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Krüppel target gene *knockout* participates in the proper innervation of a specific set of *Drosophila* larval muscles

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***Krüppel* (*Kr*) encodes a zinc finger-type transcription factor expressed in spatially and temporally restricted patterns during *Drosophila* embryogenesis. Molecular targets and the mechanism of *Kr* action have been studied extensively during the early segmentation process, but only little is known about *Kr* requirement during later development. We report the identification of a target gene of *Kr*, termed *knockout* (*ko*), isolated by virtue of Krüppel *in vitro* binding sites. Loss and gain of function experiments show that *Kr* activity maintains *ko* expression in a subset of muscles. *ko* encodes a novel protein expressed in several embryonic tissues including *Kr*-expressing muscles. Movements of embryos deficient for *ko* activity are uncoordinated. Their muscle pattern is normal, but the patterns of neuromuscular innervation are specifically disarranged. The results suggest that the *Kr* target gene *ko* is required for proper innervation of specific muscles by RP motoneurons.**

Keywords: *Drosophila*/embryo/*knockout*/*Krüppel*/muscle

Introduction

The *Drosophila* gene *Krüppel* (*Kr*) was initially identified as a member of the gap class of segmentation genes. It encodes a zinc factor binding to the consensus sequence AAAA$^{C}/_{G}$GGGTTAA (Pankratz *et al.*, 1989; Stanojevic *et al.*, 1989; Treisman, 1989). In both tissue culture and *in vitro* transcription studies, the *Kr* protein (Krüppel) was shown to act as a concentration-dependent activator and repressor of transcription when acting from a single binding site close to a basal promoter (Sauer and Jäckle, 1991). At low concentrations, the Krüppel monomer causes activation while Krüppel dimers, formed at high concentrations, function as repressors (Sauer and Jäckle, 1993). *In vivo* studies indicated that Krüppel functions mainly as a repressor of adjacently expressed gap genes and of subordinate pair-rule genes within the segmentation gene cascade (Howard and Struhl, 1990; Stanojevic *et al.*, 1991; Klingler and Gergen, 1993; Pankratz and Jäckle, 1993) by acting on far-upstream enhancer elements. In addition, *Kr* activity plays a role in the development of the larval visual system (Schmucker *et al.*, 1992), the kidney-like Malpighian tubules (Gaul and Weigel, 1991), the central nervous system (CNS) (Romani *et al.*, 1996), the stomatogastric nervous system (Gonzalez *et al.*, 1995) and the muscle pattern of the embryo (Ruiz-Gomez *et al.*, 1997).

The body wall musculature of *Drosophila* embryos and larvae consists of a stereotyped arrangement of 30 muscles in each of the abdominal hemisegments A2–A7, with minor variations in more anterior and posterior segments (reviewed in Bate, 1990, 1992, 1993). Each muscle is a syncytium which is generated as neighboring myoblasts fuse to form a multinucleate muscle precursor which then differentiates to form a mature muscle fiber. While all muscles share a set of general muscle properties such as contractile proteins, receptors for neurotransmitters and the capacity to form epidermal attachment sites, each muscle has unique properties which are acquired by the contributing myoblasts and manifested as each muscle precursor differentiates to form a particular muscle fiber. There is good evidence that the acquisition of muscle-specific properties during the process of myogenesis depends on the prior specification of a special class of mesoderm-derived myoblasts called founder cells. During normal embryogenesis, founder cells express genes which are characteristic of distinct muscle subsets (reviewed in Abmayr *et al.*, 1995). Among the genes so far described as being expressed in founders are *Kr* and its likely target *S59* (Ruiz-Gomez *et al.*, 1997). *Kr* is necessary for the acquisition of ventral acute muscle 2 (VA2) characteristics (Ruiz-Gomez *et al.*, 1997). Here we show that *Kr* is also necessary for normal muscle innervation by acting in the temporal control of a newly identified target gene required for motoneuron projection in the muscle field.

In *Drosophila*, most if not all of the muscle fibers of the abdominal segments A2–A7 are innervated by one or only a few motoneurons (reviewed by Goodman and Doe, 1993). The stereotyped projection pattern of motoneurons, which rests on the ability of motoneuron growth cones to find and recognize their correct muscles, can be observed with a single antibody reagent (van Vactor *et al.*, 1993). This antibody mAb 1D4 labels all of the motoneuron branches expressing the transmembrane form of fasciclin II (Grenningloh *et al.*, 1991). Motoneurons exit the CNS via two pathways: the segmental (SN) and the intersegmental (ISN) nerve roots (Sink and Whitington, 1991). The ISN projects to the dorsal muscles, whereas the four SN branches SNa, SNb, SNc and SNd project to distinct groups of lateral and ventral muscles. The growth cones that pioneer each of these nerve routes and peripheral motor nerve branches make stereotyped contact with a variety of cell surfaces. As they navigate out of the CNS, they pass through the exit junction where they encounter a specialized glial cell, the exit glia. At this junction, four so-called RP axons leave the ISN and switch to the SNb branch which innervates the group of ventral lateral muscles (VL). The RP1 and RP4 motoneurons innervate

the ventral lateral muscle 2 (VL2), the RP3 motoneuron innervates VL3 and VL4, and the RP5 motoneuron innervates VL1. Their growth cones initially contact and extend processes over a number of inappropriate muscles as well as contacting their correct targets. The incorrect processes are later withdrawn, generating the mature pattern of neuromuscular innervation (Sink and Whitington, 1991) by late embryonic stage 16 (stages according to Campos-Ortega and Hartenstein, 1985).

Using a molecular approach, we have identified a *Kr* target gene, termed *knockout* (*ko*). It encodes a protein of novel sequence. *ko* is expressed from gastrulation onwards in a number of different tissues including a subset of *Kr*-expressing muscles. *ko* expression is initiated normally in these muscle cells when they fail to express *Kr*. However, *ko* expression is not maintained in these cells and terminates prematurely. In the absence of *Kr* and/or *ko* expression, RP neurons fail to innervate ventral lateral muscles normally. The results indicate that *Kr* activity is required to maintain *ko* expression in specific muscles and that *ko* activity is required for the proper innervation of a subset of muscles.

Results

Isolation of putative Kr target genes

Recent studies on embryonic muscle development revealed that *Kr* activity is required for the acquisition of specific muscle characteristics (Ruiz-Gomez *et al.*, 1997). In order to identify putative direct target genes of Krüppel which are expressed during muscle development, we performed a search which was based on the DNA-binding properties of the Krüppel zinc finger domain. To this end, *Drosophila* genomic DNA was digested with *Hae*III, ligated to PCR primers, incubated with recombinant Krüppel and co-immunoprecipitated with anti-Krüppel antibodies. The precipitated DNA fragments were amplified by PCR. After five rounds of repeated PCR amplifications and immunoprecipitations, the DNA fragments (between 70 and 350 bp in length) were cloned and sequenced (for details see Materials and methods). The cloned DNA fragments containing multiple Krüppel *in vitro* binding sites were used as molecular probes to isolate corresponding λ phage and cosmid clones from genomic DNA libraries. We next investigated whether the cloned DNA coded for transcripts enriched in *Kr*-expressing cells. For this, we performed *in situ* hybridization experiments using the genomic DNA inserts of the λ phage isolates to probe whole mounted embryos. Out of our initial 21 candidate genes, two transcription units were identified which are expressed during myogenesis. One of them, which was termed *knockout* (*ko*), was found to be expressed in a number of *Kr*-expressing muscle precursor cells (see below).

Localization, structure and coding properties of the ko gene

Starting from a 320 bp immunoprecipitated DNA fragment which contains three *in vitro* Krüppel-binding sites (see Figure 1), we isolated >50 kb of overlapping genomic DNA fragments covering the *ko* transcription unit. *In situ* hybridization of genomic λ phage DNA to polytene chromosomes (Figure 1A) and mapping of the DNA to cytogenetically defined chromosomal deficiencies (Figure 1B) localized the *ko* transcription unit to the chromosome region 78B1,2 on the left arm of chromosome 3, in a position close to the previously identified breakpoint of the deficiency *Df(3L)Pc-cp2* (Figure 1C).

In order to establish the molecular organization of the *ko* gene and the nature of the protein product, we isolated cDNA clones from various cDNA libraries (see Materials and methods). We sequenced the largest cDNA clone in its entirety and the relevant portions of the genomic DNA. As shown in Figure 1E, the *ko* transcription unit consists of five exons and four introns spanning a genomic region of at least 22 kb. Northern blot analysis with the *ko* cDNA revealed two transcripts of 5.3 and 5.1 kb (Figure 1D) which differ by alternating polyadenylation sites recovered in corresponding classes of cDNA (not shown). The longest isolated cDNA clone is 4210 bp long, suggesting that it represents an almost full-size transcript with the possible exception of the most 5′ untranslated sequences. It contains a single open reading frame (ORF) of *ko* which starts with the *Drosophila* translation start site consensus sequence CAGAAATG (Cavener, 1987).

The *ko* transcript encodes an 882 amino acid polypeptide of novel structure. The putative protein contains 10 *N*-glycosylation and 12 myristylation sites and is serine/threonine rich in the carboxy-terminal half (Figure 2A). Hydrophobicity analysis revealed hydrophobic regions in the N-terminal part of the protein (Figure 2B). No diagnostic protein motifs were found. Figure 2C shows an alignment with the amino acid sequences of the gene product encoded by a *Caenorhabditis elegans* gene F53B2.6 (Figure 2C) and an expressed sequence tag (EST) clone from a mouse heart library, both of unknown function, which indicates that the *ko*-encoded protein contains a novel evolutionary conserved amino acid sequence motif.

ko expression in a subset of muscle precursors

The embryonic expression patterns of *ko* were examined by whole-mount *in situ* hybridization experiments. Figure 3 summarizes the complex spatio-temporal expression patterns of *ko* during embryogenesis, which is initially expressed in stage 10 embryos (Figure 3A). Between stage 10 and 15, *ko* expression is found in a number of tissues and organs, including the pharynx and the esophageal region, cell groups in the developing CNS, the distal part of the Malpighian tubules (not shown) and the dorsal vessel (see Figure 3B–H and legend to Figure 3). In addition, *ko* expression is observed in muscle precursor cells which can be identified by their shape and position from stage 14 onwards. They include the precursors of DA1, DO1, DO4, DT1, LT1–3, VT1, VL1–4 and the ventral acute muscle VA2 in each of the abdominal hemisegments (see Figure 3C–G). While *ko* expression in the precursors of VL2–4 is transient and disappears during stage 15, it persists in the other muscle precursors until mature muscle fibers have been formed (Figure 3G; see also below).

The *cis*-acting regulatory elements responsible for the spatial expression of *ko* during embryogenesis were identified by generating transgenes containing the β-galactose reporter gene under the control of genomic DNA fragments of the *ko* gene (Figure 4A). As summarized in Figure 4,

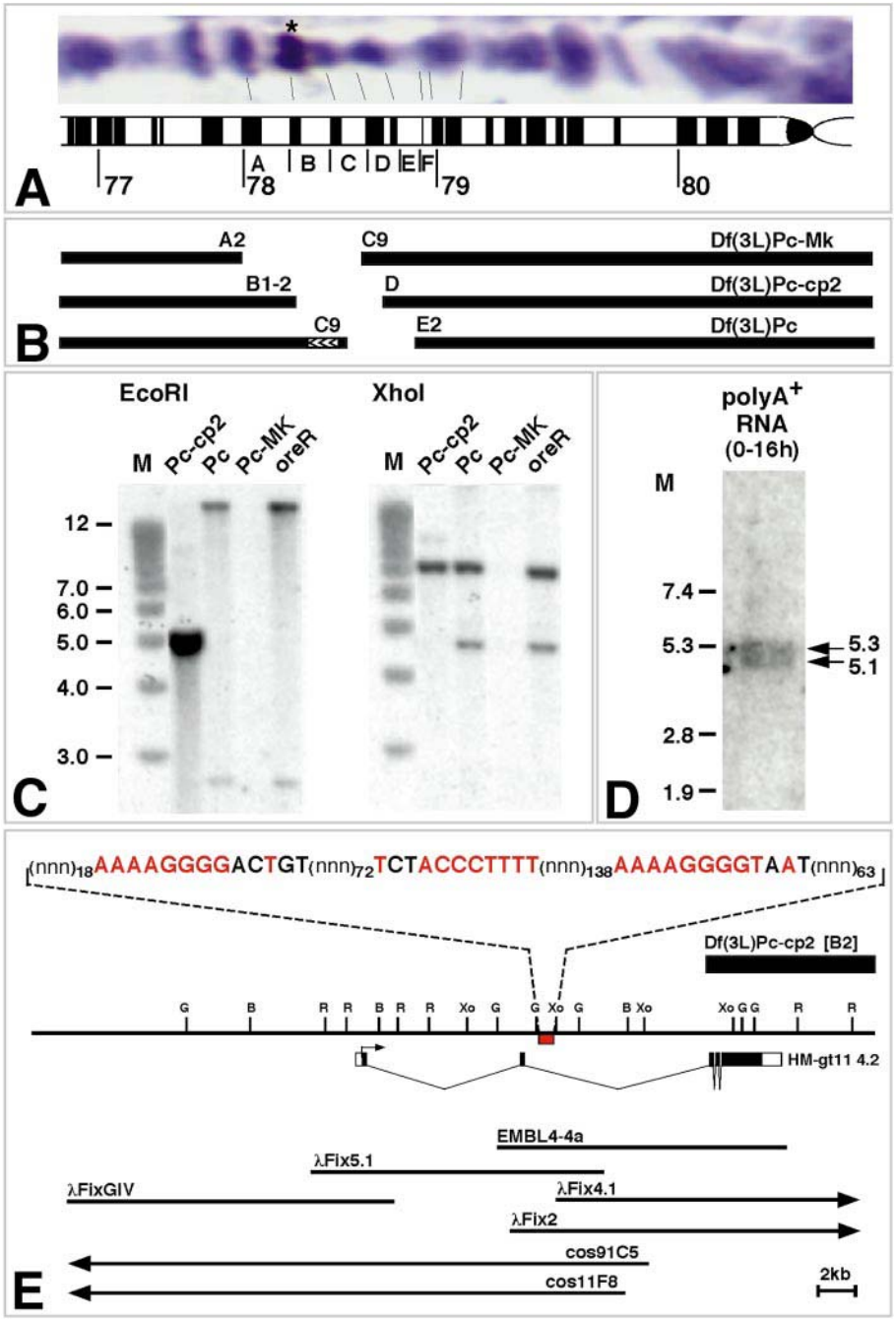


Fig. 1. Chromosomal localization and structure of the *knockout* transcription unit. (**A**) *In situ* hybridization of the EMBL4-4a DNA to polytene chromosome indicating that the *ko* gene is localized in the region 78B on the left arm of the third chromosome (asterisk). (**B**) Breakpoints of various deficiencies in the chromosomal region 78B. (**C**) Southern blots with genomic DNA of homozygous embryos of different genotypes (digested with *Eco*RI and *Xho*I), hybridized with the *Eco*RI insert of the cDNA clone HM-gt11 4.2. Note that the distal breakpoint of the deficiency *Df(3L) Pc-cp2* is located within the *ko* transcription unit, and that the *ko* transcription unit is covered by the deficiency *Df(3L) Pc*, but not by the deficiency *Df(3L) Pc-Mk*. (**D**) Northern blot showing that *ko* encodes two transcripts (5.1 and 5.3 kb; probe: 3.0 kb *Stu*I fragment of clone HM-gt11 4.2), which differ by alternative polyadenylation sites. (**E**) Genomic DNA of cosmids (cos; Hoheisel *et al.*, 1991) and λ clones (λFix, EMBL4) covering the *ko* gene. Red bar: location of the Krüppel-binding sites (see Materials and methods); abbreviations: B, *Bam*HI; G, *Bgl*II; R, *Eco*RI; Xo, *Xho*I. The sequences of the *in vitro* Krüppel-binding sites (confirmed by footprint analysis; not shown) are shown in upper case. Nucleotides matching the consensus sequence are indicated in red. Note that the *ko* transcription unit consists of at least five exons spanning a region of ~22 kb. The open reading frame is indicated by filled bars, the initiation codon ATG is indicated by the arrow, untranslated 5′ and 3′ regions by open bars. The distal breakpoint of *Df(3L) Pc-cp2* (black bar) is localized in the 3′ region of the second intron, extending towards the centromere and uncovers the transcriptional and translational start site of the *ko* transcription unit. Note that the orientation of the genomic organization of the *ko* transcription unit shown in (E) is inverted in comparison with (A) and (B).

the *cis*-acting regulatory region of the *ko* gene is composed of a modular array of separate *cis*-acting elements (Figure 4A) conducting reporter gene expression that covers various aspects of the embryonic *ko* expression patterns (Figure 4B and C), except for the muscles. The *cis*-acting elements controlling muscle expression (Figure 4D and E) must be spread over the 15 kb of DNA contained within the 15 N/Sp–*lacZ* reporter gene (Figure 4A),

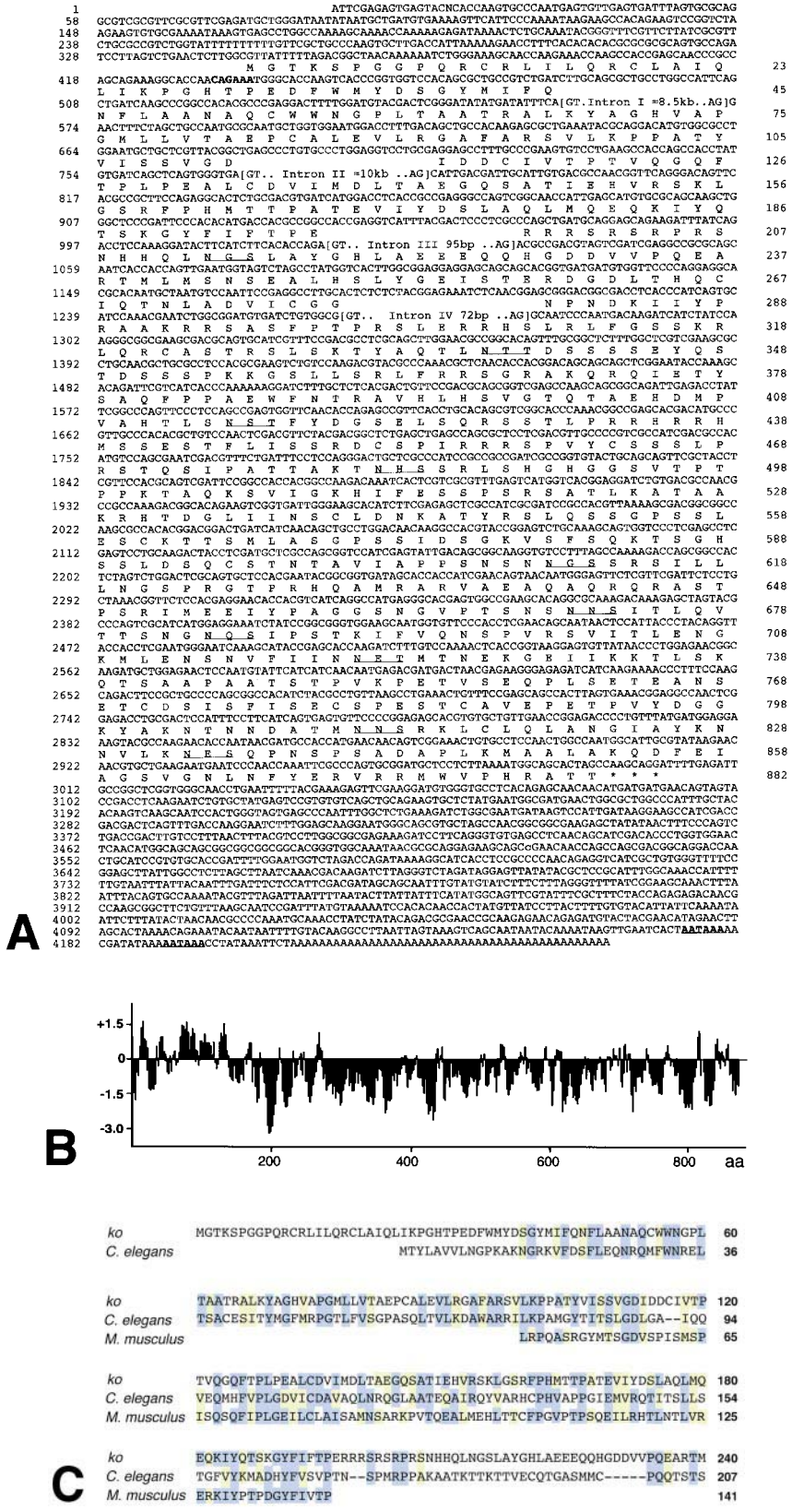


Fig. 2. (**A**) Nucleotide sequence of the cDNA clone HM-gt11 4.2 and the deduced amino acid sequence of the Ko protein. The sequence of the translational start site is shown in bold letters. The alternatively used polyadenylation sites are underlined and in bold. The positions of the introns I–IV are indicated by brackets, and 10 putative *N*-glycosylation sites of the Ko protein are underlined. (**B**) Analysis of the Ko amino acid sequence with the Kyte–Doolittle hydrophobicity plot (+, hydrophobic; –, hydrophilic) showing that the N-terminal region of the Ko protein is hydrophobic. (**C**) Alignment of the amino acid sequence of Ko with the gene product of the *C.elegans* gene F53B2.6 (DDBJ/EMBL/GenBank accession No. Z73908) and the putative protein encoded by a mouse EST clone (DDBJ/EMBL/GenBank accession No. AA138518). Note the conserved region in the N-terminal part of the Ko protein. Regions of conserved amino acids between the proteins are indicated by blue boxes, similar amino acids by yellow boxes.

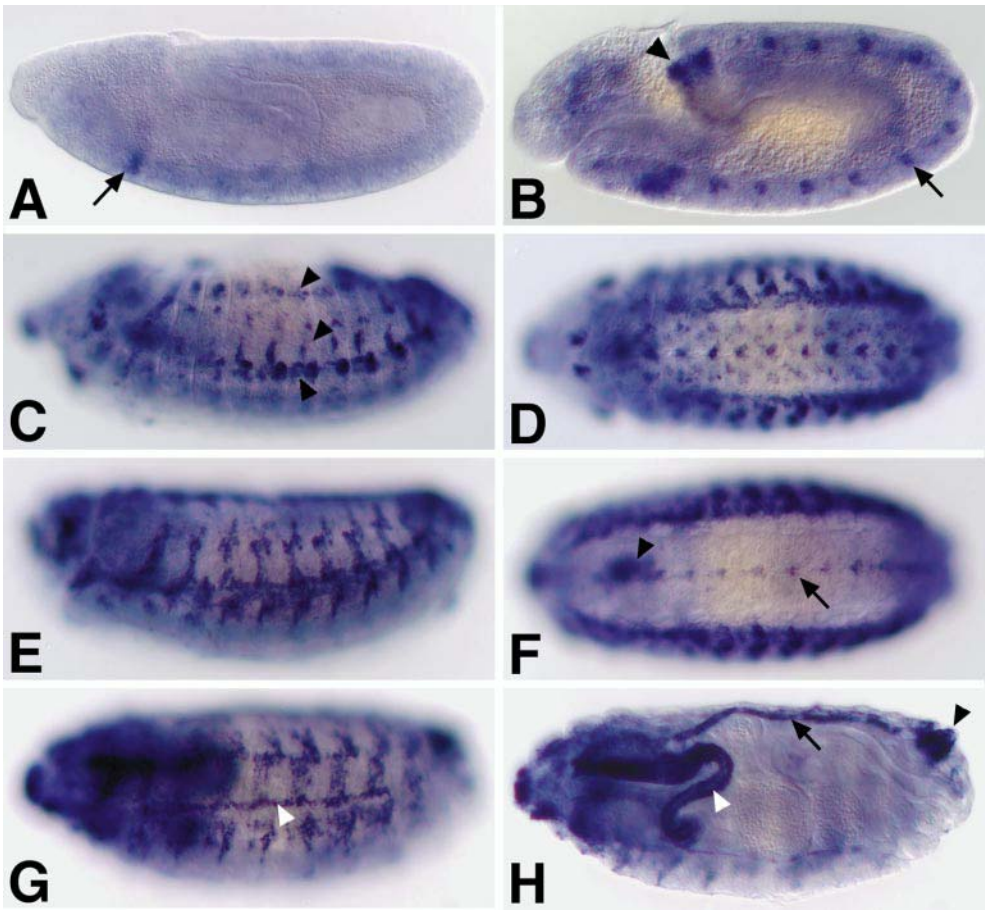


Fig. 3. Expression pattern of *ko* in wild-type embryos at various stages of embryogenesis examined by *in situ* hybridization. (**A**) Initial *ko* expression in a ventro-lateral stripe on each side of the procephalic region at stage 10 (arrow). (**B**) *ko* expression in a segmentally repeated manner along the extended germband (arrow) and in the hindgut at stage 11 (arrowhead). (**C**) *ko* expression in a subpopulation of muscle precursor cells of dorsal, lateral and ventral muscles at stage 14 (arrowheads). (**D**) *ko* expression in a subset of cells of the CNS at stage 14. (**E**) *ko* expression in the larval musculature at mid stage 15. (**F**) *ko* expression in the CNS becomes restricted to cells of the midline at stage 15 (arrow) and in the esophagus (arrowhead). (**G**) *ko* expression in the larval musculature at stage 16. Note that *ko* transcripts are expressed at this stage only in one of the ventral longitudinal muscles (VL1, white arrowhead) forming a lateral row along the embryo. (**H**) *ko* expression in the pharynx and the esophagus (white arrowhead), the dorsal vessel (arrow) and in the posterior spiracles (black arrowhead). Lateral views of embryos in (A), (B), (C), (E), (G) and (H); dorsal side is up, ventral down. Ventral views of embryos in (D) and (F). Embryos are oriented anterior to the left, posterior to the right.

since smaller subfragments failed to drive reporter gene expression in muscles.

Kr-dependent maintenance of activated ko expression in muscle precursor cells

During muscle development, *Kr* is expressed in DA1, DO1, LL1, LT1 and 2, LT4, VL2–4, VA2, VO2, VO5 and DT1 (Ruiz-Gomez *et al.*, 1997). The expression of *Kr* and *ko* therefore overlaps in the precursors of muscles DA1, DO1, LT1, LT2, DT1, VL2–4 and VA2. We next asked whether *ko* expression indeed depends on preceding *Kr* activity. For this, we examined the *ko* expression pattern in embryos which lack *Kr* activity. However, *Kr* lack-of-function alleles cause strong segmentation defects and, thus, any muscle defects observed in *Kr* embryos could be a consequence of the abnormal segmentation process. To circumvent this difficulty in assessing the role of *Kr* for the expression of *ko* during muscle development, we examined the muscle pattern in recently described '$Kr^{CD+}Kr^{1}$ embryos', which carry a *Kr* transgene providing early *Kr* expression corresponding to the *Kr* segmentation function. Provision of the transgene specifically rescues the segmentation defect of *Kr* lack-of-function mutants (Romani *et al.*, 1996) and thereby permits the study of segmentation-unrelated aspects of *Kr* requirement.

In $Kr^{CD+}Kr^{1}$ embryos, we found that *ko* expression was always strongly reduced in the group of precursors giving rise to muscles LT1, 2 and 3 (compare Figure 5A and B). From mid-stage 15 onwards, however, *ko* expression is observed in only one of the LT muscles and is absent from VA2 (compare Figure 5C and D). Thus, lack of *Kr* activity consistently causes the premature termination of *ko* transcription in the VA2 precursor and in two LT muscles, and affects *ko* expression in other muscle precursors variably. The latter observation suggests that although *Kr* is required to maintain *ko* expression in a distinct subset of muscles, it is also necessary in others where its role is not as decisive.

We also performed gain-of-function experiments expressing *Kr* ectopically under heat shock control (for details see Materials and methods). Embryos which were heat shocked at stage 14 showed ubiquitous Krüppel expression (data not shown). In such embryos, we found that *ko* expression is maintained in two VL muscle precursors

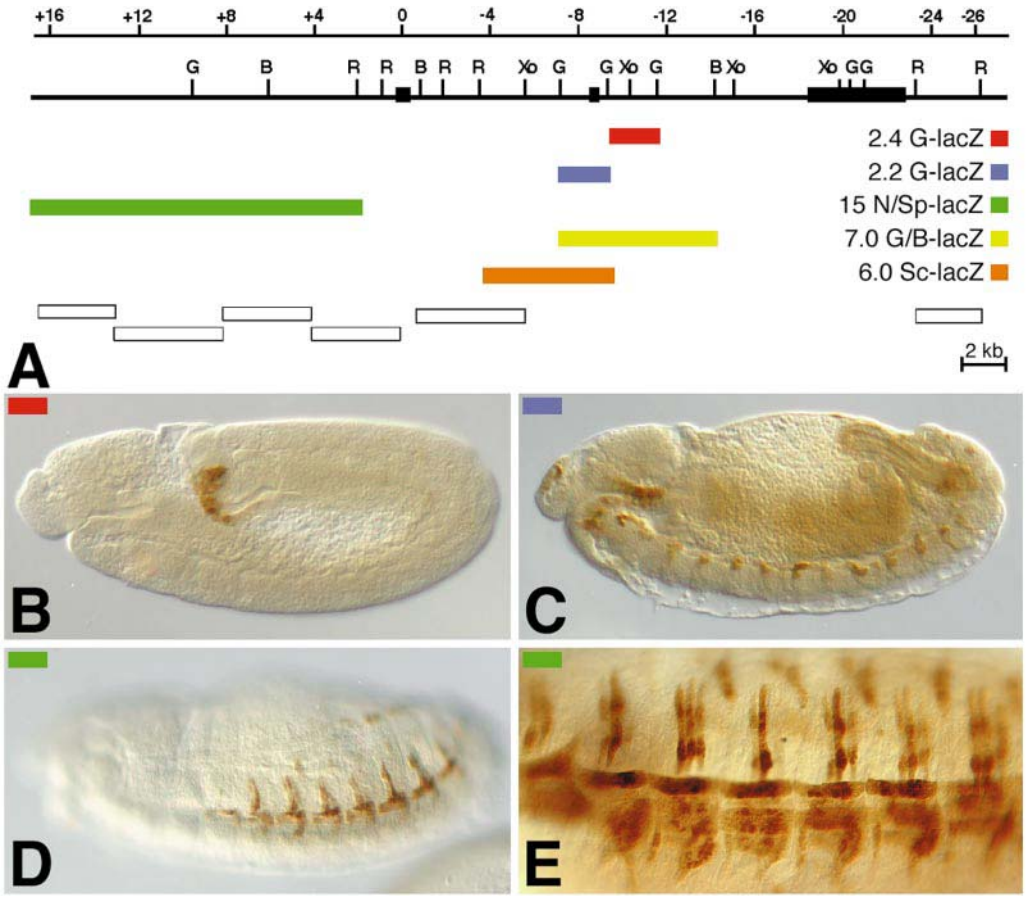


Fig. 4. Reporter gene constructs and their corresponding β-galactosidase expression patterns. (**A**) Schematic overview showing the localization of genomic DNA fragments of different reporter gene constructs. Colored bars indicate genomic regions driving reporter gene expression in tissues expressing *ko*. The reporter gene 7.0 G/B–*lacZ* drives a β-galactosidase expression pattern similar to the 2.4 G–*lacZ* construct, but does not display expression in the CNS. The spatial aspects of the β-galactosidase expression pattern of the reporter gene 6.0 Sc–*lacZ* is similar to that of 2.2 G–*lacZ*, but starts slightly later at stage 14. Open bars indicate DNA fragments which fail to drive reporter gene expression. For abbreviations, see Figure 1. (**B**) β-Galactosidase expression pattern of the reporter gene 2.4 G–*lacZ* (including the Krüppel-immunoprecipitated sequences) in the invaginating proctodeum in a stage 11 transgenic embryo. (**C**) β-Galactosidase expression pattern of the reporter gene 2.2 G–*lacZ* (contains three *in vitro* Krüppel consensus binding sites) in the esophageal region and the CNS at stage 13. (D and E) β-Galactosidase expression pattern of the reporter gene 15 N/Sp–*lacZ* in dorsal, lateral and ventral muscles at stage 14 (**D**) and at stage 16 (**E**) showing β-galactosidase expression in the abdominal segments in the lateral and ventral muscles DT1, DO5, LT1–3, VL1–4, VA2 and VT1.

during stage 15 (compare Figure 5E and F). We detected no other deviation from the wild-type pattern of *ko* expression. Thus, both the gain-of-function and lack-of-function experiments are consistent with the argument that *Kr* is required to control the temporal aspects of *ko* transcription by maintaining expression that has been activated in response to one or several other transcription factor(s).

Normal development of motor innervation pattern requires ko activity

In order to assess *ko* function during muscle development, we examined the phenotype of *ko*-deficient embryos (see Materials and methods). Such embryos develop into normal looking larvae, which fail to hatch. Larvae removed from the vitelline membrane are immobile but respond with slow, uncoordinated movements to pokes with a needle. The larval muscle pattern is indistinguishable from wild-type (data not shown), suggesting that the uncoordinated movements of the mutants are caused by factors other than improper muscle development. We therefore examined the patterns of muscle innervation in wild-type and *ko*-deficient embryos using antibodies directed against fasciclin II (van Vactor *et al.*, 1993). Figure 6 summarizes the finding that the lack of *ko* activity generates specific innervation defects of the VL muscles.

In wild-type, the muscles VL1–4 are innervated by the segmental nerve branch SNb, composed of the axons of the motoneurons RP1, RP3, 4 and 5. The SNb nerve follows a stereotyped pathway into the ventral muscle field (see Figure 6A and B). Along its path, it contacts two choice points, one between muscles VO3 and VO2 and a second one near muscle VO1, where the distal SNb growth cones shift their trajectory to extend along the interior side of the internal longitudinal muscle layer. At late stage 16, the final motoneuronal innervation pattern is established, with the axons of the RP3 motoneuron arborizing in the cleft between muscles VL4 and VL3, the RP1 and the RP4 motoraxons branching at the proximal edge of muscle VL2 and the RP5 axons innervating muscle VL1 (Figure 6A–E; van Vactor *et al.*, 1993).

In *ko*-deficient embryos, the initial path of the SNb nerve is normal. It reaches its second choice point.

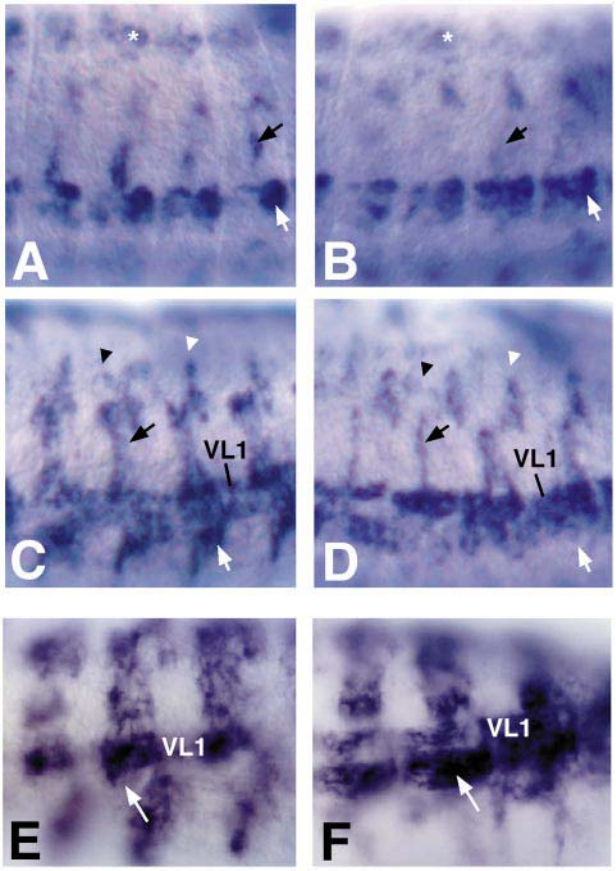


Fig. 5. Expression pattern of *ko* in the muscle precursors of wild-type (A, C and E) and $Kr^{CD+}Kr^{1}$ (B and D) embryos and its alteration in response to ectopic *Kr* expression (F) shown by *in situ* hybridization. (**A**) Late stage 14 embryo showing the pattern of wild-type *ko* expression in the precursors of the two dorsal muscles DA1 and DO1 (asterisk), the lateral transverse muscles (LT; black arrow), the ventral longitudinal muscles (VL) and the ventral acute muscle VA2 (white arrow). (**B**) Corresponding patterns in the $Kr^{CD+}Kr^{1}$ embryo. Note a reduction of *ko* expression in the precursors of the dorsal muscles (asterisk), the LT muscles (black arrow) and the VA2 muscle (white arrow). (**C**) Early stage 15 embryo showing wild-type *ko* expression in the muscles DT1 (white arrowhead), DO4 (black arrowhead), LT1–3 (black arrow), VA2 (white arrow) and the ventral longitudinal muscles (VL1–4). Note the lower amounts of *ko* transcripts in the muscles VL2–4 as compared with VL1. (**D**) Corresponding *ko* expression pattern in $Kr^{CD+}Kr^{1}$. Note that *ko* expression is reduced in the muscles LT1–3 (black arrow) and absent from VA2 muscles (white arrow). (**E**) *ko* expression in a late stage 15 control embryo which was heat shocked (see Materials and methods) during stage 13/14. As in wild-type, *ko* transcripts can be detected in the muscles DT1, LT1–3, VA2 and VL1 and are absent from the more ventral longitudinal muscles VL2–4 (white arrow). (**F**) Corresponding *ko* expression in an embryo which received heat shock-induced ubiquitous *Kr* transgene expression (see Materials and methods) during stage 13/14. *ko* expression is consistently maintained at a high level in at least one of the more ventral longitudinal muscles such as VL3 (white arrow).

However, after having contacted muscle VO1, it ends and, consequently, it fails to innervate the *ko*-expressing VL1 muscles properly via the RP1 motoraxons. In addition, RP3 motoraxons fail to innervate muscles VL3 and 4; occasionally, small neurites are observed to contact the cleft between VL4 and VL3 (Figure 6F–H). No additional defects in the innervation pattern were apparent and no aberrations were found in the CNS or the peripheral nervous system (PNS) as revealed by mAb BP102 (Seeger *et al.*, 1993) and mAb 22C10 antibody stainings (Fujita *et al.*, 1982). This suggests that *ko* activity is particularly important for the establishment of the SNb projection beyond the second choice point.

ko-deficient embryos were generated by using the chromosomal deficiency *P[Pc^{+}] Df(3L)Pc-cp2* (see Materials and methods). To show that the muscle innervation defect was caused by the lack of *ko* and not by the lack of other genes uncovered by the deficiency, we performed *ko* rescue experiments with the deficiency mutant embryos. Unfortunately, the transcribed region of the *ko* gene is already >22 kb in length (see above) and thus unsuitable for performing P-element-mediated germ line transformation. To overcome this problem, we expressed a *ko* cDNA-containing transgene under the control of the heat shock-inducible *hsp70* promotor in homozygous *P[Pc^{+}] Df(3L)Pc-cp2* embryos and monitored whether transgene-dependent *ko* activity provided by heat shock pulses during stage 13/14 (see Materials and methods) would rescue the innervation defect of the VL muscles specifically. In 58% of 72 examined hemisegments of 12 homozygous stage 16 *P[Pc^{+}] Df(3L)Pc-cp2* embryos containing the hsp70-*ko*$^{+}$ transgene, the innervation of VL muscles by the SNb neurons was found to be intact (Figure 7). Since VL muscle innervation in homozygous *Df(3L)Pc-cp2* embryos was 100% defective in the absence of the *ko*-expressing transgene (see above), this result indicates that the defective innervation of the deficiency mutants is due to the lack of *ko* activity, and that *ko* activity is necessary during stage 13/14 when the transgene is expressed. To substantiate this point further, we performed 'antisense' experiments by expressing *ko* antisense RNA from a heat shock-inducible transgene.

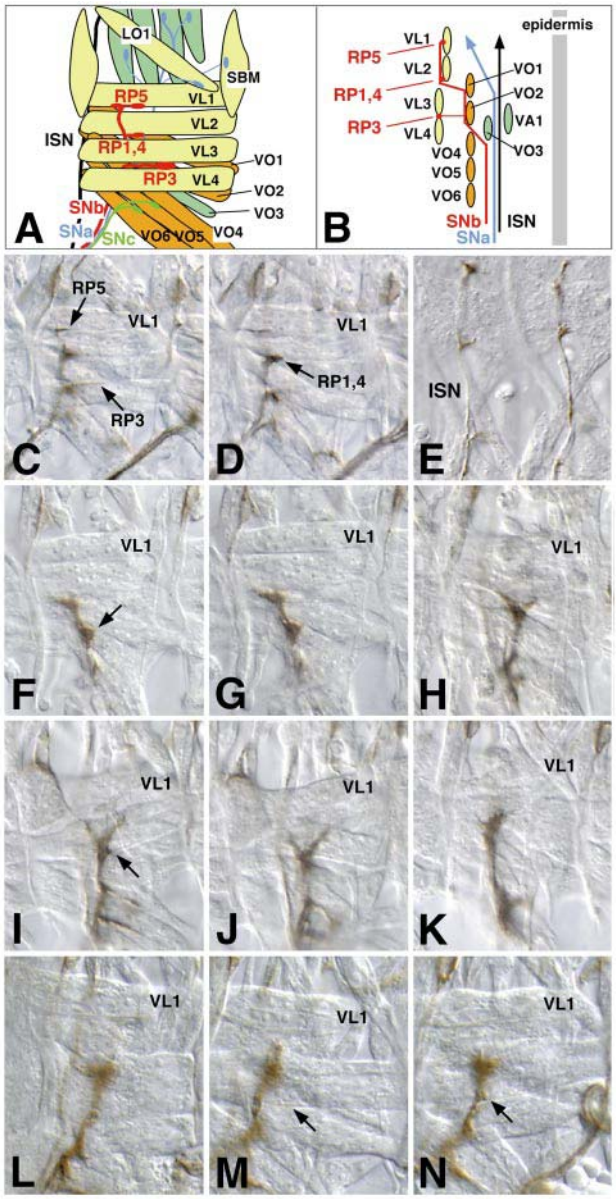
ko antisense RNA expression causes phenocopies

ko antisense RNA expression was induced by multiple heat shocks during stage 13/14 (see Materials and methods) and embryos were examined once they reached late stage 16. Figure 6I–K shows that embryos expressing the *ko* antisense RNA transgene develop the same defects as observed with *ko*-deficient embryos. However, the defects were not fully penetrant, meaning that in 177 examined hemisegments, 60% of the VL1 muscles were not innervated by RP5 axons and, in 72%, the RP3 axons failed to arborize into VL3/VL4. In heat-shocked control embryos and in heat shock-induced *ko* sense RNA-expressing embryos, only 20 and 19% of the 220 examined hemisegments showed such defects, respectively. We note that heat shock-induced ectopic expression of *ko* sense RNA did not result in axon branching or in other consistent muscle innervation defects. This suggests that although *ko* activity is necessary for proper innervation of the VL muscles, its ectopic activity is not sufficient to interfere with the normal pathfinding of the SNb motoraxons.

Since heat shock-induced *ko* antisense RNA expression causes reliable phenocopies of the *ko* mutant phenotype, we next asked whether the absence of *ko* expression in muscle precursors causes innervation defects. For this, we induced mesodermal expression of *ko* antisense RNA under the control of a Gal4 driver line (see Materials and methods). The corresponding embryos developed the same innervation defects as observed in the absence of *ko* (Figure 6L–N), although at a lower frequency. Only 45%

of the VL1 muscles were not innervated by the RP5 axon in 85 examined hemisegments; the RP3 axons failed to arborize into the cleft between muscles VL3 and VL4 in 58% of the cases.

The finding of Krüppel-binding sites within the *ko* gene and the observation that *Kr* activity is required for maintaining *ko* expression in VL muscles are consistent with the argument that *ko* is a direct target gene of Krüppel. This suggests that the absence of *Kr* causes a *ko*-like innervation phenotype involving the SNb nerve. We therefore examined the motoneuronal pattern in $Kr^{CD+}Kr^{1}$ embryos and found that the neurons RP1, RP3 and RP4 are missing in many cases (Landgraf, 1996). This observation is consistent with the finding that *Kr* acts in several genetic pathways during neural and muscle development (Romani *et al.*, 1996; Ruiz-Gomez *et al.*, 1997), suggesting that the absence of the RP neurons in *Kr* mutants involves a different and earlier primary defect than the absence of *ko* activity.



Discussion

We provide evidence that the transcription factor Krüppel acts upstream of the *ko* gene and that *ko* activity in turn is required to establish very reproducible patterns of neuromuscular contacts between motoneurons and a subset of muscles. Recent studies on muscle development have already identified the requirement for *Kr* activity in establishing distinct muscle fates (Ruiz-Gomez *et al.*, 1997). However, although *Kr* expression had been noted in the ventral lateral muscles VL2–4, no corresponding phenotype had been noted with respect to a change of these muscles in position, size or shape when *Kr* is absent, except for a consistent *Kr*-dependent transformation of the V02 muscle to a VL fate (Ruiz-Gomez *et al.*, 1997). This suggested that *Kr* activity might serve other functions different from determining the fate of muscles apparent in the pattern of muscle fibers. Our results establish that *Kr* is indeed necessary to control genes that are required to participate in the formation of specific neuromuscular contacts. The common innervation phenotype of *Kr*- and *ko*-deficient VL muscles strongly suggests that the two genes act in the same regulatory pathway, *ko* being a direct target of *Kr*. This proposal is supported by three arguments. First, we have isolated *ko* by a DNA-binding assay using Krüppel. Secondly, *ko* is expressed in a subset of *Kr*-expressing muscles. Finally, *Kr* is necessary for the maintenance of *ko* expression in the corresponding precursors.

ko mutant embryos exhibit a wild-type muscle pattern.

Fig. 6. Motoneural innervation patterns of wild-type and *ko* mutant embryos. (**A**) Schematic representation of the stereotyped innervation pattern of the SNb motoraxons showing an abdominal hemisegment at late stage 16/17. Internal muscles (yellow), intermediate muscles (orange) and external muscles (green) are shown. The motoraxons RP1 and RP3–5 of the SNb nerve are marked in red. (**B**) Corresponding cross-section showing the trajectory of SNb and the specific innervation pattern of RP1 and RP3–5. (The trajectory of the SNa and ISN nerves are indicated for reference; for details, see van Vactor *et al.*, 1993). (C–N) Photomicrographs showing abdominal hemisegments of late stage 16 filleted embryos which were stained with anti-fasciclin II antibodies. (C–E) Different focal planes of the SNb and ISN motoneuron projections in wild-type, showing the terminal arbor of the RP3 growth cone in the cleft between the VL muscles 3 and 4 and the innervation of the muscle VL1 by RP5 (**C**), the terminal arbors of RP1 and 4 on muscles VL2 and VO1 (**D**) and the dorso-lateral projections of the ISN (**E**). (F–H) SNb motoneuronal projections in *ko* mutant embryos showing the absence of a normal terminal arbor of RP3 into the cleft between muscles VL4 and 3 (arrow in **F**); the RP3 growth cones terminate on the VO2 muscles and growth cones of RP1, 4 and 5 terminate at the second choice point (**G**, see details in the text). (**H**) A different hemisegment of a *ko* mutant embryo showing a more severe phenotype; there is no arborization of the RP3 growth cone at muscle VO2. (I–K) Projections of the SNb axons after heat shock-induced expression of an antisense *ko* transgene affecting *ko* activity. (**I** and **J**) Different focal planes of the same hemisegment showing defects similar to those in *ko* mutant embryos, i.e. no arborizing of RP3 axons into the cleft between muscles VL4 and 3 (arrow in I) and the SNb growth cone splits after reaching the second choice point. (**K**) In this hemisegment, no arborizing of the RP3 motoraxons occurs; the SNb growth cone stopped at the second choice point. (**L**–**N**) Abnormalities in the projection pattern of the SNb motoraxons in three hemisegments of embryos in which the *ko* activity had been reduced in the mesoderm by expressing a UAS-antisense *ko* construct under the control of the mesoderm-specific GAL4 driver line 24B (Brand and Perrimon, 1993). No arborizing of RP3 axons occurs into the cleft between muscles VL4 and 3; occasionally small neurites can be seen (arrow in N) and the SNb growth cones stop at the second choice point.

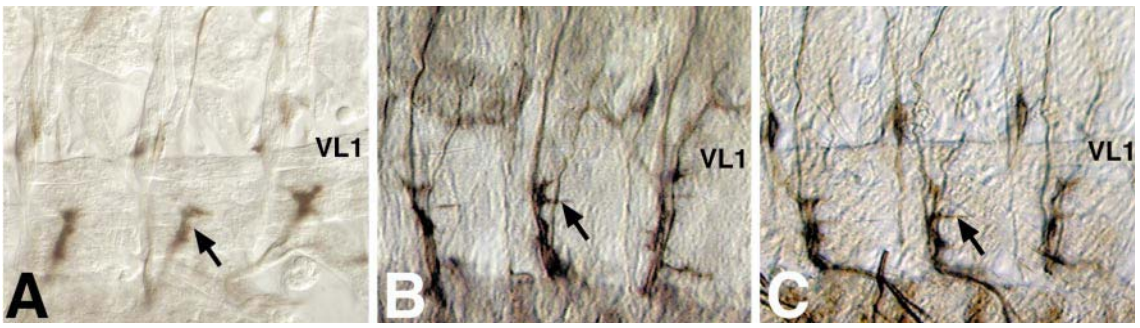


Fig. 7. Motoneuronal innervation pattern in the abdominal segments of *ko*-deficient, transgene-rescued and wild-type late embryos as revealed by anti-fasciclin II antibody staining. (**A**) SNb axonal projections in *ko* mutant embryos showing the absence of a normal terminal arbor of RP3 into the cleft between muscles VL4 and 3 (arrow) and the growth cones of RP1, 4 and 5 which terminate at the second choice point. (**B**) Restoration of the SNb projections in response to heat shock-induced ubiquitous *ko* expression. The RP3 motoraxons arborize normally into the cleft between muscles VL4 and 3 (arrow) and the RP5 growth cones innervate muscle VL1 (out of focus). (**C**) SNb axonal projections in the hemisegments of a wild-type embryo showing the terminal arbor of the RP3 growth cone in the cleft between the VL muscles 3 and 4 (arrow).

However, they develop into larvae which lack coordinated movement. This phenotype is consistent with the finding that the absence of *ko* activity and its reduction by *ko* antisense RNA expression in the mesoderm affect the innervation pattern of the muscles in a highly specific manner. No consistent *ko* mutant effects were seen with respect to the stereotyped roots and specific contacts of the intersegmental nerve and the segmental nerve branches SNa, SNc and SNd, and along the initial path of SNb, and no mutant effect was observed in the other non-neural locations where the *ko* gene is expressed. This suggests that *ko* expression might not be functional in such locations or that the lack of *ko* expression is then compensated for by the activity of other genes that may act in a redundant fashion. In contrast, the SNb nerve branch stalls after having reached its second choice point near muscle VO1, and the motoneurons fail to contact their correct VL muscle targets in both *ko*-deficient embryos and in embryos expressing *ko* antisense RNA. This indicates that *ko* activity provides the muscle cells with a unique property necessary for the recognition by or the attraction of the correct motoneurons. Although we cannot distinguish between central and peripheral effects of *ko*, the innervation patterns that we observed strongly suggest that the *ko*-dependent defect concerns exclusively some late aspect of motoneuronal and/or muscle differentiation, leading to failures of connectivity in the neuromuscular system.

Muscles and neurons are generated in remarkably similar ways, given the very different organization of the mesoderm and ectoderm from which they are derived. Both depend on the prior segregation and specification of progenitors from groups of equivalently competent neighboring cells. In the case of the muscles, these progenitors give rise to muscle founder cells that condition the general process of myogenesis by seeding the formation of muscles with specific properties and patterns of gene expression that specify the shape and the position of individual muscle fibers (Bate, 1992; Rushton *et al.*, 1995). We recently have found that *Kr* functions during the specification of these properties by regulating patterns of gene expression in the founders and the muscles to which they give rise. Similarly, *Kr* has been shown to act in a subset of neural cells and determines the specific fates of both glial and neuronal cells (Romani *et al.*, 1996). Thus, it may be that, as for the muscles, *Kr* acts in the nerves in those cells in which it is expressed to regulate patterns of gene expression that determine some aspects of cell specificity which are not apparent in their shape, size or position but in physiological or cell surface characteristics. These characteristics undoubtedly include the shared attributes of muscles and nerves which enable them both to act as substrates for oriented axon growth and which contribute to the stereotypic outgrowth and targeting of motor axons within the muscle field.

The *ko*-like sequences in other organisms such as *C.elegans* and mouse suggest that *ko* represents a founding member of a novel gene family or, at least, contains conserved protein motifs of novel structure. Also, Ko shows some features similar to those recently described for a conserved family of proteins, the netrins. Netrins are secreted molecules that play important and conserved roles in circumferential axon guidance (Hedgecock *et al.*, 1990; Kennedy *et al.*, 1994; Serafini *et al.*, 1994). As observed with *ko*, two netrin family members of *Drosophila* were shown to be expressed in both the CNS and muscles (Harris *et al.*, 1996; Mitchell *et al.*, 1996). Also, *netrin* mutants show defects in the innervation pattern which were similar to, but less specific than, those of *ko* mutants. These formal similarities argue for similar functions of the gene products in establishing a proper innervation pattern. However, unlike netrins (Harris *et al.*, 1996; Mitchell *et al.*, 1996), the ectopic expression of *ko* does not cause abnormal branching of motoraxons. Thus, in contrast to netrins, *ko* activity is only necessary for neuronal connectivity but not sufficient to interfere with it. Also, the deduced Ko protein lacks a diagnostic signal sequence that allows it to be classified unambiguously as a secreted protein, although the hydrophobic residues in the N-terminal part of the Ko protein as well as the putative sites for *N*-glycolsylation and myristylation would be consistent with its function as an extracellular matrix protein. Attempts to produce antibodies directed against the recombinant Ko protein have failed so far. Thus, we cannot infer from the subcellular localization whether the protein acts inside or outside the cell.

Materials and methods

Drosophila strains

The following fly strains were used in this work: Oregon R, Kr^{CD+} *Kr / CyO hb-lacZ*; *ry* (a transgenic line that rescues the *Kr* segmentation

phenotype, described in Romani *et al.*, 1996), *P(ry$^+$ HSKr)/CyO hb-lacZ*; *ry* (a gift from G.Struhl) and *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s/TM3*, *Sb*, *e^s*, *hb-lacZ* which was generated by recombining the *Pc* rescue transgene from the homozygous viable *Polycomb* rescue strain *P[Pc$^+$ 62B] X-7*, *Pc^2*, *h*, *sr*, *e^s* (kindly provided by R.Paro, Heidelberg) and the *Pc* deficiency of the strain *Df(3L)Pc-cp2* (obtained from M.Fuller). To exclude the possibility that the phenotype of this stock is due to additional mutations in the recombinant chromosome other than that caused by the deletion, transheterozygous embryos were obtained by crossing *Df(3L)Pc-MK/TM3*, *Sb*, *e^s*, *hb-lacZ* (uncovering the *ko* transcription unit) to the *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s/TM3*, *Sb*, *e^s*, *hb-lacZ* stock. Transheterozygous embryos of the genotype *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s/Df(3L)Pc-MK* were analyzed and similar defects were observed. Transheterozygous embryos obtained from a cross with the deficiency strain *Df(3L)Pc* which covers the *ko* transcription unit are viable (they die as larvae) and do not display similar defects.

Isolation of genomic DNA containing Krüppel-binding sites

DNA fragments containing *in vitro* binding sites for Krüppel were isolated by whole genome PCR immunoprecipitation (Kinzler and Vogelstein, 1989). For this, 5 µg of genomic DNA of Oregon R flies were incubated with the restriction endonuclease *Hae*III to yield 70–350 bp long DNA fragments. They were ligated to catch linkers of the sequence 5′-GAGTAGAATTCTAATATCTC-3′. After *Xho*I digestion (which serves to cleave tandems of the ligated linkers) followed by a phenol extraction, ~200 ng of DNA were incubated (30 min on ice) with 10–50 ng of bacterially produced, purified *Kr* protein extract (Pankratz *et al.*, 1989) in a total volume of 50 µl of binding buffer [150 mM NaCl, 100 mM Tris pH 7.5, 30 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 10 µM $ZnSO_4$, 500 µg/ml bovine serum albumin (BSA), 500 ng of poly(dI/dC)]. Twenty µl of polyclonal rabbit anti-Krüppel antibodies (diluted 1:100; Gaul *et al.*, 1987) pre-coupled to protein A–Sepharose beads were added. After incubation (30 min on ice), DNA fragments bound to the Krüppel–antibody complex were spun down by centrifugation (500 *g*). Unbound DNA fragments were removed by five washes in 1 ml of binding buffer containing 400 mM instead of 150 mM NaCl (see above). The remaining DNA-containing pellet was treated with 200 µl of proteinase K solution (500 µg/ml 500 mM Tris pH 9, 20 mM EDTA, 10 mM NaCl, 1% SDS) and the DNA was phenol extracted. The purified DNA fragments were amplified by PCR using catch linkers (see above) as primers. One-fifth of the PCR reactions was used to repeat the above-described immunoprecipitation and DNA purification steps. After five rounds of immunoprecipitation–PCR amplification, the amplified DNA fragments were digested with *Eco*RI (cleavage site within the catch linkers; see above) and cloned into the pBstKS II vector. DNA fragments were sequenced by the chain termination method (Sanger *et al.*, 1977) using the USB sequencing kit to verify that they contain Krüppel-binding sites.

Cloning of the knockout gene

A 320 bp DNA fragment containing three *in vitro* Krüppel-binding sites was used to isolate genomic phages from an EMBL4 genomic *Drosophila* DNA library. Whole-mount *in situ* hybridizations (Tautz and Pfeifle, 1989) to wild-type embryos using digoxigenin-labeled DNA fragments of the phage insert DNA revealed transcripts present in *Kr*-expressing cells which alter their distribution pattern in *Kr* mutant embryos (see Results). Corresponding cDNAs were isolated from a λgt 11 cDNA phage library prepared from 0- to 15-h-old embryos. Further genomic DNA fragments from the *ko* transcription unit (see Figure 1E) were isolated from a λFix genomic *Drosophila* DNA library (Canton S; Stratagene) and from a cosmid library (Hoheisel *et al.*, 1991). This newly identified transcription unit was termed *knockout* (*ko*). Localization of the *ko* gene in polytene chromosomes, the mapping of the cloned DNA and of the deficiency breakpoints, the location of the Krüppel-binding DNA fragment within the transcription unit and the structural analysis of the *ko* gene and its transcripts (see Figure 1) were done as described by Wimmer *et al.* (1993). The DNA sequence has been submitted to DDBJ/EMBL/GenBank (accession No. AF006601). Northern blot analysis with 10 µg of poly(A)$^+$ RNA of 0- to 16-h-old embryos prepared as described (Sambrook *et al.*, 1989) using a 3.0 kb *Stu*I DNA fragment of the cDNA clone HM-gt11 4.2 as a probe.

Construction of β-galactosidase fusion genes

For the identification of regulatory elements of the *ko* transcription unit, the various genomic DNA fragments (see Figure 4A) were cloned into the pCaSpeR AUG β-gal vector (Thummel and Pirotta, 1992). The 2.4 and 2.2 kb *Bgl*II DNA fragments as well as the 7.0 kb *Bgl*II–*Bam*HI DNA fragments were cloned into the *Bam*HI site of the vector. The 6.0 kb *Sac*I DNA fragment was isolated using the isoschizomer *Ecl*136II and cloned into the repaired *Eco*RI site of the vector. The 15 kb *Not*I–*Spe*I fragment was isolated from the lambda phage λFix GIV (*Not*I site from the polylinker of the phage vector), the ends of the DNA fragment were repaired and it was cloned into the repaired *Eco*RI site of the vector. Constructs that contained inserts in 5′–3′ orientation were injected into *w*, *sn^3* embryos. The embryonic progeny of the transgenic flies were assayed for β-galactosidase expression by antibody staining. With the exception of the 15 N/Sp–*lacZ* construct (see Figure 4A) for which only one transgenic strain was obtained, at least three independent lines were established and analyzed for all constructs.

Histochemistry

In situ hybridizations in whole-mount embryos were performed with minor modifications to the protocol of Tautz and Pfeifle (1989). Immunological stainings of whole-mount embryos, using the Vectastain ABC Elite Kit from Vectalabs, were made as described (Macdonald *et al.*, 1986). Stained embryos were embedded in Canada Balsam (Sigma) or, in the case of flat preparations, in 70% glycerol, 100 mM Tris pH 7.4, 50 mM NaCl. The primary antibodies anti-β-galactosidase (Cappel) and anti-fasciclin II (van Vactor *et al.*, 1993) were used. Stained embryos were examined and photographed using a Zeiss Axiophot microscope.

Ectopic expression and phenotypic rescue

To monitor the effects of *Kr* on *ko* expression, transient *Kr* expression was induced in 9.5- to 10.5-h-old *P(ry$^+$ HSKr)/CyO hb-lacZ*; *ry* embryos (stage 13/14) by three mild 15 min heat shock pulses at 37°C with a 15 min recovery at room temperature between pulses. Embryos were allowed to continue development at room temperature until the desired stage before fixation.

For the ectopic expression of the *ko* gene, the 4.2 kb *Eco*RI insert of the cDNA clone HM-gt11 4.2 (Figure 1) was inserted (in sense orientation) 3′ of the heat shock-inducible *hsp70* promoter in 5′–3′ orientation into the *Eco*RI site of the pCaSpeR HS vector (Thummel and Pirotta, 1992). For the ubiquitous expression of the antisense *ko* transcripts, a 3.0 kb *Stu*I fragment of the cDNA clone HM-gt11 4.2 was cloned in 3′–5′ orientation behind the heat shock-inducible *hsp70* promotor into the *Stu*I site of the pCaSpeR HS vector. The resulting constructs were injected into *w*, *sn^3* embryos. Several independent lines were established. Ubiquitous expression of *ko* sense and antisense transcripts was induced by three heat shocks as described above for embryos containing the *hsp70-Kr* transgene using collections of 9.5- to 10.5-h-old embryos bearing the *hsp70-ko* or the *hsp70*-anti-*ko* transgene constructs. As a control, *w*, *sn^3* embryos were treated and analyzed in parallel. Ectopic expression of the transgenes was confirmed by immunostaining in the case of *Krüppel* or by *in situ* hybridization with a DNA probe of the *ko* gene.

Ectopic expression of antisense *ko* transcripts in the mesoderm was carried out using the GAL4/UAS system. For this, homozygous females of the 24B-GAL4 driver line (Brand and Perrimon, 1993) were mated to males bearing two copies of a UAS-antisense *ko* transgene. For this, a 3.0 kb *Stu*I DNA fragment (see above) was inserted into the repaired *Eco*RI site of the UAST-pCaSpeR3 vector (Brand and Perrimon, 1993). Embryo collections of 0–2 h were allowed to develop at 29°C until stage 16/17 and fixed for immunostaining.

Rescue of the *ko* phenotype in homozygous *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s* embryos was performed by ubiquitous expression of the *ko* gene under the control of a heat shock-inducible promotor (see above). For this, a fly stock of the genotype *w*, *P[w$^+$ hsp70-ko]*; *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s/TM3*, *Sb*, *e^s*, *hb-lacZ* was established and the transient ubiquitous expression of *ko* was induced by three heat shocks as described above using collections of 9.5- to 11.5-h-old embryos. To distinguish embryos homozygous for the deficiency chromosome *P[Pc$^+$]Df(3L)Pc-cp2*, *e^s* from those containing the balancer chromosome *TM3*, *Sb*, *e^s*, *hb-lacZ* embryo collections were stained for β-galactosidase activity by X-gal staining prior to the immunostaining with anti-fasciclin II antibodies.

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