than in amorphic embryos and up to three abdominal segments are formed in the head region (Figure 2V) (LEHMANN and NÜSSLIN-VOLLHARD 1987). However, we find that in contrast to these drastic effects, the segmental partitioning itself is reasonably well established at the level of the pair-rule genes, though the picture is not directly comparable to the effects of the other hb alleles (Figure 2, W and X) (see below).

Target gene analysis: Three of the presumed direct target genes of hb at the gap gene level, namely Kr, kni and hb itself, were chosen to analyze potential differential effects of the hb alleles on their expression. It was previously shown that Kr is repressed and activated by hb, while kni is only repressed (HÜLSKAMP et al. 1990; STRUHL et al. 1992). Both effects are likely to be direct, since the regulatory regions of these genes show binding sites for hb (HOCH et al. 1991; PANKRATZ et al. 1992). In addition, we show here that hb regulates also its own secondary expression in the region of parasegment 4 (the PS4 stripe). Using in situ hybridization with the hb probe in amorphic alleles of hb we found that the PS4 stripe is lacking (compare Figure 4). On the other hand, this stripe is shifted and expanded toward posterior in embryos mutant for Kr (Figure 3A). Thus, the PS4 stripe is formally repressed by Kr and activated by hb. To rule out the possibility that the apparent activation by hb is an effect of the expansion of the Kr domain toward anterior in hb mutant embryos (GAUL and JÄCKLE 1987; HÜLSKAMP et al. 1990), we have also stained embryos double mutant for Kr and hb and found that the PS4 stripe is still missing (Figure 3B). This suggests that the inferred regulatory effects on this stripe are direct. This is further supported by the fact that the respective enhancer element that drives the PS4 expression contains evolutionary conserved consensus binding sites for Kr and hb (LUKOWITZ et al. 1994).

Figure 4 summarizes the different effects of the hb alleles on the regulation of the three target genes hb, Kr and kni. A comparison between wild-type and amorphic hb embryos shows the specific changes of the expression pattern in the mutant background. In amorphic hb embryos the PS4 stripe is missing (see above), while the anterior patches of hb expression and the posterior stripe are unaffected. The central domain of Kr and the posterior domain of kni are shifted toward anterior due to lack of repression by the zygotic hb expression (HÜLSKAMP et al. 1990; STRUHL et al. 1992).

While mutations in the first finger domain cause the same changes of hb, Kr and kni expression as amorphic hb alleles, mutations in the second finger domain have a less severe effect. Only the Kr expression is expanded toward anterior in the same way as in amorphic alleles, while the kni expression is not affected. In addition, the activation of the hb PS4 stripe is partly restored. These observations suggest that the second finger domain has a specific function for the repression of Kr but appears not to be necessary for the repression of kni. Interestingly, a similar result was obtained for artificial alleles in which only the D box was affected (pΔPST and pPST). Again we find that the Kr domain is expanded toward anterior, while the kni domain is not. A slight difference exists, however, for the PS4 stripe in these “alleles,” which was still completely lacking. Thus it appears that two different regions in the HB protein are required for one type of regulation, namely the repression of Kr, but not for another type of regulation, namely the repression of kni.

In the light of these results, the deletion of both, the D box and the second finger domain in hb857, hb14c and psPvu results in an unexpected phenotype. While mutations in either of the domains do not influence the regulation of kni, the removal of both domains leads to an expansion and ectopic expression of kni in the anterior region. This kni expression is in fact reminiscent of the expression seen in embryos lacking both, maternal and zygotic hb product (HÜLSKAMP et al. 1990; STRUHL et al. 1992). It should be noted, however, that the three alleles not only lack the two specific domains, but also the whole C-terminal portion of the protein.

Embryos homozygous mutant for hb857, which represents the only hb allele affecting the C box, show only minor distortions in the expression pattern of hb, Kr and kni. The kni expression in hb857 is indistinguishable from wild type, while the anterior Kr border and the hb PS4 stripe are shifted slightly to more anterior positions than in wild-type embryos.

**Figure 3.**—Regulation of the hb PS4 stripe. (A) Kr’ mutant embryo at late blastoderm stage hybridized with a hb probe. The hb PS4 stripe is shifted posteriorly as compared to wild type. The borders of the hb PS4 stripe in wild-type is marked by two dashes. (B) hb14cKr’ double mutant embryo. Since Kr’ embryos do not express Kr RNA, the double mutant embryos could be unequivocally identified by double hybridization with a hb and a Kr probe. Embryos double mutant for hb14c and Kr’ did not show any hb PS4 stripe expression.
The gain of function hb alleles: Three alleles of hb were found in the genetic screens, that do not fit into the allelic series of the other alleles, since each of them shows extra abdominal denticle belts in addition to the pattern defects. A careful genetic analysis had already suggested that they fall into two classes. Two alleles (hb\(^{9K57}\) and hb\(^{14C}\)) were found to be clearly neomorphic, since they express their phenotype independently of the wild-type copy of the hb gene supplied by the embryo or the mother. The third allele (hb\(^{9K57}\)), however, shows its phenotype only in the homozygous condition, but not when it is in trans to a hb null allele (LEHMANN and NÜSSELINK-VOLHARD 1987). In the following we want to show that the different phenotypes are in fact caused by effects at different levels of the segmentation gene hierarchy. The two classes will be discussed in turn.

The finding of ectopic anterior expression of kni in the neomorphic alleles of the first class suggests that their protein product interferes in some way with the function of the maternal hb product. The reason to assume this is that it is clear from previous experiments that the maternal expression of hb alone is sufficient to prevent high levels of kni expression in the anterior region (HÜLSKAMP et al. 1990; STRUHL et al. 1992). Thus, since embryos zygotically mutant for one of the neomorphic alleles still carry a wild-type copy of the maternal hb, we must assume that there is an interference of the neomorphic alleles with the function of this protein. To support this conclusion, we have sought to create another mutant combination where this effect can also be observed. nos controls abdominal segmentation by repressing the translation of the maternal hb RNA. Thus, in embryos lacking nos activity, maternal HB protein is expressed in the posterior region where it acts as a repressor of abdominal segmentation. We found previously that this repression effect is weakened in nos mutant embryos that are at the same time maternally heterozygous mutant for hb (HÜLSKAMP et al. 1989). However, this rescue effect is only observed at high temperatures (29°). At 18°, embryos derived from mothers homozygous for nos and heterozygous for an amorphic hb allele show the nos phenotype. In contrast, when hb\(^{9K57}\) was tested in a nos mutant background at 18°, all embryos developed three abdominal segments (not shown), suggesting that the activity of the maternal wild-type copy of hb was partly suppressed by this neomorphic hb allele.

This interference of the neomorphic alleles with the maternal function of hb would explain why the head defects are more severe in these alleles than in amorphic alleles. However, it cannot explain the neomorphic phenotype itself, since this is independent of a maternal wild-type copy of hb (LEHMANN and NÜSSELINK-VOLHARD 1987). The neomorphic effect is dependent on BX-C gene expression (LEHMANN and NÜSSELINK-VOLHARD 1987) and could therefore partly be due to a misregulation of the respective genes, which can indeed be observed (WHITE and LEHMANN 1986). However, this misregula-
tion occurs only at late stages, but is not yet evident at blastoderm stage (result not shown). Yet, even this observation can not sufficiently explain the observed phenotype and a full explanation must therefore remain open at this point.

The nature of the *hb*\(^{K49}\) allele is very different from that of the two neomorphic alleles. At the cuticle level, homozygous *hb*\(^{K49}\) embryos lack the meso- and metathorax and an additional segment A1 is formed in many, but not all embryos. The formation of this additional segment is dosage dependent, since it can not be seen when the allele is in trans to a null allele of *hb* (LEHMANN and NÜSSELIN-VOLHARD 1987). The phenotypic effect of the allele can be traced back to the level of the pair-rule and segment polarity genes. As shown in Figure 5, the expression of the pair-rule genes *eve*, *ftz* and of the segment polarity gene *en* are affected, whereby the effect is somewhat variable, similar as it is seen at the cuticle level. In the case of *eve*, embryos mutant for *hb*\(^{K49}\) either lack the third *eve* stripe completely, or express it as a weak and slightly shifted stripe (Figure 5, B and C). The complementary pattern is seen for *ftz* where embryos mutant for *hb*\(^{K49}\) leave a partly fused second and third *ftz* stripe leaving sometimes a small gap in between (Figure 5G). In the case of *en*, the fifth stripe is lacking in some embryos, but is visible in others, though it is slightly disturbed (Figure 5, D-F). A further effect of this allele is seen at the level of *Ubx* expression, where the PS6 stripe expression appears to be broadened toward anterior (Figure 5H). This is best seen in the double staining with *ftz*, which shows that the gap between *ftz* stripes 2 and 3 is filled by *Ubx* in *hb*\(^{K49}\) mutant embryos (Figure 5I) and thus occupies now both PS5 and PS6. Finally, we find that the PS4 stripe of *hb* is also shifted toward anterior in this allele, as it is evident from the double staining with *eve*. Normally, the *hb* PS4 stripe is located exactly between *eve* stripes 2 and 3 (Figure 5A), but overlaps *eve* stripe 2 in *hb*\(^{K49}\) mutant embryos and thus lies formally now in PS3. We have tested the possibility that the effect of the *hb*\(^{K49}\) allele may be connected with the secondary expression of *hb* in the PS4 stripe. We have therefore devised a P element rescue construct that would express this allele under the normal *bcd* dependent promoter, but not in the PS4 stripe. We found that this construct (*p9K57*) leads to a partial rescue in the anterior region, i.e., the gnathal segments and T1 of the amorphic *hb* phenotype, but does not lead to the duplication of A1, even not when two doses are present. Note that this behavior is different from the one observed with the *p9K57* construct, which does show the same neomorphic phenotype as the respective allele (compare Table 1).

In view of these results, we interpret the phenotype of *hb*\(^{K49}\) as follows. The mutant HB protein from this allele causes in some way a disturbance of the phasing of both, the *hb* PS4 and the *Ubx* PS6 stripe, but does not affect the primary setting of the pair-rule stripes. However, once the HB protein in the PS4 stripe becomes expressed, it appears to be capable of locally reestablishing a segmental subdivision leading to the weak third *eve* stripe. The new segment would now be covered by the *Ubx* expression domain and assumes therefore an A1 quality. This interpretation would suggest that the initial setting of the parasegmental boundaries and the setting of the expression domains of the homeotic genes may not be as stringently coupled as it is usually assumed (see for example MÜLLER and BIENZ 1992). **Stability of the HB protein:** We have analyzed the different *hb* alleles whether they might have a differential

### Figure 5.—Regulation of *hb* dependent genes in *hb*\(^{K49}\). (A) Wild type; double *in situ* hybridization with a *hb* and an *eve* probe. The PS4 stripe of *hb* lies exactly within the space between the second and third *eve* stripe. The posterior *hb* domain serves as a control for the relative staining intensities. (B) *hb*\(^{K48}\), double *in situ* hybridization with a *hb* and an *eve* probe. Note that the PS4 stripe of *hb* is shifted to a more anterior position, now lying on top of the second *eve* stripe. (C) *hb*\(^{K49}\); some mutant embryos show ventrally a third *eve* stripe (labeled with arrowhead). (D-F) *en* expression pattern at blastoderm stage. (D) Wild type. (E) *hb*\(^{K49}\) mutant embryo in which the fifth *en* stripe is missing. (F) *hb*\(^{K49}\) mutant embryo with the fifth *en* stripe formed (labeled with arrowhead). (G) *hb*\(^{K49}\), *in situ* hybridization with a *ftz* probe. The second and third *ftz* stripe are still separated at the ventral side (see arrowhead) but fused at the dorsal side. (H) *hb*\(^{K49}\), *in situ* hybridization with an *Ubx* probe. (I) *hb*\(^{K49}\), double *in situ* hybridization with an *Ubx* and a *ftz* probe. The second and third *ftz* stripes appear to be fused in such double stainings, suggesting that the first *Ubx* domain spans this region (see text).
effect on protein stability, which could point to regions within the protein that might be involved in the regulation of protein turnover. For this purpose, we have generated an antiserum which should stain all alleles with the same intensity. This was achieved by producing an affinity column for the protein product of the shortest allele found (hb78) and absorbing the antiserum originally produced against the full HB protein against this column. To provide an internal standard in the staining reactions, we have also included a defined amount of KR antisera in all stainings. The relative staining of the KR and the hb antisera was balanced such that they would produce a similar signal within a certain staining time in wild-type embryos (Figure 6). The mixture of the two sera was then used to stain the different hb alleles. For most of the alleles we observed that a quarter of the embryos showed only KR staining but no HB staining and a further half showed reduced HB staining when compared to the KR domain. Surprisingly, even the allele with a single point mutation in the second finger domain (hb70) showed this behavior. Only two alleles with a point mutation in the first finger domain (hb72, hb149) and in the C box (hb9849) showed normal staining. These results suggest that the presence and the integrity of the second finger domain of hb are required for the normal stability of the protein. The two fingers at the C terminus of hb can be expected to form normally a tight globular structure, which would not be present, or would be disrupted in any of the alleles showing reduced stability. It should be noted that our experiments assay only the relative stability of hb compared to Kr, but not the absolute stability. All alleles show some residual staining with our antiserum, if stained for longer periods. Nonetheless, the experiment indicates clearly that the C terminus of the protein is in some way involved in defining the relative stability of the protein.

**DISCUSSION**

hb provides a good case to analyze the function of individual protein domains for different target genes. First, based on evolutionary comparisons, several subregions in the protein can be distinguished, namely the two separate DNA-binding finger domains and four boxes, the A, B, C and D box (Figure 1). Second, several of the regulatory interactions between hb and other segmentation genes, as well as some homeotic genes are well understood.

Most easy to explain are the effects of the amino acid replacements found in the finger domains, since there is a good structural model for zinc-fingers (Pavletich and Pabo 1991). The alleles hb70 and hb149 in the second finger domain lead to an exchange of the crucial cysteine residues which are required for binding the structural zinc ion. It can therefore be expected that the respective fingers loose their DNA-binding capabilities. Each allele would affect only one of the two fingers in the second finger domain. Nonetheless, the comparison with the phenotype of the hb110 allele, which leads to a stop codon before the second finger domain, suggests that loss of any of the two fingers in the domain results in a complete loss of the function for this domain. A similar mutation in the zinc-finger gene Kr leads also to a complete loss of function of the whole domain (Redemann et al. 1988). The alleles hb72 and hb149 affect amino acids that are likely to be directly involved in DNA recognition. They occur at positions within the α-helix of the fingers which are supposed to be in direct contact with the bases in the DNA (Pavletich and Pabo 1991). Moreover, DNA-binding experiments with the finger carrying the hb149 mutation have shown that this mutation results in a strong loss of affinity for the consensus binding site of the HB protein (Sommer et al. 1992).

The mutations in the two finger domains have different effects on their target genes. While mutations in the first finger domain cause a loss of function phenotype for all three target genes analyzed, mutations in the second finger domain affect only the Kr and hb regulation. Thus, the regulation of kni does not require an intact second finger domain. Interestingly, a similar result was
obtained for mutations in the D box, suggesting that the D box is also only necessary for the regulation of Kr and hb but not for kni. The molecular function of the D box, however, is still unclear. Although the D box is almost fully conserved as a 56-amino acid stretch in Musca domestica, no similarities to any other gene were found so far. Genetic evidence, however, suggests that this region may be involved in the mediation of protein-protein interactions. This assumption is based on the observed interference of the neomorphic hb alleles $h^bK49$ and $h^bK57$ with the maternal wild-type function of hb. This effect could either be interpreted as an indication that hb acts normally as a dimer, or that it interacts with another unknown component of the system to provide the full function. It should be pointed out, however, that this kind of “interactive” function of the D box was observed only in the absence of the second finger domain.

Our finding that most hb alleles lead to a general destabilization of the residual HB protein is somewhat surprising. The HB protein has already a fairly high turnover rate in the early embryo. A rough estimate of its half life can be obtained from the comparison of the RNA and protein expression patterns which suggests that the protein can be seen for about 1–2 h longer than the RNA, suggesting a half life 30–40 min. Such short half-lives are possibly conveyed by particular regions of the protein (Rogers et al. 1986) and we might have expected that deletion of these regions in some alleles would have resulted in a longer half life. However, we find the opposite. All alleles lead to a reduction in the half-life, even a point mutation in the second finger domain. This suggests that the regulation of half life of the protein is more due to stabilizing rather than destabilizing effects.

The different hb alleles were previously classified according to the strength and nature of their phenotype (Lehmann and Nüsslein-Volhard 1987). We show here that the molecular lesions found in the respective alleles can be roughly correlated with the thus established allelic series. We have in addition conducted a systematic comparison between the cuticular phenotypes of the alleles and their effects on the pair-rule genes h and ftz. This allows a more detailed description of the early events that are eventually responsible for the cuticular phenotype. We find that, at the level of the parasegments, the phenotype of the amorphic alleles and the hypomorphic alleles can best be described as a fusion of parasegments 2–6 or 4–6, respectively. This area corresponds to the region in which $h^b$ and Kr show a broad overlap of their expression domains. At early stages, this overlap is due to the overlap between the protein gradients of the two genes, rather than to the primary expression domains. A more direct overlap between the primary expression domains occurs a little later when the secondary stripe of hb in the region of PS4 is expressed. Our results shed some light on the function of this PS4 stripe on the segmentation process. Two lines of evidence suggest that the PS4 stripe is necessary to achieve the correct initiation of parasegments 4, 5 and 6. In rescue experiments with a rescue construct containing all regulatory elements for the early expression of hb but lacking the PS4 stripe, the second and third ftz stripes are not completely resolved and the third h stripe is either very weak or missing. A very similar result on the pair-rule gene regulation was seen in the $h^bK49$ allele in which the specification of parasegment 5 depends on the presence of the PS4 stripe of hb. Although a functional requirement of the PS4 stripe for the initiation of parasegments 4, 5 and 6 is suggested by these experiments, it may not be the exact position of this stripe rather than a general local increase of hb activity in a critical region, namely the overlap between the hb and Kr expression domains that confers the effect. Such a more general requirement of hb activity is also supported by the fact that constructs providing only the bed-dependent expression of hb still show a low frequency of completely rescued embryos (see Lukowitz et al. 1994).

Molecular analysis of the enhancer elements of the Ubx gene have shown that hb acts as a direct regulator on these elements (Zheng et al. 1991; Qian et al. 1991; Müller and Bienz 1992; Qian et al. 1993). It would therefore be conceivable that the effect of the neomorphic alleles on the misregulation of Ubx (White and Lehmann 1986) is a direct one. However, an effect on Ubx expression is only seen at the extended germ band stage where the blastodermal hb protein has presumably already decayed. Qian et al. (1993) discuss the possibility that the initial repression effect of hb on Ubx expression becomes locked into this state by Pc group genes. In this scenario one could expect that there exists at least a transient stage, where hb itself interacts directly with products of these genes and it seems conceivable that this interaction is in some way affected by the residual protein products produced from the two neomorphic alleles. However, the detailed mechanism of how this may be achieved must remain open at this point.

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**LITERATURE CITED**


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