

# *jkk-1* and *mek-1* regulate body movement coordination and response to heavy metals through *jnk-1* in *Caenorhabditis elegans*

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Although *in vitro* evidence suggests two c-Jun N-terminal kinase (JNK) kinases, MKK4 and MKK7, transactivate JNK, *in vivo* confirmation is incomplete. In fact, JNK deficiency may differ from the composite deficiency of MKK4 and MKK7 in *Drosophila* and mice. Recently, the *Caenorhabditis elegans* homolog of human JNK, *jnk-1*, and two MKK-7s, *mek-1* and *jkk-1*, were cloned. Here we characterize *jnk-1*, which encodes two isoforms JNK-1 $\alpha$  and JNK-1 $\beta$ . A null allele, *jnk-1(gk7)*, yielded worms with defective body movement coordination and modest mechanosensory deficits. Similarly to *jkk-1* mutants, elimination of GABAergic signals suppressed the *jnk-1(gk7)* locomotion defect. Like *mek-1* nulls, *jnk-1(gk7)* showed copper and cadmium hypersensitivity. Conditional expression of JNK-1 isoforms rescued these defects, suggesting that they are not due to developmental errors. While *jkk-1* or *mek-1* inactivation mimicked *jnk-1(gk7)* locomotion and heavy metal stress defects, respectively, *mkk-4* inactivation did not, but rather yielded defective egg laying. Our results delineate at least two different JNK pathways through *jkk-1* and *mek-1* in *C.elegans*, and define interaction between MKK7, but not MKK4, and JNK.

**Keywords:** body movement/*Caenorhabditis elegans*/heavy metals/*jkk-1/mek-1*

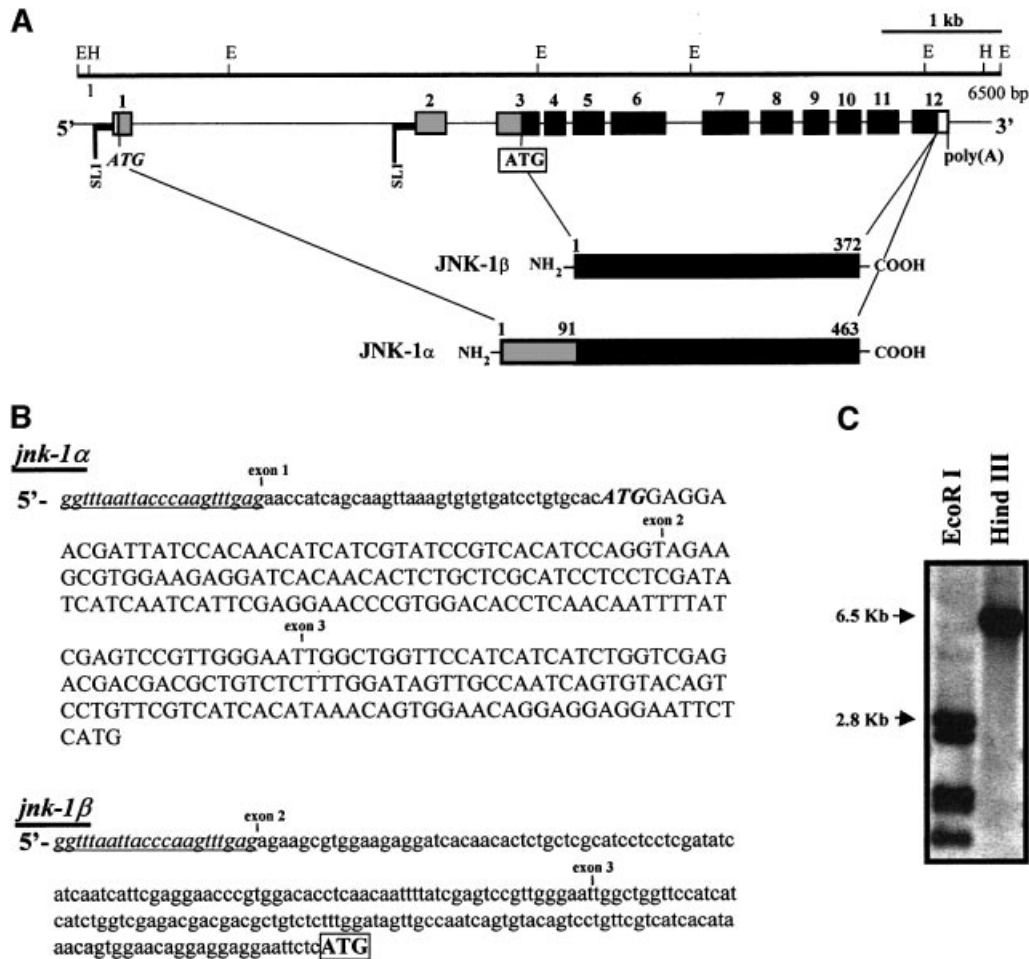
## Introduction

Mitogen-activated protein kinases (MAPKs) are components of signaling cascades that regulate normal development, mitogenesis and stress responses (Robinson and Cobb, 1997). Further, these pathways are co-opted during oncogenic transformation and tumor progression (Kyriakis and Avruch, 1996). Three subgroups of MAPKs have been identified: the extracellular signal-regulated kinase (ERK) (Davis, 1993), c-Jun N-terminal (JNK also referred to as stress-activated protein kinase) (Derijard *et al.*, 1994) and p38 kinases (also called MpK2) (Han *et al.*, 1994). MAPKs are activated via dual phosphorylation of threonine and tyrosine residues in a Thr-X-Tyr motif present within their activation loops (kinase subdomain VIII). X corresponds to glutamic acid in

ERK, proline in JNK and glycine in p38 (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). The specific MAPK kinases (MAPKKs or MEKs) that carry out this reaction are themselves phosphorylated and activated by specific MAPKK kinases (MAPKKKs). In mammalian cells, evidence suggests that two MAPKKs, MKK4 and MKK7, activate JNK. While MKK4 also functions as an activator of p38, MKK7 probably functions as a specific activator of JNK (Cavigelli *et al.*, 1995; Derijard *et al.*, 1995; Tournier *et al.*, 1997).

The ERK cascade is highly responsive to signals initiated by the binding of growth factors, such as epidermal growth factor or platelet-derived growth factor, to their tyrosine kinase receptors (Marshall, 1995; Whitmarsh *et al.*, 1995). JNK activity is strongly stimulated in vertebrate cell culture by inhibitors of protein biosynthesis, such as cycloheximide and anisomycin, by inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), and by heat, osmotic shock, UV light or other DNA-damaging agents (Derijard *et al.*, 1994; Cavigelli *et al.*, 1995; Coso *et al.*, 1995). When JNK is activated, it migrates from the cytoplasmic compartment into the nucleus and stimulates the activity of several transcription factors including c-Jun, ATF-2, ELK-1 and p53 (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Cavigelli *et al.*, 1995; Gupta *et al.*, 1995; Whitmarsh *et al.*, 1995; Fuchs *et al.*, 1998).

Although a substantial body of biochemical and cell biological evidence supports this model, genetic proof remains incomplete. Based on the current hypothesis, it is anticipated that JNK knockouts in developmental models would represent a composite of the cell type-specific expression of MKK4 and MKK7. The best defined system is that of *Drosophila*. Conserved elements of the *Drosophila* JNK pathway include *D-jun*, *basket* (*bsk*) and *hemipterous* (*hep*), which encode homologs of c-Jun, JNK and MKK7, respectively (Glise *et al.*, 1995; Riesgo-Escovar *et al.*, 1996; Sluss *et al.*, 1996; Noselli, 1998). These gene products are all required for the normal morphogenetic process of dorsal closure beginning 12 h after fertilization. Biochemical evidence shows that HEP phosphorylates D-JNK, which in turn phosphorylates D-JUN (Noselli, 1998). Further support for these genes acting in a linear pathway is derived from epistasis studies which show that activated D-Jun can rescue the dorsal closure defect caused by *hep* and *bsk*. In contrast, the *Drosophila* p38 MAPKs (*D-p38a* and *-b*) may be involved in insect immunity, as well as in the response to environmental stresses (Han *et al.*, 1998). *Drosophila* also contains a homolog of MKK4, *D-mkk4*, for which mutations have not yet been isolated. Whatever its role in development, it is clear that *Drosophila* MKK4 is not sufficient to substitute for MKK7 and prevent the lethal dorsal closure phenotype (Noselli, 1998).



**Fig. 1.** *jnk-1* encodes two different transcripts. (A) Structure of *C.elegans jnk-1*. Exons are indicated by boxes, while introns are represented as lines. Numbers refer to amino acids. RACE was performed using two different sets of primers as described in Materials and methods. In 11/15 clones (using primers based on sequences from exons 7 and 8) and in 6/10 clones (using primers from exon 12), we detected two transcripts, which we termed *jnk-1α* and *jnk-1β* containing 5' SL1 *trans*-splicing leader sequences (Bektesh *et al.*, 1988). For *jnk-1α*, the SL1 *trans*-spliced sequence was followed by exon 1, whereas for *jnk-1β* the SL1 *trans*-spliced sequence was followed by exon 2. The translation initiation site, ATG (in bold and italicized) of *jnk-1α* is located in exon 1 while the translation initiation site of *jnk-1β* is located in exon 3 (in bold and marked by a box). JNK-1α has a 91 amino acid extended N-terminal region (in gray), fused in-frame with 372 amino acid residues (in black) that are identical in JNK-1α and JNK-1β. Poly(A), polyadenylation site; SL1, *trans*-splicing leader sequence; E, *EcoRI* and H, *HindIII* restriction sites; NH<sub>2</sub>, N-terminus; COOH, C-terminus. (B) Nucleotide sequence of the 5' regions of *jnk-1α* and *jnk-1β* transcripts. SL1 *trans*-splicing leader sequences (underlined and italicized) are located at the beginning of both *jnk-1* transcripts. Non-translated sequences are in lower case and translated sequences are in upper case. (C) Southern blot analysis using full-length [ $\alpha$ -<sup>32</sup>P]dCTP-labeled *jnk-1α* cDNA as a probe shows a pattern of DNA restriction compatible with that predicted from cosmid B0478.

Recent investigations in the mouse have extended our understanding of the role of the JNK pathway in development. The mammalian JNK protein kinases are encoded by three genes, which are alternatively spliced to create 10 JNK isoforms. While *jnk1* and *jnk2* are ubiquitously expressed, *jnk3* has a limited pattern of expression and is largely restricted to brain, heart and testis (Davis, 2000). Disruption of the *jnk1* (Dong *et al.*, 1998), *jnk2* or *jnk3* (Yang *et al.*, 1998) genes in mice causes no obvious phenotypic abnormalities. However, *jnk1*<sup>-/-</sup> or *jnk2*<sup>-/-</sup> mice are immunodeficient due to defects in T-cell function (Constant *et al.*, 2000), and *jnk3*<sup>-/-</sup> animals are defective in stress-induced AP-1 transcriptional activity in the hippocampus (Yang *et al.*, 1997b). Mice with compound deletion in *jnk1* plus *jnk3* or *jnk2* plus *jnk3* alleles are also viable (Kuan *et al.*, 1999). In contrast, *jnk1*<sup>-/-</sup>/*jnk2*<sup>-/-</sup> double mutant animals display dysregulated

developmental apoptosis in the brain and die by day E11.5 (Kuan *et al.*, 1999).

In mice, both *mkk4/sek1* (Yang *et al.*, 1997a) and *mkk7* (Dong *et al.*, 2000) are required for embryonic development. While a *mkk4/sek1* deficiency in mice results in early embryonic lethality due to defective hepatogenesis (Ganiatsas *et al.*, 1998), the cause of embryonic death in *mkk7*<sup>-/-</sup> mice is unclear (Dong *et al.*, 2000). As *mkk4/sek1*<sup>-/-</sup> mice do not manifest the central nervous system (CNS) abnormalities observed in *jnk1*<sup>-/-</sup>/*jnk2*<sup>-/-</sup> mice (Lenord Zon, personal communication), normal CNS development probably requires MKK7. Consistent with the observation that deficiency of MKK7 or JNK delivers similar phenotypes, Flavell and co-workers reported that T cells from both *mkk7*<sup>-/-</sup> and *jnk1*<sup>-/-</sup>/*jnk2*<sup>-/-</sup> mutant mice display increased IL-2 production and proliferation after anti-CD3/CD28, and stimulated *mkk7*<sup>-/-</sup> T cells display

low JNK activity (Dong *et al.*, 2000). Further, *mkk7*<sup>-/-</sup> mice did not manifest defective hepatogenesis, and *mkk4*<sup>-/-</sup> T cells displayed normal JNK activation and defective IL-2 production after anti-CD3/CD28 (Dong *et al.*, 2000). These developmental models suggest association of JNK with MKK7 signaling but not with MKK4.

In *Caenorhabditis elegans*, *jnk-1*, a *jnk* homolog, *mek-1*, which is highly homologous to mammalian *mkk7*, and *jkk-1*, a new member of the MAPKK superfamily which has 41.6% amino acid identity with MKK7 in the kinase domain, have recently been identified (Kawasaki *et al.*, 1999; Koga *et al.*, 2000). While *jnk-1* and *jkk-1* are co-expressed in the cell bodies and the axons of most neurons (Kawasaki *et al.*, 1999), *mek-1* is expressed in pharyngeal muscles, uterus, a portion of intestine and neurons in the ring, ventral and anal ganglia (Koga *et al.*, 2000). Although *jkk-1* or *mek-1* mutants did not show developmental defects, *jkk-1* disruption altered coordination of body movement via type-D GABAergic motor neurons (Kawasaki *et al.*, 1999), while *mek-1* disruption resulted in hypersensitivity to heavy metals and starvation (Koga *et al.*, 2000). The *C.elegans* genome also contains *mkk-4* (corresponding to the cosmid F42G10.2), which is highly homologous to mammalian *mkk4/sek1*, about which nothing presently is known.

To determine whether JNK-1 acted genetically distal to these gene products, we isolated a *jnk-1* null allele, *jnk-1(gk7)*. Worms homozygous for this mutant allele did not show obvious development defects. However, *jnk-1(gk7)*, showed defects in body movement coordination similar to *jkk-1(km2)* and, like *mek-1(ks54)*, were hypersensitive to copper and cadmium ions. In contrast, inactivation of *mkk-4* in N2 or *jnk-1(gk7)* worms resulted in an egg-laying defect in adult hermaphrodites. Our results delineate at least two different JNK signaling pathways through *jkk-1* and *mek-1* in *C.elegans*. Further, three distinct genetic models, *C.elegans*, *Drosophila* and mice, now define interaction between MKK7, but not MKK4, and JNK.

## Results

### ***Caenorhabditis elegans jnk-1* encodes two different transcripts generated by SL1 trans-splicing**

The mammalian JNK genes, *jnk1*, *jnk2* and *jnk3* encode at least 10 different JNK isoforms (four JNK1, four JNK2 and two JNK3) generated by two different mechanisms: alternative *cis*-splicing that yields different C-termini or the utilization of alternate sequences within protein kinase subdomains IX and X (Gupta *et al.*, 1996). Recently, *C.elegans jnk-1* has been cloned (Kawasaki *et al.*, 1999). It is localized to the left arm of chromosome IV. To determine whether *jnk-1* encodes different isoforms or whether *C.elegans* contains related genes, we reverse transcribed total RNA from mixed stages N2 animals to cDNA, applied the rapid amplification of cDNA ends (RACE) technique, and detected and sequenced two transcripts (Figure 1A and B). Both transcripts contained 5' SL1 *trans*-splicing leader sequences (Bektesh *et al.*, 1988), defining the limits of their 5' ends. In *jnk-1 $\alpha$* , the SL1 leader sequence was *trans*-spliced to exon 1, whereas in *jnk-1 $\beta$* , *trans*-splicing occurred at the beginning of exon

2. The larger transcript *jnk-1 $\alpha$*  is identical to the transcript of *jnk-1* reported by Kawasaki and co-workers (Kawasaki *et al.*, 1999), and contains an open reading frame coding for a 463 amino acid protein (Figure 2). The smaller transcript *jnk-1 $\beta$*  is predicted to use an in-frame ATG in exon 3 (corresponding to amino acid 92 of JNK-1 $\alpha$ ) as the translation start site, coding for a protein of 372 amino acids (Figures 1A and B, and 2). The 91 amino acid N-terminal sequence that distinguishes JNK-1 $\alpha$  from JNK-1 $\beta$  is unique, while the amino acid sequence comprising JNK-1 $\beta$  is conserved almost entirely in human JNK1 (70% identity, 83% homology; Figure 2). Both JNK-1 isoforms contain the conserved protein kinase subdomains I–XI found in all kinases, as well as the TPY motif located in subdomain VIII of all JNKs (Galcheva-Gargova *et al.*, 1994; Kyriakis *et al.*, 1994; Gupta *et al.*, 1996). *Cis*-spliced isoforms that might yield different C-termini in the *jnk-1* gene were not detected by RACE, nor were alternative sequences in kinase subdomains IX and X detected by RT-PCR/single strand conformation polymorphism (SSCP). In both *jnk-1* transcripts, the polyadenylation consensus site (AATAAA) was detected, indicating that we cloned complete cDNAs.

Screening the *C.elegans* database using the BLAST program did not identify any other potential *jnk* genes. Furthermore, Southern blot analysis of *C.elegans* genomic DNA hybridized with full-length *jnk-1 $\alpha$*  cDNA at high and low stringency yielded a pattern of digestion identical to that predicted from the B0478 cosmid, which contains the *jnk-1* gene (Figure 1C). Together, these results suggest that *C.elegans* generates two JNK-1 isoforms by alternative transcription or translation of a single *jnk* gene.

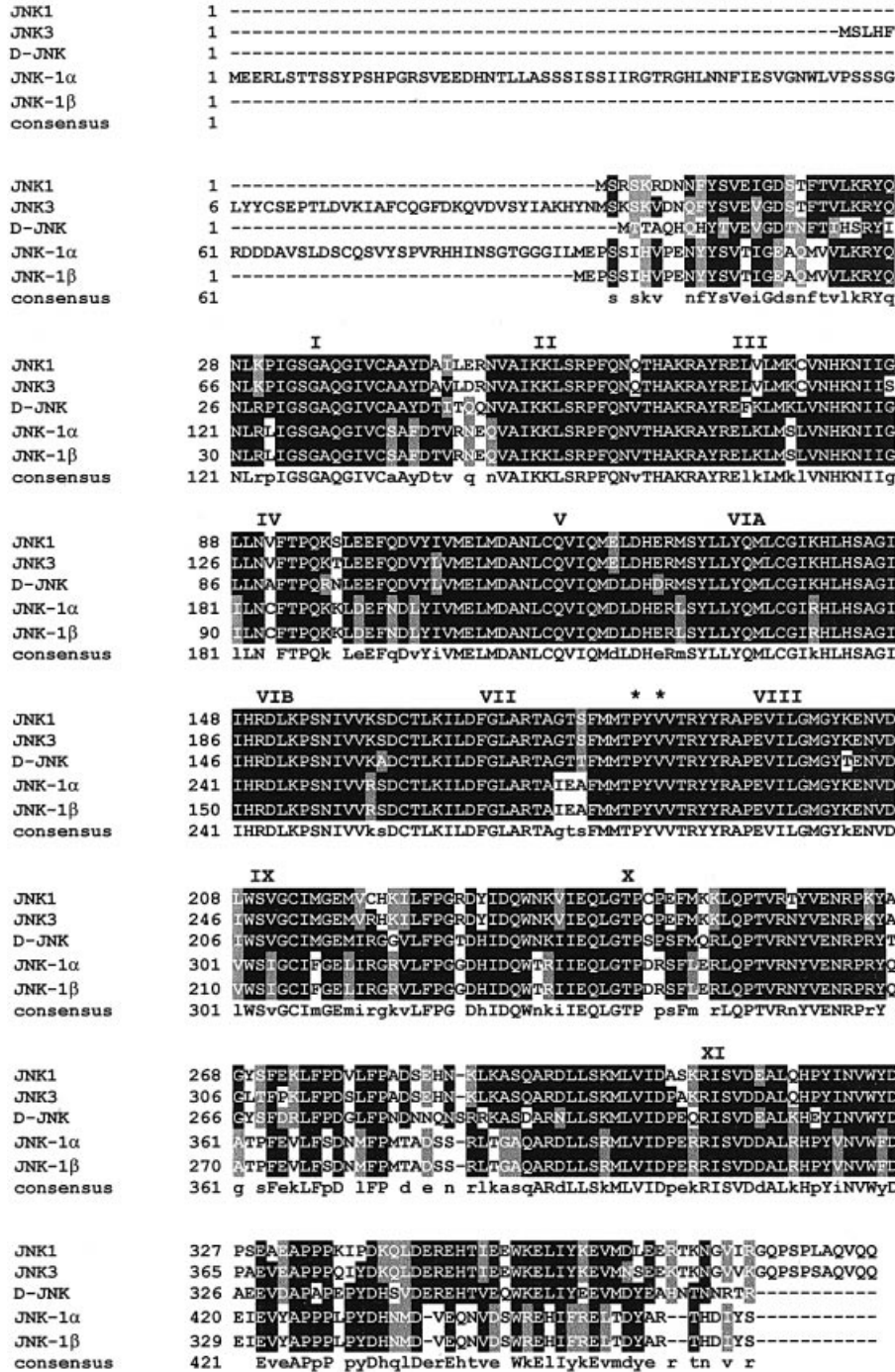
### ***jnk-1* is expressed throughout development**

To determine the RNA expression pattern of *jnk-1* during development, northern blot analysis was carried out using membranes containing total RNA from embryos, L1–L2, L3, L4 and young adult worms. High stringency hybridization, using the complete coding region of the *jnk-1 $\beta$*  cDNA as a probe, showed a single band of 1.5 kb (Figure 3A). It should be noted that this probe recognizes both *jnk-1* isoforms, which differ by only 82 bp and hence cannot be distinguished on a northern blot. The *jnk-1* message was most abundant during embryonic (E) and L1/L2 stages, and decreased gradually from early L3 larva to the young adult worm (Figure 3A). The same result was obtained using the 82 bp fragment only present in *jnk-1 $\alpha$* , as a probe (not shown). In both cases, the transcript size was the same as that predicted by the sizes of the cloned cDNAs, confirming that full-length cDNAs had been isolated. Semi-quantitative RT-PCR using primers that selectively amplify the individual isoforms confirmed that both isoforms had abundant RNA expression during embryonic and L1 stages (Figure 3B). To identify the individual JNK-1 isoforms, we performed western analysis on *C.elegans* lysates using a monoclonal anti-human JNK1 antibody. The antibody recognized two bands of the expected sizes for JNK-1 $\alpha$  (46 kDa) and JNK-1 $\beta$  (38 kDa) (Figure 3C). To provide evidence that the bands represent bona fide isoforms rather than a single form post-translationally modified, we showed that the migration pattern of the endogenous JNKs corresponded to the Flag-tagged versions from COS-7 cells. (The small size

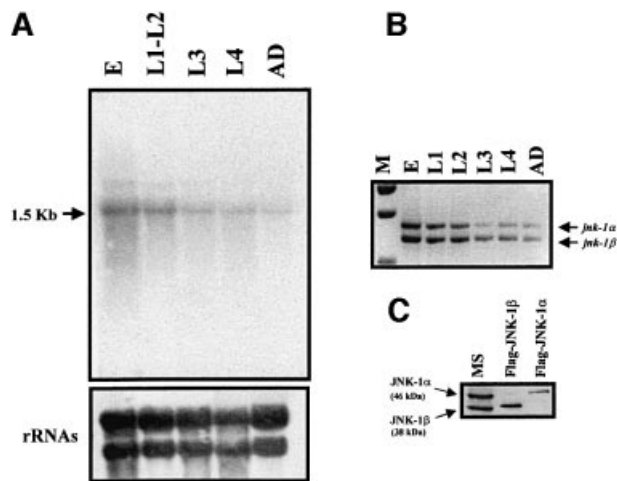
difference between endogenous *C.elegans* isoforms and epitope-tagged proteins is due to 25 amino acids included in the cloning process.) A close correlation between mRNA and protein levels at L1 to adult stages was detected using this antibody (not shown). However, at the embryo stage, low levels of protein were detected, suggesting that post-transcriptional or post-translational mechanisms regulate JNK-1 levels in embryos.

**Transiently expressed JNK-1 $\alpha$  and JNK-1 $\beta$  are activated in response to stress stimuli**

Mammalian JNKs are activated in response to diverse stress stimuli (Derijard *et al.*, 1994, 1995; Galcheva-Gargova *et al.*, 1994; Kyriakis *et al.*, 1994). To investigate activation of JNK-1 isoforms, COS-7 cells were transiently transfected with mammalian expression plasmids encoding Myc-tagged JNK-1 $\alpha$ , Flag-tagged JNK-1 $\beta$  or



**Fig. 2.** Primary structure of the *C.elegans* JNK-1 $\alpha$  and JNK-1 $\beta$  proteins. Alignment of amino acid sequences of human JNK1 and JNK3 (DDBJ/EMBL/GenBank accession Nos L26318 and U34820, respectively), *Drosophila* D-JNK (DDBJ/EMBL/GenBank accession No. U49180), and JNK-1 $\alpha$  (DDBJ/EMBL/GenBank accession No. AB024085) and JNK-1 $\beta$  protein kinases using the PILE-UP program. Identical amino acid residues are highlighted and similar residues are shaded. A consensus sequence is presented. The conserved TPY motif is marked with asterisks and the kinase subdomains are marked with Roman numerals. Gaps (-) were introduced into the sequences to optimize alignment. Note that not all groups have reported the long N-terminus of JNK3 (Kyriakis *et al.*, 1994).



**Fig. 3.** Expression pattern of *jnk-1* during development. (A) Northern blot and (B) RT-PCR analysis using total RNA from different *C.elegans* developmental stages. Northern blot was probed with *jnk-1β* full-length cDNA and RT-PCR was performed using a specific set of primers that amplified both isoforms. Ethidium bromide staining was used to control for RNA quality and loading (lower panel). (C) Western blot analysis of 100  $\mu$ g of total protein from mixed *C.elegans* stages using a monoclonal anti-human JNK1 antibody (sc-1648, Santa Cruz Biotechnology) shows expression of both JNK-1 proteins. Flag-tagged JNK-1 $\alpha$  or - $\beta$ , isolated by immunoprecipitation from COS-7 lysates using anti-FLAG monoclonal antibody, served as controls. E, embryos; L1-L2, L1 and L2 larvae; L3, L3 larvae; L4, L4 larvae; AD, young adult worms; MS, mixed stages; M, DNA molecular weight marker.

vector alone. After 48 h, cells were left untreated or stimulated with UV light (40 J/m<sup>2</sup>), hyperosmotic shock (400 mM sorbitol), heat shock (42°C for 20 min), TNF- $\alpha$  (100 ng/ml) or lipopolysaccharide (LPS; 1  $\mu$ g/ml). Epitope-tagged JNK-1 $\alpha$  and JNK-1 $\beta$  were isolated by immunoprecipitation with anti-Myc and anti-FLAG antibodies, respectively, and kinase activity was determined in an immune complex kinase assay using glutathione *S*-transferase (GST)-c-Jun as substrate. These stimuli similarly activated both JNK-1 $\alpha$  (not shown) and JNK-1 $\beta$  (Figure 4A, top panel). Similar results were obtained with  $\gamma$ -radiation (20 Gy) or H<sub>2</sub>O<sub>2</sub> (75  $\mu$ M) (not shown). In parallel, a control experiment was performed in cells transfected with human hemagglutinin (HA)-tagged JNK1 and yielded quantitatively similar results (not shown). Western blot analysis of COS-7 cells extracts showed that the epitope-tagged proteins were expressed at comparable levels (Figure 4A, lower panel).

The different human JNK isoforms can phosphorylate c-Jun, ATF-2 and ELK-1 transcription factors (Gupta *et al.*, 1995, 1996; Jacobs *et al.*, 1999). In a separate set of studies, both *C.elegans* JNK-1 isoforms also phosphorylated human recombinant GST-ATF-2 and GST-ELK-1, when supplied as substrates (Figure 4B, not shown for JNK-1 $\alpha$ ).

#### ***Caenorhabditis elegans* JNK-1 isoforms are activated by phosphorylation on residues corresponding to Thr183 and Tyr185 of mammalian JNK1**

In vertebrates and *Drosophila*, evidence suggests that JNK is activated by two MAPKKs, MKK7 and MKK4/SEK1,

in response to diverse stimuli (Derijard *et al.*, 1995; Tournier *et al.*, 1997). Recently, two MAPKKs, *mek-1* (Koga *et al.*, 2000) and *jkk-1* (Kawasaki *et al.*, 1999), have been cloned in *C.elegans* and a third, *mkk-4*, has been identified (corresponding to cosmid F42G10.2). *mek-1* and *jkk-1* encode two distinct MKK7 homologs. MEK-1 shows 53.5% amino acid identity in the kinase domain to MKK7, while JKK-1 shows 41.6% identity (Koga *et al.*, 2000). JKK-1 specifically stimulates the kinase activity of JNK-1 $\alpha$  but not *C.elegans* p38 in transfected 293 cells (Kawasaki *et al.*, 1999). These data are consistent with the notion that JKK-1 is a MAPKK specific for JNK.

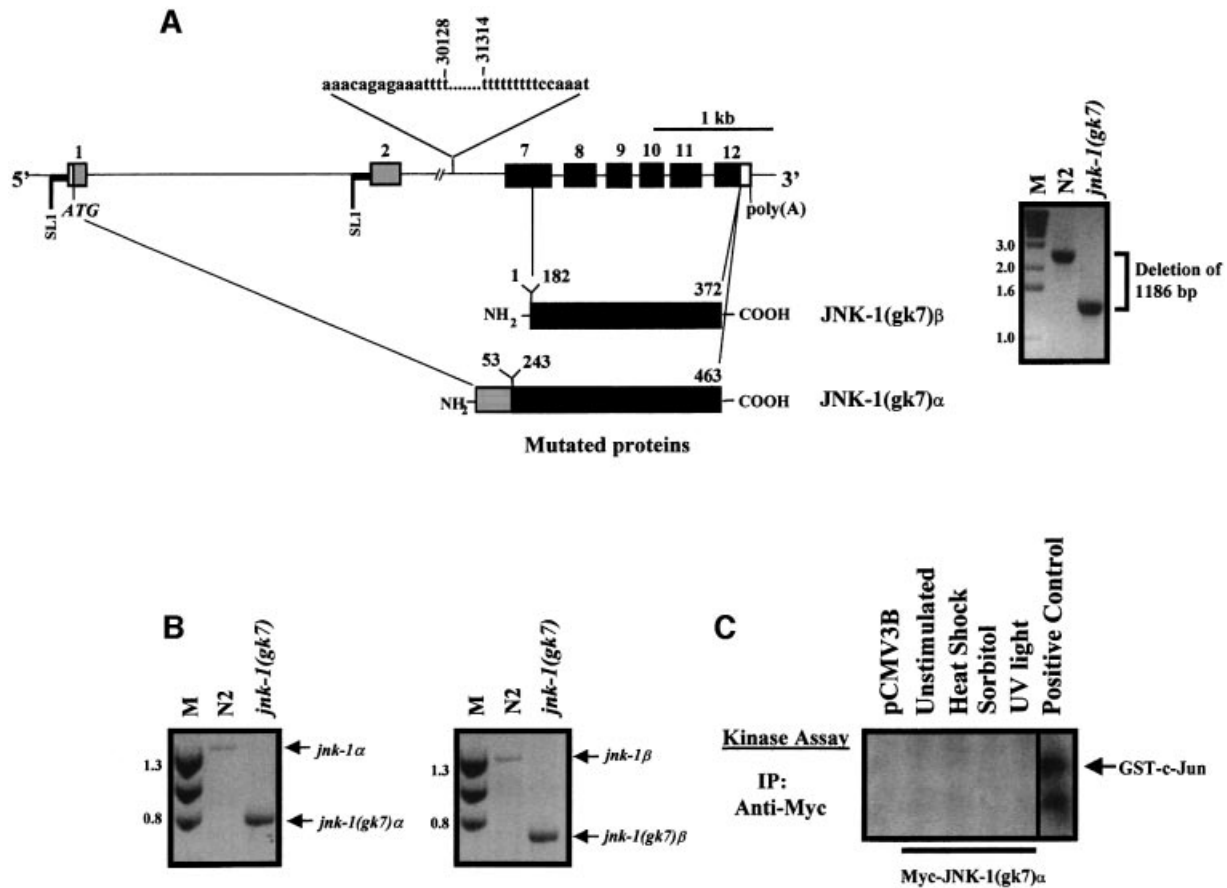
To investigate whether MKK4/SEK1 might also activate *C.elegans* JNK-1 isoforms, we transiently transfected COS-7 cells with plasmids encoding Myc-tagged JNK-1 $\alpha$  or Flag-tagged JNK-1 $\beta$  either alone, or in combination with mouse GST-tagged MKK4/SEK1 or dominant-negative kinase-inactive GST-tagged MKK4/SEK1(K129R) (Verheij *et al.*, 1996). As shown in Figure 4C, wild-type GST-MKK4/SEK1 activated Flag-JNK-1 $\beta$  in the absence of any stress stimuli, while dominant-negative GST-MKK4/SEK1 was not activating. Similar results were obtained with Myc-tagged JNK-1 $\alpha$  (not shown). COS-7 cells were also co-transfected with expression vectors encoding *C.elegans* HA-tagged MEK-1 and Flag-tagged JNK-1 $\alpha$  or - $\beta$ . Co-expression with HA-MEK-1 resulted in strong Flag-JNK-1 $\alpha$  activation (Figure 4D). Consistent with a requirement for the kinase function of MEK-1, expression of kinase-inactive HA-tagged MEK-1(K99R) did not result in Flag-JNK-1 $\alpha$  activation. Similar results were obtained with Flag epitope-tagged JNK-1 $\beta$  (not shown).

Mammalian JNK proteins are activated by dual phosphorylation on threonine and tyrosine residues. In particular, JNK1 is phosphorylated on Thr183 and Tyr185, located within its activation loop (kinase subdomain VIII) (Galcheva-Gargova *et al.*, 1994). To investigate whether the corresponding residues are also phosphorylated in *C.elegans* JNK-1 isoforms in response to stress stimuli, we transiently transfected COS-7 cells with plasmids encoding Flag-tagged JNK-1 $\beta$  or mutant Flag-tagged JNK-1 $\beta$ (AF) lacking the requisite phosphorylation sites. Kinase activity measured in an immune complex assay using GST-c-Jun as substrate showed that substitution of amino acids Thr185 and Tyr187 (corresponding to human JNK1 Thr183 and Tyr185) for alanine and phenylalanine blocked the activation of JNK-1 $\beta$  in response to UV light (Figure 5). Western blot analysis using an anti-human phospho-JNK (Thr183/Tyr185) antibody (New England Biolabs) that also recognizes the phosphorylated form of *C.elegans* JNK-1 confirmed that phosphorylated isoforms were only present in immunoprecipitates obtained from stimulated cells, or from cells co-expressing GST-tagged MKK4/SEK1 or HA-tagged MEK-1 (not shown). Similar results were obtained using Myc-tagged JNK-1 $\alpha$  and mutant Myc-tagged JNK-1 $\alpha$  (AF) (not shown).

#### **Isolation of a *jnk-1* loss-of-function allele**

To investigate the physiological role of *jnk-1*, we undertook a reverse genetic approach to isolate loss-of-function mutations in the *jnk-1* gene locus. Using a PCR-based screening strategy, we detected and isolated an individual worm with a deletion of 1186 bp (Figure 6A, right panel)





**Fig. 6.** Structure of the mutant *jnk-1(gk7)* $\alpha$  and *jnk-1(gk7)* $\beta$  transcripts generated from the *jnk-1(gk7)* allele. (A) Exons are indicated by boxes, while introns are represented as lines. DNA sequencing of the PCR product from *jnk-1(gk7)* showed an in-frame deletion of 1186 bp involving exons 3–6 (right panel), corresponding to nucleotides 30 128–31 314 of the cosmid B0458 (left panel). Sequences encoding JNK-1 $\alpha$  amino acids 53–243 and JNK-1 $\beta$  amino acids 1–182 are deleted, respectively, in the *gk7* mutant. (B) RT-PCR using specific primers that amplified each mutant isoform confirms the existence of two mutant transcripts expressed in *jnk-1(gk7)*. (C) COS-7 cells were transfected with the plasmid encoding mutated Myc-tagged JNK-1( $\alpha$ ) and, after 48 h, cells were left unstimulated or stress stimulated for 20 min, as indicated. Myc-JNK-1( $\alpha$ ) was immunoprecipitated and protein kinase activity measured in an immune complex kinase assay using GST-c-Jun. The positive control is from COS-7 cells expressing wild-type Myc-JNK-1 $\beta$  stimulated with UV light. M, DNA molecular weight marker. The other abbreviations are as in Figure 1.

lawn. Similarly to *jkk-1(km2)* knockout worms (Kawasaki et al., 1999), *jnk-1(gk7)* mutants left tracks with increased wave amplitude, approximately double that of a wild-type N2 animal (Table I; Figure 7A). The wavelength, however, was unchanged. The locomotion of *jnk-1(gk7)* animals was slightly sluggish and hence the distance covered during a fixed time (5 min) was reduced substantially. The *jnk-1* knockout worms seldom moved in a straight trajectory and instead moved in small circles, while wild-type worms covered straight long distances (Figure 7B). Compatible with the notion that both kinases function in the same pathway, *jnk-1(gk7);jkk-1(km2)* manifested movement defects equivalent to those of either *jnk-1(gk7)* or *jkk-1(km2)*.

*jnk-1(gk7)* worms also displayed modest reductions in some mechanosensory responses: light touch to the nose was 45% reduced and harsh touch to the body 40% reduced when compared with N2 worms (Table I). Consistent with the concept that touch responses through JNK-1 were regulated via a JKK-1 independent mechanism, abnormalities in touch responses were not detected in *jkk-1(km2)*, while *jnk-1(gk7);jkk-1(km2)* still displayed the

modest reductions in mechanosensory responses observed in *jnk-1(gk7)* (not shown).

In contrast, *jnk-1(gk7)* worms manifested normal responses to a variety of attractive and repulsive water-soluble and volatile compounds (Table I). Indeed, no changes in the uptake of the lipophilic dye DiO by the amphid and phasmid neurons were detected (not shown), suggesting that the anatomy of these sensory structures is normal in *jnk-1* mutants.

Other behaviors including pharyngeal pumping, egg laying, dauer formation, male mating and defecation were also normal in the *jnk-1(gk7)* strain. Consistent with the proposed loss-of-function nature of the *jnk-1(gk7)* deletion, we found that *jnk-1(gk7)/+* heterozygous animals did not show any of the behavioral defects observed in the homozygous mutant animals (not shown).

#### **Conditional overexpression of JNK-1 $\alpha$ or JNK-1 $\beta$ reverses movement and mechanosensory response defects in *jnk-1(gk7)***

To determine whether the defects observed in *jnk-1(gk7)* mutants are due to abnormal development

or cell function, we generated transgenic animals in the *jnk-1(gk7)* background harboring, as extrachromosomal arrays, plasmids encoding Myc epitope-tagged JNK-1 $\alpha$  or Flag-tagged JNK-1 $\beta$  under control of the heat shock promoters *hsp16-2* and *hsp16-41*. These promoters direct protein expression in the majority of tissues, including neurons (Stringham *et al.*, 1992; Mello and Fire, 1995). Transgenic animals exhibited the same locomotion defects and modest reductions in mechanosensory responses in the absence of heat treatment as the parental strain. When heat treated at the young adult stage, movement defects (wave amplitude, locomotion and trajectory) and mechanosensory responses (light touch to the nose and harsh body touch) were rescued by either isoform within 12–24 h (Table I), demonstrating that these defects are not due to developmental abnormalities, but rather to defects in functions regulated by JNK-1 protein isoforms. It should be noted that we did not detect low levels of *jnk-1* cDNA by RT-PCR in the absence of heat (not shown). In contrast, heat treatment of worms carrying empty vector did not rescue the mutant phenotype (Table I).

**Increased amplitude phenotype in *jnk-1(gk7)* mutants requires GABAergic synaptic transmission**

Recently, it has been demonstrated that JNK-1, like JKK-1, is expressed in most or all neurons and their processes, including the nerve ring, head ganglion, dorsal and ventral nerve cords and tail ganglion (Kawasaki *et al.*, 1999). *Caenorhabditis elegans* body wall muscle contraction depends on a balance between inhibitory GABAergic inputs from the DD and VD neurons (McIntire *et al.*, 1993a,b) and excitatory cholinergic inputs (Lewis *et al.*, 1980). The six DD neurons (which innervate the dorsal body wall muscles) and the 13 VD neurons (which innervate the ventral body wall muscles) coordinate muscle relaxation during locomotion. Animals in which the DD and VD neurons have been destroyed by laser ablation, or genetic mutations in the genes *unc-25*, *unc-30*, *unc-46*, *unc-47* and *unc-49*, which regulate GABA synthesis/action (McIntire *et al.*, 1993a; Eastman *et al.*, 1999), manifest simultaneous contraction of antagonistic dorsal and ventral body muscles, causing these animals to ‘shrink’ (White *et al.*, 1976). To investigate the genetic relationship between *unc-25* (which encodes glutamic acid decarboxylase), the GABA biosynthetic enzyme (Jin *et al.*,

**Table I.** Behavioral analysis

Locomotion assays							
Strain	Amplitude		Wavelength		Locomotion		
Wild type (N2)	0.10 ± 0.04 (40)		0.42 ± 0.02 (40)		+		
<i>jnk-1(gk7)</i>	0.22 ± 0.02 (40)		0.48 ± 0.05 (40)		slightly sluggish		
<i>jnk-1(gk7)</i> [Myc-JNK-1 $\alpha$ ] <sup>a</sup>	0.14 ± 0.02 (20)		0.43 ± 0.05 (20)		+		
<i>jnk-1(gk7)</i> [Flag-JNK-1 $\beta$ ] <sup>b</sup>	0.12 ± 0.03 (20)		0.44 ± 0.04 (20)		+		
<i>jnk-1(gk7)</i> [vector alone] <sup>c</sup>	0.23 ± 0.03 (10)		0.44 ± 0.03 (10)		slightly sluggish		
<i>unc-25(e156)</i>	0.09 ± 0.04 (20)		0.36 ± 0.04 (20)		shrinker <sup>d</sup>		
<i>jnk-1(gk7);unc-25(e156)</i>	0.12 ± 0.02 (20)		0.48 ± 0.05 (20)		shrinker		

Sensory responses					
Strain	Osmotic avoidance escape time (min)	Foraging	Defecation (s)	Light nose touch (% responding)	Harsh body touch (% responding)
Wild type (N2)	9 ± 1 (100, 5)	+	44 ± 2 (10, 10)	80 ± 5 (20, 10)	85 ± 2 (20, 10)
<i>jnk-1(gk7)</i>	8 ± 1 (100, 5)	slow	45 ± 3 (10, 10)	44 ± 6 (20, 10)	51 ± 5 (20, 10)
<i>jnk-1(gk7)</i> [Myc-JNK-1 $\alpha$ ] <sup>a</sup>	ND	+	ND	75 ± 5 (10, 10)	80 ± 4 (10, 10)
<i>jnk-1(gk7)</i> [Flag-JNK-1 $\beta$ ] <sup>b</sup>	ND	+	ND	71 ± 5 (10, 10)	82 ± 2 (10, 10)
<i>jnk-1(gk7)</i> [vector alone] <sup>c</sup>	ND	slow	ND	47 ± 4 (10, 10)	58 ± 3 (10, 10)

Volatile chemical odorants and salt chemotaxis assays (% responding)							
Strain	Chemical odorants <sup>e</sup>					Salts	
	Alcohol (isoamyl alcohol)	Ketone (acetone)	Ester (ethyl acetate)	Aromatic compound (benzonitrile)	Ether (diethyl ether)	NH <sub>4</sub> Cl	NaCl
Wild type (N2)	95	72	77	69	71	+	+
<i>jnk-1(gk7)</i>	90	77	70	72	76	+	+

ND, not determined.

Errors indicate SEM. The number and trials per animal are shown in parentheses.

Young adult *jnk-1(gk7)* harboring as extrachromosomal arrays: <sup>a</sup>pPD49.78-Myc-JNK-1 $\alpha$  and pPD49.83-Myc-JNK-1 $\alpha$ ; <sup>b</sup>pPD49.78-Flag-JNK-1 $\beta$  and pPD49.83-Flag-JNK-1 $\beta$ ; and <sup>c</sup>pPD49.78 and pPD49.83 as control plasmids, were heat treated for 30 min at 33°C, and individual worms were placed on NGM plates seeded with *E.coli* at 20°C. Animals were monitored every 4 h to select worms showing a wild-type movement pattern. After 24 h, amplitude, wavelength and locomotion were scored. Amplitude and wavelength were measured in millimeters. Transgenic animals exhibited the same locomotion defects and modest reductions in mechanosensory responses in the absence of heat as the parental strain.

<sup>d</sup>An animal was scored as a shrinker if it failed to move backwards and decreased in body length (McIntire *et al.*, 1993a,b).

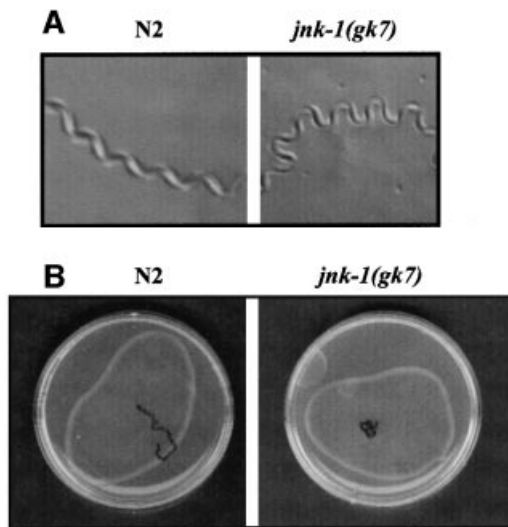
<sup>e</sup>Chemical odorant and salt chemotaxis studies were performed as described (Culotti and Russell, 1978; Bargmann *et al.*, 1993).



1999) and *jnk-1*, an *unc-25(e156);jnk-1(gk7)* double mutant was constructed. Similarly to results obtained with *unc-25(e156);jkk-1(km2)* worms (Kawasaki et al., 1999), loss of *unc-25* function reversed the *jnk-1(gk7)* movement phenotype; the *unc-25(e156);jnk-1(gk7)* double mutant transversed the bacterial lawn in a manner similar to *unc-25(e156)* single mutants, and the amplitude of the wave of motion was normalized (Table I). In order to investigate if other genes involved in the GABA signaling pathway were also genetically related to *jnk-1*, we used double-stranded RNA-mediated interference (RNAi) (Fire et al., 1998) to inactivate *unc-25*, *unc-30* (which encodes a homeobox gene that controls the fate of 19 type-D GABAergic neurons) (Eastman et al., 1999), and *unc-47* (which encodes a vesicular GABA transporter) (McIntire et al., 1997). *unc-25*, *unc-30* or *unc-47* inactivation by RNAi resulted in a shrinker phenotype in

both wild-type and *jnk-1(gk7)* animals, indicating that RNAi was an effective technique for inactivation of these genes (Table II). Further, inactivation of *unc-30* or *unc-47*, like *unc-25*, reversed the *jnk-1(gk7)* movement defect, whereas injection of a non-specific dsRNA for green fluorescent protein (GFP), was without effect. These investigations provide additional support for the concept that the locomotion defect resulting from loss of JNK-1 function is mediated through a GABAergic pathway. Unfortunately, *unc-49* (which encodes three distinct GABA<sub>A</sub> receptor-like subunits) (Bamber et al., 1999), like other genes in the nervous system (Tavernarakis et al., 2000), could not be inactivated by RNAi, and thus is not included in this analysis.

The modest mechanosensory deficits reported above, however, could still be observed in the *unc-25(e156);jnk-1(gk7)* double mutant (not shown), as well as in *jnk-1(gk7)* worms subjected to *unc-25*, *unc-30* or *unc-47* RNAi (Table II). These results suggest that JNK-1 proteins are also involved in the regulation of functions independent of GABA action in *C.elegans*.



**Fig. 7.** *jnk-1(gk7)* mutants worms exhibit defects in coordination of body movement. Single young adult N2 and *jnk-1(gk7)* worms were spotted in the center of NGM plates and wave forms (A) and trajectory (B) evaluated at 5 min. Tracks inscribed in the bacterial lawn (B) were traced using a black pen.

### *jnk-1(gk7)* are hypersensitive to heavy metal stress

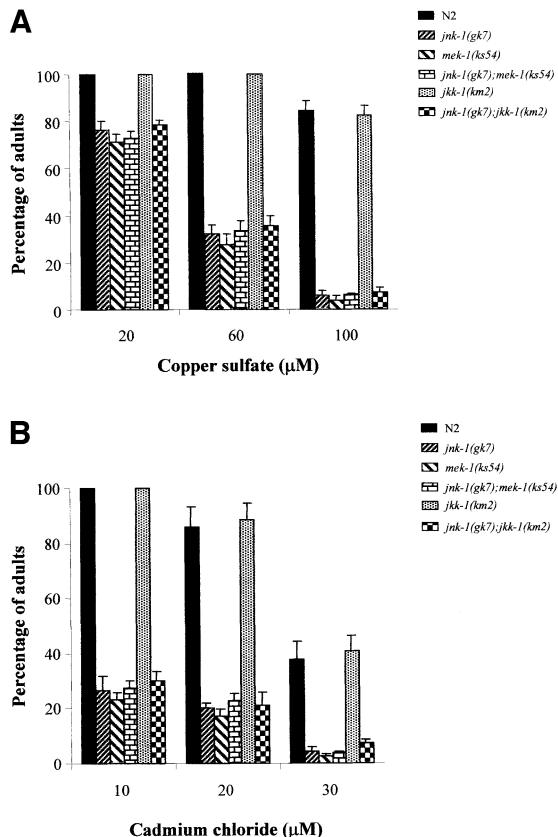
There is little genetic evidence validating the role of the JNK signaling pathway in stress response signaling in whole animals. Recently, it has been shown that the *C.elegans* MKK7 homolog MEK-1 is involved in some forms of stress response (Koga et al., 2000). Similarly to the *mek-1(ks54)* null allele, we found that *jnk-1(gk7)* mutants were also hypersensitive to copper and cadmium ions (Figure 8A and B). N2 animals grew well, becoming adults by 4 days in plates containing low concentrations of copper (Cu; <60  $\mu$ M) or cadmium (Cd; <10  $\mu$ M), while *jnk-1(gk7)* animals grew poorly. In fact most of the animals failed to reach the adult stage within 4 days. At high doses of Cu (100  $\mu$ M) or Cd (20–30  $\mu$ M), some N2 animals were viable whereas virtually all *jnk-1(gk7)* animals were arrested at young larvae stages. Furthermore, *jnk-1(gk7);mek-1(ks54)* displayed heavy metal sensitivity identical to that detected in *mek-1(ks54)* or *jnk-1(gk7)* (Figure 8A and B), suggesting that these kinases act in a common pathway. *jnk-1(gk7);mek-1(ks54)*, like *jnk-1(gk7);jkk-1(km2)*, continued to display

**Table II.** Inactivation of GABAergic regulatory genes in *jnk-1(gk7)* rescues the locomotion defect

RNAi	Strain	Locomotion assays			Sensory responses	
		Locomotion	Amplitude	Wavelength	Light nose touch (% responding)	Harsh body touch (% responding)
Uninjected	wild type (N2)	+	0.11 $\pm$ 0.03 (15)	0.45 $\pm$ 0.04 (15)	84 $\pm$ 4 (15, 10)	87 $\pm$ 4 (15, 10)
Uninjected	<i>jnk-1(gk7)</i>	slightly sluggish	0.24 $\pm$ 0.04 (15)	0.46 $\pm$ 0.04 (15)	42 $\pm$ 5 (15, 10)	53 $\pm$ 3 (15, 10)
<i>unc-30</i>	wild type (N2)	shrinker <sup>a</sup>	0.12 $\pm$ 0.04 (20)	0.42 $\pm$ 0.04 (20)	85 $\pm$ 3 (20, 10)	84 $\pm$ 3 (20, 10)
<i>unc-30</i>	<i>jnk-1(gk7)</i>	shrinker <sup>a</sup>	0.14 $\pm$ 0.03 (20)	0.43 $\pm$ 0.03 (20)	47 $\pm$ 4 (20, 10)	53 $\pm$ 5 (20, 10)
<i>unc-47</i>	wild type (N2)	shrinker <sup>a</sup>	0.09 $\pm$ 0.03 (20)	0.43 $\pm$ 0.03 (20)	82 $\pm$ 3 (20, 10)	85 $\pm$ 4 (20, 10)
<i>unc-47</i>	<i>jnk-1(gk7)</i>	shrinker <sup>a</sup>	0.12 $\pm$ 0.03 (20)	0.46 $\pm$ 0.05 (20)	48 $\pm$ 6 (20, 10)	55 $\pm$ 4 (20, 10)
<i>unc-25</i>	wild type (N2)	shrinker <sup>a</sup>	0.10 $\pm$ 0.03 (20)	0.40 $\pm$ 0.03 (20)	86 $\pm$ 4 (20, 10)	85 $\pm$ 2 (20, 10)
<i>unc-25</i>	<i>jnk-1(gk7)</i>	shrinker <sup>a</sup>	0.15 $\pm$ 0.04 (20)	0.45 $\pm$ 0.04 (20)	48 $\pm$ 5 (20, 10)	56 $\pm$ 4 (20, 15)
<i>gfp</i>	wild type (N2)	+	0.10 $\pm$ 0.03 (10)	0.44 $\pm$ 0.02 (10)	83 $\pm$ 3 (10, 10)	86 $\pm$ 5 (10, 10)
<i>gfp</i>	<i>jnk-1(gk7)</i>	slightly sluggish	0.23 $\pm$ 0.03 (10)	0.47 $\pm$ 0.04 (10)	43 $\pm$ 4 (10, 10)	53 $\pm$ 4 (10, 10)

Errors indicate SEM. The number and trials per animal are shown in parentheses. Amplitude and wavelength were measured in millimeters.

<sup>a</sup>An animal was scored as a shrinker if it failed to move backwards and decreased in body length (McIntire et al., 1993a,b).

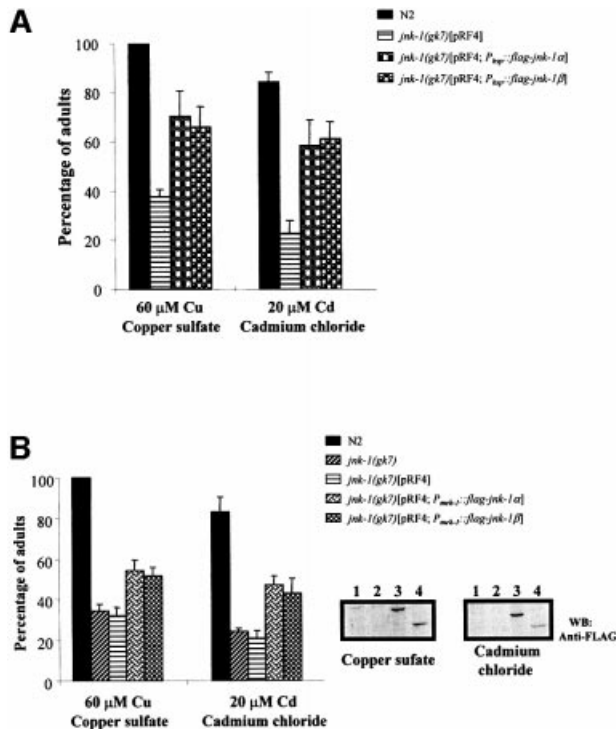


**Fig. 8.** *jnk-1(gk7)* mutant worms show similar hypersensitivity to heavy metals as *mek-1(ks54)*. Effect of copper sulfate (CuSO<sub>4</sub>) (A) and cadmium chloride (CdCl<sub>2</sub>) (B) in N2, *jnk-1(gk7)*, *mek-1(ks54)*, *jkk-1(km2)*, *jnk-1(gk7);mek-1(ks54)* and *jnk1(gk7);jkk-1(km2)*. Toxicity assays were performed as described by Koga *et al.* (2000). Briefly, four worms were allowed to lay eggs in normal NGMSR plates containing the indicated concentrations of heavy metals. After 6 h, worms were removed, and the number of eggs counted. The percentage ( $\pm$  standard errors) of worms reaching adulthood 4 days after egg laying is compiled from three experiments.

the modest reductions in touch responses observed in *jnk-1(gk7)* (not shown).

To determine whether restoring JNK-1 activity in *jnk-1(gk7)* reverts the heavy metal sensitivity, transgenic *jnk-1(gk7)* animals were generated harboring Flag-JNK-1 isoforms under the control of heat shock promoters. After heat shock induction, copper and cadmium sensitivity in *jnk-1(gk7)* transgenic worms was significantly, but not completely, rescued (Figure 9A). While only 30–35% of *jnk-1(gk7)* survived at 60  $\mu$ M copper, 65% of the transgenic worms survived. Similarly, only 20–25% of *jnk-1(gk7)* survived at 20  $\mu$ M cadmium while 55% of the transgenic worms survived (Figure 9A).

To investigate whether *jnk-1* expression in cells, where *mek-1* is normally expressed, rescues the heavy metal sensitivity, Flag-JNK-1 $\alpha$  or - $\beta$  were expressed in *jnk-1(gk7)* under the control of the *mek-1* promoter (*P<sub>mek-1</sub>*). *P<sub>mek-1</sub>::flag-jnk-1 $\alpha$*  or - $\beta$  transgenes were maintained as extrachromosomal arrays. As shown in Figure 9B, the increased sensitivity to copper (60  $\mu$ M) and cadmium (20  $\mu$ M) was partially rescued by expression of Flag-JNK-1 $\alpha$  and - $\beta$  proteins driven by the *mek-1*



**Fig. 9.** Rescue of *jnk-1(gk7)* heavy metal hypersensitivity. (A) Transgenic *jnk-1(gk7)* were generated harboring plasmids expressing Flag-tagged JNK-1 isoforms under the control of heat shock promoters, and pRF4 (dominant *rol-6*) plasmid as a selection marker. Young adult worms were heat treated for 30 min at 33°C, after 16 h allowed to lay eggs for 6 h onto plates containing cadmium (20  $\mu$ M) and copper (60  $\mu$ M), and survival assessed as in Figure 8. (B) *P<sub>mek-1</sub>::flag-jnk-1 $\alpha$*  or - $\beta$  transgenes were co-injected with the plasmid pRF4 into *jnk-1(gk7)*; progeny with a Roller phenotype were selected, and heavy metal sensitivity determined as in (A) (left panel). Expression of Flag-JNK-1 $\alpha$  or - $\beta$  in transgenic worms was confirmed at the end of these experiments by western blot (right panel). 1, *jnk-1(gk7)*; 2, *jnk-1(gk7)[pRF4]*; 3, *jnk-1(gk7)[pRF4; P<sub>mek-1</sub>::flag-jnk-1 $\alpha$* ; 4, *jnk-1(gk7)[pRF4; P<sub>mek-1</sub>::flag-jnk-1 $\beta$* .

promoter. Furthermore, after 4 days treatment with 10  $\mu$ M cadmium, an increase in phospho-JNK-1 was detected in N2, while *mek-1(ks54)* showed a phospho-JNK-1 level similar to untreated N2 (not shown).

***jnk-1(gk7)* reproduces the loss-of-function phenotypes of *jkk-1* and *mek-1* but not that of *mkk-4***

Although biochemical evidence supports the model that two MAPK kinases, MKK4 and MKK7, mediate activation of JNK, genetic evidence supporting this model is incomplete. While *jkk-1(km2)* and *mek-1(ks54)* null alleles each show isolated defects detected in *jnk-1(gk7)*, inactivation by RNAi of *mkk-4*, the *C.elegans* homolog of mammalian MKK4/SEK1, caused an egg-laying defect in N2 worms (Table III). No egg-laying defect was observed in *jnk-1(gk7)*, *jkk-1(km2)*, *mek-1(ks54)*, *jnk-1(gk7);jkk-1(km2)* or *jnk-1(gk7);mek-1(ks54)*. To investigate if the egg-laying defect observed in N2 worms where *mkk-4* was inactivated occurs independently of *jnk-1*, we inactivated *mkk-4* by RNAi in *jnk-1(gk7)*. As in N2, inactivation of *mkk-4* in a *jnk-1*-deficient worm still resulted in the egg-laying defect

**Table III.** *In vivo* effects of inactivation of *jkk-1*, *mek-1* and *mkk-4* genes by RNAi in wild-type and *jnk-1(gk7)* worms

RNAi	Strain	Development	Locomotion		Touch responses		% egg-laying defective
			Amplitude		Light nose (% responding)	Harsh body (% responding)	
Uninjected	wild type (N2)	normal	0.09 ± 0.03 (10)		85 ± 3 (10, 10)	84 ± 6 (10, 10)	0 (150)
Uninjected	<i>jnk-1(gk7)</i>	normal	0.22 ± 0.04 (10)		46 ± 4 (10, 10)	49 ± 3 (10, 10)	0 (150)
<i>jkk-1</i>	wild type (N2)	normal	0.17 ± 0.02 (15)		82 ± 6 (15, 10)	83 ± 5 (15, 10)	0 (150)
<i>jkk-1</i>	<i>jnk-1(gk7)</i>	normal	0.21 ± 0.02 (15)		42 ± 5 (15, 10)	53 ± 4 (15, 10)	0 (150)
<i>mek-1</i>	wild type (N2)	normal	0.11 ± 0.03 (15)		86 ± 4 (15, 10)	88 ± 6 (15, 10)	0 (150)
<i>mek-1</i>	<i>jnk-1(gk7)</i>	normal	0.21 ± 0.02 (15)		47 ± 5 (15, 10)	50 ± 5 (15, 10)	0 (150)
<i>mkk-4</i>	wild type (N2)	normal	0.10 ± 0.03 (15)		82 ± 5 (15, 10)	85 ± 4 (15, 10)	32 (48/150)
<i>mkk-4</i>	<i>jnk-1(gk7)</i>	normal	0.23 ± 0.02 (15)		44 ± 6 (15, 10)	51 ± 6 (15, 10)	28 (42/150)

Values indicated mean ± SEM. The number of animals and, when indicated, trials per animal are shown in parentheses, respectively. Locomotion and touch responses were performed as in Table I. *jkk-1* and *mek-1* correspond, respectively, to F35C8.3 and K08A8.1 genes predicted by BLAST. *mkk-4*, which is highly homologous to mammalian *mkk4/sek1*, corresponds to the F42G10.2 gene predicted by BLAST. For *mkk-4*, RNAi was performed with three different RNA fragments described in Materials and methods. All three yielded similar results. Data from *mkk-4(1)* are shown.

(Table III) further dissociating MKK-4 from JNK-1 in *C.elegans*.

## Discussion

In this study, we characterize the recently cloned *C.elegans jnk-1* gene (Kawasaki *et al.*, 1999) at the biochemical and genetic level. In mammalian cells, JNK protein kinases are encoded by three genes, which are alternatively spliced to create 10 JNK isoforms (Gupta *et al.*, 1996). In *C.elegans*, the system is simpler, a single gene, *jnk-1*, encodes two different protein isoforms generated either by alternative transcription start site selection or the use of alternative SL1 *trans*-splice sites. The JNK-1 $\alpha$  isoform described previously (Kawasaki *et al.*, 1999) has a unique N-terminus of 91 amino acids fused in-frame to the methionine residue that serves as the JNK-1 $\beta$  N-terminus. In contrast, the amino acid sequence of JNK-1 $\beta$  is conserved almost entirely within mammalian JNKs. Similarly to the insect and mammalian JNKs (Galcheva-Gargova *et al.*, 1994), diverse stimuli, murine MKK4/SEK1 as well as *C.elegans* MKK7 homologs activate both JNK-1 isoforms by dual phosphorylation on threonine and tyrosine residues within a TPY motif. Further, both JNK-1 isoforms appear capable of phosphorylating human c-Jun, ATF-2 and ELK-1 recombinant proteins, consistent with conservation of function. In contrast to knockouts of the conserved genes of the *Drosophila* JNK pathway and *jnk1/jnk2* in mice, a null mutation for *C.elegans jnk-1* is viable. Animals harboring the null allele, *jnk-1(gk7)*, did not display obvious developmental abnormalities. However, *jnk-1(gk7)* exhibited defects in locomotion and hypersensitivity to several heavy metals, phenotypes found in *C.elegans* mutants null for the *mkk7* homologs, *jkk-1(km2)* (Kawasaki *et al.*, 1999) and *mek-1(ks54)* (Koga *et al.*, 2000), respectively. *jnk-1(gk7)* also exhibited some mechanosensory defects that were not mimicked by *jkk-1(km2)* or *mek-1(ks54)*, suggesting the existence of additional mechanisms of *jnk* regulation in this species. Inactivation of *mkk-4* by RNAi also did not yield these mechanosensory deficits but rather resulted in an egg-laying defect in hermaphrodites. Despite biochemical activation of JNK-1 by murine MKK4/SEK1, this *mkk-4*-

deficient phenotype was not detected in *jnk-1(gk7)* worms. These results demonstrate that at least two distinct MKK7s regulate functions through JNK-1. Further, they suggest that MKK-4 acts through a kinase other than JNK-1. In this regard, an analysis of the 19 099 predicted proteins for *C.elegans* demonstrates the existence of 14 MAPKs (Plowman *et al.*, 1999), including three p38 homologs or potential homologs (Kawasaki *et al.*, 1999; A.Villanueva and R.N.Kolesnick, unpublished data).

Recent reports characterizing mice mutants for the JNK signaling pathway genes suggest that differential expression patterns of these genes may influence the role of this pathway in various tissues. In *C.elegans*, *jnk-1* and *jkk-1* appear to be co-expressed in most neurons and their processes, including the nerve ring, the head and tail ganglia and the dorsal and ventral nerve cords (Kawasaki *et al.*, 1999). In contrast, *mek-1* is expressed preferentially in pharyngeal muscles, uterine and intestine cells, and in some neurons in the nerve ring and ventral and anal ganglia (Koga *et al.*, 2000). These different expression patterns, together with the different phenotypes of *jkk-1* and *mek-1* mutant worms, suggest that the JNK pathway is regulated through compartmentalization of function in *C.elegans*.

In *C.elegans*, body muscle contraction depends on a balance between excitatory cholinergic (Lewis *et al.*, 1980) and inhibitory GABAergic inputs (McIntire *et al.*, 1993a,b). Animals in which DD and VD neurons have been killed by laser ablation (McIntire *et al.*, 1993b) or which carry mutations in several genes in the GABAergic pathway (McIntire *et al.*, 1993a) simultaneously contract the normally antagonistic dorsal and ventral body muscles, causing these animals to 'shrink' (White *et al.*, 1976). Recently, Kawasaki and co-workers reported that *jkk-1* regulates coordinated movement in D-type motor neurons by acting as a suppressor of GABA action (Kawasaki *et al.*, 1999). The present study shows that loss of JNK-1 function in *C.elegans* delivers an identical body movement phenotype as loss of JKK-1 function, and shows that the loss of UNC-25 (glutamic acid decarboxylase) activity suppresses this movement defect. Moreover, inactivation of *unc-30* or *unc-47* by RNAi also suppressed the movement phenotypes. Further, Kawasaki *et al.* demonstrated that selective *jkk-1* expression in the D-type motor neurons using the *unc-30* promoter rescues the *jkk-1(km2)*

movement defects. Since the UNC-30 homeodomain protein coordinates transcriptional regulation of *unc-25* and *unc-47* genes, taken together the results suggest that functional GABAergic synaptic transmission in D-type motor neurons is required for expression of the *jnk-1* locomotion defect. One attractive possibility is that normal action of JNK-1 is to negatively regulate GABA action, and the increased wave amplitude observed in *jnk-1* mutants is the result of increased GABAergic signaling. For example, the *C.elegans* JNK signaling pathway could regulate the quantity of GABA generated by the nervous system, the activity of the UNC-25 protein or the timing of GABA release. However, our results do not exclude other, less direct interactions between JNK-1 function and GABAergic transmission.

There is a little genetic evidence in mice or *Drosophila* regarding the significance of the JNK signaling pathway in stress responses in whole animals. The embryonic lethality of these mutants in different species makes it difficult to determine whether the JNK signaling pathway is involved in stress responses later in life. In mice, disruption of *mkk4*, *mkk7* or *jnk1/jnk2* cause early embryonic lethality (Yang *et al.*, 1997a; Kuan *et al.*, 1999; Dong *et al.*, 2000), while the *jnk3* knockout displays defects in stress-induced AP-1 transcriptional activity and apoptosis in the hippocampus (Yang *et al.*, 1997b). The non-essential nature of *jnk-1*, *jkk-1* (Kawasaki *et al.*, 1999), *mek-1* (Koga *et al.*, 2000) and *mkk-4* for embryogenesis allows an evaluation of the role of these proteins in stress responses. So far, we have shown that the animals harboring a null allele for *jnk-1*, like *mek-1* null mutants, are hypersensitive to the heavy metals cadmium and copper. Consistent with the pattern of *mek-1* expression in the intestine, Koga *et al.* suggested possible explanations for the hypersensitivity of *mek-1(ks54)* mutant worms to heavy metals: (i) animals might fail to arrest pumping in response to these toxic metals and consequently be exposed to higher doses; (ii) animals might take up more metal into the intestine independent of pumping; or (iii) these metals may alter nutritional status unfavorably (Koga *et al.*, 2000). Further studies will be needed to determine the role of MEK-1 and JNK-1 in the normal disposal/metabolism of these heavy metals to gain insight into this issue.

In conclusion, our results confirm the existence of a highly conserved JNK signaling pathway in *C.elegans*. This pathway is involved in the regulation of body movement coordination and the response to heavy metals, apparently via compartmentalized expression of two MKK7s, JKK-1 and MEK-1, respectively. However, as in mice and *Drosophila*, we do not find genetic evidence for MKK4 signaling through JNK in *C.elegans*. Investigations into functional and genetic associations between JNK-1 and other conserved components of this pathway, the relationship to other MAPKs and the delineation of JNK-1 substrates should allow for a more complete understanding of the *C.elegans* JNK pathway in the near future.

## Materials and methods

### Growth and synchronization of *C.elegans*

All strains were maintained as hermaphrodites at 20°C on NGM standard plates fed with the *Escherichia coli* OP50 bacterial strain, as described

(Brenner, 1974). Worms were observed either under a dissecting microscope (Leica MZ8) or using a Zeiss Axioplan with Nomarski optics. Worms from each developmental stage were obtained from synchronized liquid cultures: L1 larvae were collected 7 h after hatching, L2 larvae at 17 h, L3 larvae at 27 h, L4 larvae at 38 h, and adult worms were harvested at 45 h.

### RNA preparation, RT-PCR, RACE, northern blot and RT-SSCP analysis

Total RNA was extracted by a single-step method (Chomczynski and Sacchi, 1987).

**RT-PCR.** Two micrograms of total RNA were reverse transcribed to cDNA using pd(N<sub>6</sub>) random hexamers (Amersham-Pharmacia Biotech) as described (Villanueva *et al.*, 1998). Full-length transcripts of *jnk-1α* and *jnk-1β* were amplified using the 5' primers 5'-CCCAAGTTT-GAGAACCATCA-3' and 5'-CCCAAGTTT-GAGAGAAGCGTG-3', respectively, and the common 3' primer 5'-TCAGGAATAAATGTC-ATGGGTTCTTG-3'. *mek-1* cDNA was amplified using 5'-ATGGAG-AGAGACTTCGACCTT-3' and 5'-TTATCCGCACTCGCCCATCAC-ATC-3' primers. The reactions were performed in a thermal cycler for 30 cycles (94°C, 60 s; 59–66°C, 90 s; 72°C, 120 s) in a final volume of 30 μl. Identities of the amplified fragments were confirmed by sequencing.

**RACE.** For analysis of the 5' end, two rounds of RACE were performed using a RACE Kit (Gibco-BRL) following the manufacturer's directions. Briefly, for the first round, total RNA was reverse transcribed to cDNA using a primer, 5'-TCCTAGTCCACTGATCGATA-3', derived from sequence within exon 8, and amplified using AAP (Gibco-BRL) and 5'-ACGTATCAGCTCTCCAAATA-3' primers. Nested PCR amplification subsequently was performed using UAP (Gibco-BRL) and 5'-TTCGAGCCAATCCGAAATCCAA-3' primers. A second round of RACE was performed using the sequence 5'-AGCACAAAA-GGAGAC-3' derived from exon 12 for reverse transcription, AAP and 5'-TAGAGATCGAAATGTGACC-3' primers for amplification, and UAP and 5'-AAAACATGGAATCAGGAAT-3' primers for nested PCR. For analysis of the 3' end, total RNA was reverse transcribed to cDNA using a commercial AP primer, and cDNA was amplified using the 5'-TTGGATTTCGGATTGGCTCGAA-3' and 3' UAP primers. PCR products were cloned directly into the TOPO system (Invitrogen) and individual clones were selected and sequenced.

**Northern blot.** Thirty micrograms of total RNA from different developmental stages were electrophoresed in 1.2% agarose gels containing formaldehyde, transferred onto a Hybond N+ Nylon membranes (Amersham-Pharmacia Biotech) following the manufacturer's instructions, and the RNA was UV-cross-linked to the membrane using a Stratalinker (Stratagene). The blots were pre-hybridized with ExpressHyb hybridization solution (Clontech) for 1 h, and then probed with random-primed (Prime-it II, Stratagene), [ $\alpha$ -<sup>32</sup>P]dCTP (NEN Life Science)-labeled full-length *jnk-1β* cDNA at 68°C for 1 h. The blots were washed under stringent conditions (65°C and 0.1% SSC/0.1% SDS) and exposed for 48 h to Kodak XAR-5 film at -70°C.

**RT-PCR/SSCP.** The cDNA region coding for protein kinase subdomains VIII–XI was amplified using 5'-ACTATCGGGCACCGG-AAGTTA-3' and 5'-TGTGACCGTAAAACATGGAATCAGGA-3' primers, and SSCP was performed as described (Villanueva *et al.*, 1998).

### Plasmid construction

**Mammalian expression vectors.** Epitope-tagged *jnk-1* isoforms and *mek-1* were generated by PCR. The Flag epitope MDYKDDDK sequence was introduced at the C-terminus of *jnk-1β* using 5'-GGTACCG-CCACCATGGAACCATCCTTATTTCAT-3' and 5'-CTCGAGTCT-ACTTGTCATCGTCTCCTTGTAGTCGGAATAAATGTCATGGGT-TCT-3' primers and cloned into the mammalian expression vector pCEP4 (Invitrogen) to create pCEP4-Flag-JNK-1β. *jnk-1α* was amplified using 5'-GGATCCATGGAGGAACGATTATCCACA-3' and 5'-AAGCTTT-CAGGAATAAATGTCATGGGT-3' primers, and cloned into the mammalian expression vector pCMV3B (Stratagene), that contains the c-Myc epitope MEQKLISE sequence in the N-terminus, to create pCMV3B-Myc-JNK-1α. *mek-1* was amplified using 5'-GGTACCATGGAGA-GAGACTTCGAC-3' and 5'-GCGGCCGCTTATCCGCACTCGCC-3' primers and cloned into the mammalian expression vector pHM6 (Boehringer) that contains the HA epitope YPYDVPDYA sequence in the N-terminus to create pHM6-HA-MEK-1. Amino acids Thr185 and Tyr187 of JNK-1 (corresponding to human JNK1 Thr183 and Tyr185) were substituted with alanine and phenylalanine, respectively, using pCMV3B-Myc-JNK-1α and pCEP4-Flag-JNK-1β mammalian expression vectors as templates, by oligonucleotide mutagenesis using 5'-TCGAAGTGGATTGAGGCATTCATGATGGCTCTTTTCGTTGTG-

ACAAGATACTATCGGGC-3' and 5'-GCCCGATAGTATCTTGTCACAACGAAAGGAGCCATCATGAATGCCTCAATCGCAGTTCCGA-3' primers and the One Site Mutagenesis kit (Stratagene). Similarly, a kinase-inactive form of MEK-1, in which the conserved Lys99 in the ATP-binding domain was substituted with arginine, was generated using pHM6-HA-MEK-1 as a template and the set of primers 5'-GTGATCATGGCTGTGAATACGATGCCTCGGACG-3' and 5'-CGTCCGAGGCATCGTATTACAGCCATGATCAC-3'. Mutant *jnk-1* isoforms, *jnk-1(gk7) $\alpha$*  and *jnk-1(gk7) $\beta$* , were amplified by RT-PCR from the *jnk-1(gk7)* knockout and cloned into pCMV3B to generate pCMV3B-Myc-JNK-1(*gk7*) $\alpha$  and Myc-JNK-1(*gk7*) $\beta$ .

*Escherichia coli* expression vectors. The open reading frames of the *jnk-1 $\alpha$*  and *jnk-1 $\beta$*  isoforms were cloned into the prokaryotic expression vector pTrcHisB (Invitrogen) to create pTRCHis-JNK-1 $\alpha$  and pTRCHis-JNK-1 $\beta$ . Expression and purification of His-tagged JNK-1 proteins were carried out using the TALON system (Clontech) according to the manufacturer's instructions. Purified proteins were used to validate the specificity of antibody binding.

*Caenorhabditis elegans* expression vectors. *flag-jnk-1 $\alpha$*  and *flag-jnk-1 $\beta$*  cDNAs were amplified by PCR using mammalian expression vectors as templates and cloned into the heat shock-inducible vectors pPD49.78 and pPD49.83, placing them under control of heat shock promoters. These promoters direct protein expression in the majority of *C.elegans* tissues (Jones et al., 1986; Stringham et al., 1992). *P<sub>mek-1</sub>::flag-jnk-1 $\alpha$*  or *- $\beta$*  transgenes, in which *flag-jnk-1 $\alpha$*  or *- $\beta$*  cDNAs are under control of the *mek-1* promoter (*P<sub>mek-1</sub>*), were constructed using a PCR fusion approach in several steps. Initially, a 4750 bp DNA fragment, corresponding to the putative *mek-1* promoter and initial ATG was amplified from N2 genomic DNA using 5'-AAATCTCCGTAGATCTT-AAATGC-3' and 5'-GTCATCCTTGTAAATCCATCTTTTACCTTAA-AATTA-3' primers. The 3' primer contains the nucleotide sequences of the Flag epitope tag. Next, DNA fragments including the complete *flag-jnk-1 $\alpha$*  or *- $\beta$*  cDNA, respectively, were amplified using mammalian expression vectors as templates, and 5'-TGGAGCTCCACCGCGG-TGGC-3' and 5'-TGACCGTAAAACATCAGGAATAAATG-TCATG-3' primers. A 2100 bp segment of the *jnk-1* 3'-untranslated region (UTR), which include 1900 bp beyond the polyadenylation site, were amplified using N2 genomic DNA as a template and 5'-CATGAC-ATTATCTCTGATTCATGTTTTACGGTC-3' and 5'-CTAGTG-CATTCTCACCGAGACTG-3' primers. Fusion between *flag-jnk-1* cDNAs and the *jnk-1* 3'-UTR was performed by combing 1.5  $\mu$ l of each PCR using 5'-ATGGATTACAAGGATGACGACGATAAG-3' and 5'-TGTCCATCAGTTGCTATAATGC-3' primers. Finally, PCR products [*P<sub>mek-1</sub>::flag* and *flag-jnk-1* 3'-UTR] were fused by combing 1.5  $\mu$ l of each PCR and subsequent amplification with 5'-GGTACCCTGGTGGTCTTAAGACTGGC-3' and 5'-GCGGCCGCTTCTCGTGATGTTCTTGATGGATCC-3' nested primers from the beginning of the *mek-1* promoter and at the end of the *jnk-1* 3'-UTR region, respectively. The fusion product was inserted between the *KpnI* and *NorI* sites of TOPO vector (Invitrogen). For all PCRs, a mixture of *Taq* polymerase and proofreading *PwoI* polymerase (Boehringer Mannheim) was used to ensure fidelity of the reaction, and the fusion fragment sequences confirmed by automated DNA sequencing.

### Reversal of *jnk-1(gk7)* defects

To generate transgenic animals, young adult *jnk-1(gk7)* were injected into both gonads with 25–50 ng/ $\mu$ l DNA with or without pRF4 plasmid (dominant *rol-6* marker at 80 ng/ $\mu$ l). For analysis of the recovery of normal movement and touch responses, *jnk-1(gk7)* worms were injected with heat shock plasmids containing *flag-jnk-1 $\alpha$*  or *- $\beta$*  cDNAs without a selection marker. After 30 min at 33°C, young adults were monitored every 4 h to select worms showing a wild-type movement pattern (wave amplitude, locomotion and trajectory). For recovery from heavy metal hypersensitivity, the same heat shock constructs were co-injected into *jnk-1(gk7)* worms with pRF4 plasmid, as a selection marker, and animals with a Roller phenotype were selected, heat treated, and after 16 h allowed to lay eggs for 6 h onto plates containing heavy metals. For evaluating the expression of JNK-1 via the MEK-1 promoter, *P<sub>mek-1</sub>::flag-jnk-1 $\alpha$*  or *- $\beta$*  transgenes were co-injected with pRF4 into *jnk-1(gk7)* progeny with the Roller phenotype were selected, and heavy metal sensitivity assayed. All transgenes were maintained as extra-chromosomal arrays. The presence of the different plasmids was confirmed in individual worms at the end of each experiment by specific PCR.

### Cell culture and transient transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. Transient transfection was performed with lipofectAMINE according to the manufacturer's recommendations (Life Technologies). After 48 h, transfected cells were treated with UV light (40 J/m<sup>2</sup>) or  $\gamma$ -radiation (20 Gy) followed by incubation at 37°C for 20 min. Another set of cells was treated with sorbitol (400 mM), TNF- $\alpha$  (100 ng/ml; Promega), LPS (1  $\mu$ g/ml; Sigma), H<sub>2</sub>O<sub>2</sub> (75  $\mu$ M) or heat shocked at 42°C for 20 min. Cells were lysed in buffer [20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, 10  $\mu$ M leupeptin, 25  $\mu$ g/ml aprotinin, 100  $\mu$ M phenylmethylsulfonyl fluoride]. Insoluble material was removed by centrifugation at 14 000 g for 10 min at 4°C and total protein measured by the Bradford method using the Bio-Rad protein assay.

### Western blot analysis

To verify expression levels of the different epitope-tagged proteins transiently transfected in COS-7 cells, 15  $\mu$ g of whole-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Amersham-Pharmacia Biotech), probed with antibodies for 1 h and specific binding was detected by the enhanced chemiluminescence method (Amersham Life Science).

### Immunoprecipitation

Epitope-tagged proteins were isolated by immunoprecipitation from cell lysates. Aliquots of 0.5–1 mg of whole-cell lysate were incubated for 2 h at 4°C with 1  $\mu$ g of anti-FLAG M2 (Stratagene), anti-HA 12CA5 or anti-Myc (Roche Molecular Biochemicals) monoclonal antibodies. Immune complexes were incubated further for 1 h at 4°C with protein AG-Sepharose (Santa Cruz), recovered by centrifugation and washed six times in 1 ml of lysis buffer containing 500 mM NaCl. An additional 1 ml wash in kinase buffer was performed when the immune complexes were used for kinase assays.

### Protein kinase assays

Immune complex kinase assays were performed in a final volume of 40  $\mu$ l of kinase buffer (25 mM MOPS pH 7.4, 25 mM  $\beta$ -glycerophosphate, 25 mM MgCl<sub>2</sub>, 2 mM DTT and 100  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>). Protein kinase reactions were initiated by addition of recombinant substrate proteins [GST-c-Jun (Upstate Biotechnology), GST-ATF-2 or GST-ELK-1 (New England Biolabs)] and kinase buffer containing 250  $\mu$ M ATP and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; NEN Life Science). Reactions were incubated for 30 min at 30°C and terminated by the addition of an equal volume of Laemmli sample buffer.

### Generation of null alleles

Worms carrying a homozygous deletion of *jnk-1* [named *jnk-1(gk7)*] were isolated at the *C.elegans* Reverse Genetics Core Facility of Vancouver, British Columbia, Canada, by PCR screening of a chemical (formaldehyde) mutagenesis library using the primers 5'-CTCTGCGDCTTCTTCCATCT-3' and 5'-GAAGAGAAGTCAAACGGCG-3'. Nested PCR was performed using primers 5'-AAGACATCCAAA-CGGACAGC-3' and 5'-TCATACCTTGCCGATTTTC-3'. Mutant animals were backcrossed five times onto the wild-type Bristol N2 background before phenotypic analysis.

### Strain construction

To generate the *unc-25(e156);jnk-1(gk7)*, *jnk-1(gk7);jkk-1(km2)* and *jnk-1(gk7);mek-1(ks54)* double mutant strains, *jnk-1(gk7)* males were mated with CB156 [genotype: *unc-25(e156)*] (Brenner et al., 1974), KU2 [genotype: *jkk-1(km2)*] (Kawasaki et al., 1999) or FK171 [genotype: *mek-1(ks54)*] (Koga et al., 2000) hermaphrodites, respectively. F<sub>2</sub> *unc-25(e156)/+;jnk-1(gk7)/jnk-1(gk7)* hermaphrodites, selected by PCR, were allowed to self-fertilize to generate the *unc-25(e156);jnk-1(gk7)* double mutant strain. *unc25(e156)* mutant alleles carry a point mutation in codon 383 (TGG→TAG) that generates a new *XbaI* restriction site. This site allows discrimination of the wild-type and *unc-25(e156)* alleles by restriction fragment length polymorphism (RFLP)/PCR using the 5' primer 5'-GGCACAATGATGTTTTCAAGCTTTGGTTGATCT-3' and the 3' primer 5'-GCTCACCGAAACTCACATTCTAGATAATCAACT-3'. F<sub>2</sub> *jnk-1(gk7)/jnk-1(gk7);jkk-1(km2)/+* and *jnk-1(gk7)/jnk-1(gk7);mek-1(ks54)/+* hermaphrodites were selected by PCR, and allowed to self-fertilize to generate *jnk-1(gk7);jkk-1(km2)* and *jnk-1(gk7);mek-1(ks54)*, respectively.

**Double-stranded RNA-mediated interference (RNAi)**

A 2 µg aliquot of total RNA was reverse transcribed to cDNA using pd(N<sub>6</sub>) random hexamers, as above, and either full-length cDNA for *unc-25*, *unc-30*, *unc-47* or a common N-terminus region of 717 bp including amino acids 1–239 of all *unc-49* isoforms were amplified using the following primers: *unc-25*, 5'-ATGTCCTCTGCTGCTGCTGAC-3' and 5'-TTATTCCAAAGACTCTCCAAT-3'; *unc-30*, 5'-ATGGATGACAA-TACGGCCACA-3' and 5'-CTAAAGTGGTCCACTGTACTG-3'; *unc-47*, 5'-ATGGCGTCTCGAATAGATTCAA-3' and 5'-TTAAGATCAGCAGAGTTGAT-3'; *unc-49*, 5'-ATGGCTCGTCCATTACACTTA-3' and 5'-CATTGGACACGTTGCAGTGAC-3'. *mkk-4*, which corresponds to the gene F42G10.2 predicted by BLAST, was amplified using the following different set of primers: (i) 5'-CTTTCGACAGG-CACATTA-3' and 5'-TGCAAGATACGGCTGACA-3'; (ii) 5'-TGTCAGCCGTATCTTGCA-3' and 5'-CTTAGCCTCTTCAATTCG-3'; (iii) 5'-ACATAGAGAACAATCCGC-3' and 5'-GTCTGGATGT-AGAATTGGA-3'. RNAi using the three different *mkk-4* primer sets yielded identical data. *gfp* was used as a negative control, while *bir-1* and *unc-22* were used as positive controls in the RNAi experiments. PCR products were cloned into the pCRII-TOPO dual promoter vector using the TOPO system (Invitrogen) and the identities of the amplified fragments confirmed by sequencing. RNAs were *in vitro* transcribed using T7 and SP6 RNA polymerases according to standard protocols. Sense and antisense strands were mixed prior to injection to 500 ng/µl and then annealed. Young adult wild-type (N2) and *jnk-1(gk7)* hermaphrodites were injected into both gonads. After 12 h, injected animals were transferred to fresh plates (Fire *et al.*, 1998).

**Behavioral assays**

Assays for osmolarity, volatile repellents, chemoattractants and chemorepellents, light nose touch, hard body touch, dauer formation, defecation reflex and toxicity assays were performed as previously described (Ward, 1973; Culotti and Russell, 1978; Golden and Riddle, 1982; Bargmann *et al.*, 1993; Kaplan and Horvitz, 1993; Hart *et al.*, 1995, 1999; Maricq *et al.*, 1995; Chou *et al.*, 1996; Koga *et al.*, 2000).

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