MRP-1, a member of the ABC transporter superfamily, participates in the decision of the transition to the diapause stage, called dauer larva, in *C. elegans*

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Summary

When living organisms encounter inappropriate environment for their survival and reproduction, they either escape from the environment (e.g., bird migration or herbivore migration in African Content) or adapt to the environment by physiological changes. Dormancy or diapause is a representative example of such adaptation and observed in various organisms. Studies on dormancy have been performed mostly in the fields of physiology, ecology and biochemistry. However, the molecular mechanisms of the regulation of dormancy are not well known.

The nematode *C. elegans* also has a diapause stage, which is called dauer larva. The dauer larva has common features of dormant animals, i.e., low metabolism, no feeding, accumulation of fat, and resistance to stress. Since many molecular biological and genetic techniques are available, *C. elegans* is a good model organism for studying the regulation of transition into the diapause stage at molecular and cellular levels. During the life cycle, *C. elegans* grows up to adults through 4 larval stages (L1-L4) in 2-3 days at 25°C. But under inadequate conditions for growth, that is, under reduced food availability, crowding, and high temperature, animals arrest development and form dauer larvae corresponding to the 3rd larval stage. Dauer larvae can live for several months without feeding, while the life span in normal development is about 2-3 weeks. When the environmental conditions are improved, dauer larvae molt to normal L4 larvae and resume the life cycle. Like insects, the nervous system is involved in the diapause of *C. elegans*; some neurons in a pair of head sensory organs called amphids have been shown to control dauer larva formation.

Genes that regulate dauer larva formation have been studied by isolation and characterization of mutants that show abnormality in this function. These mutants consist of two groups: dauer-constitutive (*daf-c*) mutants, which form dauer larvae even under the conditions of abundant food availability and no crowding, and dauer-defective (*daf-d*) mutants, which do not form dauer larvae even under the conditions of extreme crowding and starvation. The genetic pathways of dauer formation have been revealed by epistasis tests of these mutations and molecular cloning of the mutated genes. At least four signal transduction pathways control dauer larva formation: cGMP-related signaling pathway, TGF-ß signaling pathway, insulin signaling pathway and recently suggested steroid hormone signaling pathway.

In addition to these mutations that show abnormal phenotypes in dauer larva formation by themselves, mutations that show the dauer-constitutive phenotype only in the background of another mutation have been discovered and called synthetic dauer-constitutive mutations. A great majority of them were isolated by other phenotypes and later found to show this phenotype

when double mutants of these mutations were constructed. *unc-31* mutation is one of such mutations, while *unc-31* gene encodes a homologue of CAPS (calcium activated protein for secretion), which is required for the exocytosis of dense core vesicles, which contain neuropeptides and biogenic amines. Mutants in this gene show many phenotypes: slow locomotion, defective egg-laying, constitutive pharyngeal pumping, etc., but essentially the wild type phenotype concerning dauer larva formation except at very high temperature (27°C), at which *C. elegans* cannot reproduce.

To identify new genes regulating dauer formation and to discover new mechanisms, 44 synthetic dauer-constitutive mutants were isolated in the *unc-31(e169)* background, mapped and named *sdf* (**s**ynthetic abnormality in **d**auer **f**ormation) mutants in our laboratory.

In this study, I cloned one of the mutant genes, *sdf-14* gene, by positional cloning, and analyzed its function on dauer larva formation. *sdf-14* gene encoded MRP-1 (multidrug resistance-associated protein-1), a member of the ABC transporter superfamily. ABC transporters export or import a wide variety of substrates by directly coupling these functions with the energy of ATP hydrolysis. Human MRP1 has been reported to export unnecessary compounds (conjugates, xenobiotics and detoxification products) from inside cells to outside. Since *C. elegans* MRP-1/SDF-14 had homology to human MRP1 throughout the amino acid sequence, it was predicted that, like human MRP1, *C. elegans* MRP-1/SDF-14 consists of 3 membrane spanning domains (MSDs) and 2 nucleotide binding domains (NBDs). The two mutant alleles of *sdf-14*, *ut151* and *ut155*, had missense mutations in NBD1, while another allele, *ut153*, had a mutation at the splice acceptor site of the 4th intron. In addition to the four MRP-1 isoforms that have been reported already, I found a new isoform, e-type. These isoforms seemed to differ in their functions, because the b- and c-type isoforms rescued the dauer formation abnormality of the *unc-31(e169);sdf-14(ut153)* double mutant, but the e-type isoform did not. These isoforms had variant copies of exon 13, suggesting that exon 13 may code for amino acid sequences that contribute to substrate specificity. The wild type human *MRP1* cDNA driven by the *sdf-14* promoter, but not the mutant human *MRP1* cDNA, rescued the dauer-constitutive phenotype of the *unc-31(e169);sdf-14(ut153)* double mutant. Furthermore, the rescue was partially canceled by the addition of a human MRP1 inhibitor. Those results strongly suggested that *C. elegans* MRP-1/SDF-14 acts as an exporter like human MRP1 in the regulation of dauer larva formation. A functional *sdf-14*::GFP fusion gene was expressed in many cells, i.e., pharyngeal cells, pharynx-intestinal valve cells, intestinal cells, intestinal-rectum valve cells, vulval epithelial cells, some neurons, and hypodermal seam cells. Moreover, expression in at least two of the three types of cells (neurons, intestinal cells and pharyngeal muscle cells) was needed for the rescue of the dauer formation abnormality of the *unc-31(e169);sdf-14(ut153)* mutant.

Epistasis analysis revealed that MRP-1/SDF-14 acts neither in the cGMP-related signaling pathway nor in the TGF-ß signaling pathway. MRP-1/SDF-14 may act in the insulin or steroid hormone signaling pathway. Alternatively, it may act in an unknown pathway, or has indirect influence on many pathways. Sodium arsenite, which is a substrate of human MRP1, induced dauer formation of the *unc-31(e169)* mutant, and *sdf-14* mutations enhanced this effect, while *sdf-14* single mutants did not form dauer larvae even in the presence of sodium arsenite at 27°C. These results suggest that wild type MRP-1/SDF-14 molecules seemed to suppress dauer larva formation by exporting sodium arsenite or unidentified intrinsic substance of which accumulation may cause of dauer formation due to *sdf-14* mutations.

Contents

Introduction

When living organisms encounter inappropriate environment for their survival and reproduction, they either escape from the environment (e.g., bird migration or herbivore migration in African Content) or adapt to the environment by physiological changes. The dormant state is a representative example of such adaptation and observed in various organisms, e.g., hibernation of Mammalia, Reptilia and Amphibia, diapause of insects, dormant buds of plants, and sporulation of fungi and bacteria. The dormancy of these organisms has been studied mostly by physiological examinations, ecological observations and biochemical analyses. The studies of insects and mammals revealed that their dormancy is controlled by hormones secreted by the nervous system (Yamashita O, 1993; Agui N, 1993; Hodkova M, 1993; Kondo N, 2000). However, the molecular mechanisms of the regulation of dormancy are not well known.

The nematode *C. elegans* also has a diapause stage, which is called dauer larva. The dauer larva has common features of dormant animals, i.e., low metabolism, no feeding, accumulation of fat, and resistance to stress (Riddle, 1988; Riddle & Albert, 1997). Since many molecular biological and genetic techniques are available, *C. elegans* is a good model organism for studying the regulation of transition into the diapause stage at molecular and cellular levels.

During the life cycle, *C. elegans* grows up to adults through 4 larval stages (L1-L4) in 2-3 days (Fig. 1). But under inadequate conditions for growth, that is, under reduced food availability, crowding, and high temperature, animals arrest development and form dauer larvae corresponding to the 3rd larval stage (Cassada & Russell, 1975; Golden & Riddle, 1984; Ailion & Thomas, 2000). *C. elegans* larvae sense "crowding" through dauer-inducing pheromone produced by themselves (Golden & Riddle, 1982). When the environmental conditions are improved, dauer larvae molt to normal L4 larvae and resume the life cycle. Since dauer larvae have characteristic morphology, thin and straight shape, they can be easily distinguished from other stages by observation with a dissecting microscope. Other characteristics of dauer larvae are closed mouth, no pharyngeal pumping and feeding, accumulation of fat (which looks dark) in the intestine,

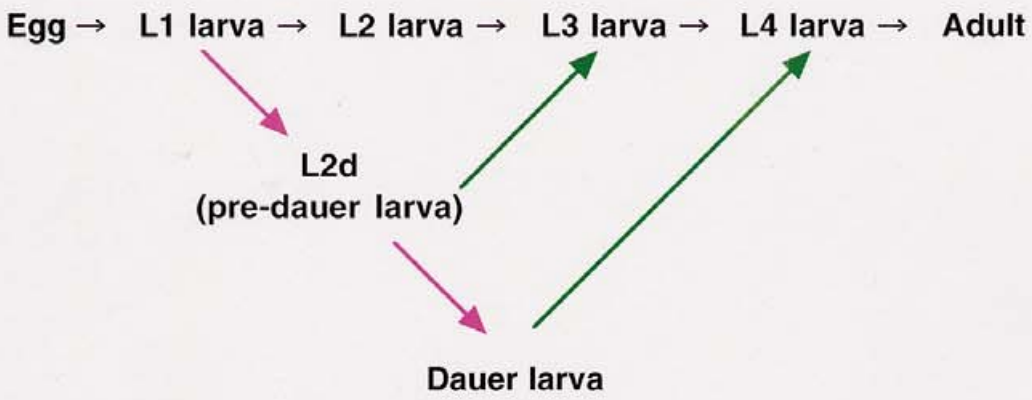

Fig. 1. Life cycle of *C. elegans*
Under appropriate environmental conditions for growth, *C. elegans* develop to adults in 2-3 days at 25°C. But they change development into dauer larva formation, when the environmental conditions are inadequate for their growth (pink allows), and after the environmental conditions are improved to appropriate conditions for growth, they can return to normal growth (green allows).

survival for several months (life span in normal development is about 2-3 weeks), resistance to 1 % SDS solution, in which normal animals are dissolved.

Like insects, the nervous system is involved in the diapause of *C. elegans*. The structure of the entire nervous system of adult hermaphrodites, including the morphology and synaptic connectivity of all the 302 neurons, has been reconstructed from electron micrographs of serial sections (White et al., 1986). There is a pair of sensory organs in the head, called amphids, each of which contains 12 neurons, including eight neurons exposed to outside. Some of these exposed neurons were shown to control dauer larva formation by laser microsurgery experiments (Bargmann & Horvitz, 1991). When neurons called ADF and ASI are killed, many animals become dauer larvae, although other amphids neurons are alive. In contrast, when either ADF or ASI is alive, animals did not become dauer larvae, even if all the other amphids neurons are killed. The dauer formation caused by killing of ADF and ASI neurons is enhanced by killing of ASG neurons, while killing of ASJ neurons prevents these dauer larvae to become L4 larvae spontaneously. Subsequent studies have shown that ASI neurons produce TGF-ß and insulins that prevent dauer formation (Ren et al., 1996; Pierce et al., 2001; Li et al., 2003), and that ASJ neurons are also required for the dauer-inducing signal in the cGMP-related signaling pathway (See the next paragraph) (Schackwitz et al., 1996). However, the molecular mechanisms of the dauer-inhibitory function of ADF and ASG neurons remain to be studied.

Genes that regulate dauer larva formation have been studied by isolation and characterization of mutants that show abnormality in this function. These mutants consist of two groups: dauer-constitutive (*daf-c*) mutants, which form dauer larvae even under non-crowded and well-fed conditions, and dauer defective (*daf-d*) mutants, which do not form dauer larvae even under crowded and starving conditions. The genetic pathways of dauer larva formation have been revealed by epistasis tests and molecular cloning of these mutations. At least three signal transduction pathways control dauer larva formation: cGMP-related signaling pathway, TGF-ß signaling pathway, and insulin signaling pathway (Fig. 2; Table 1) (Vowels & Thomas, 1992; Thomas et al., 1993; Gottlieb & Ruvkun, 1994; Malone et al., 1996; Riddle & Albert, 1997). The

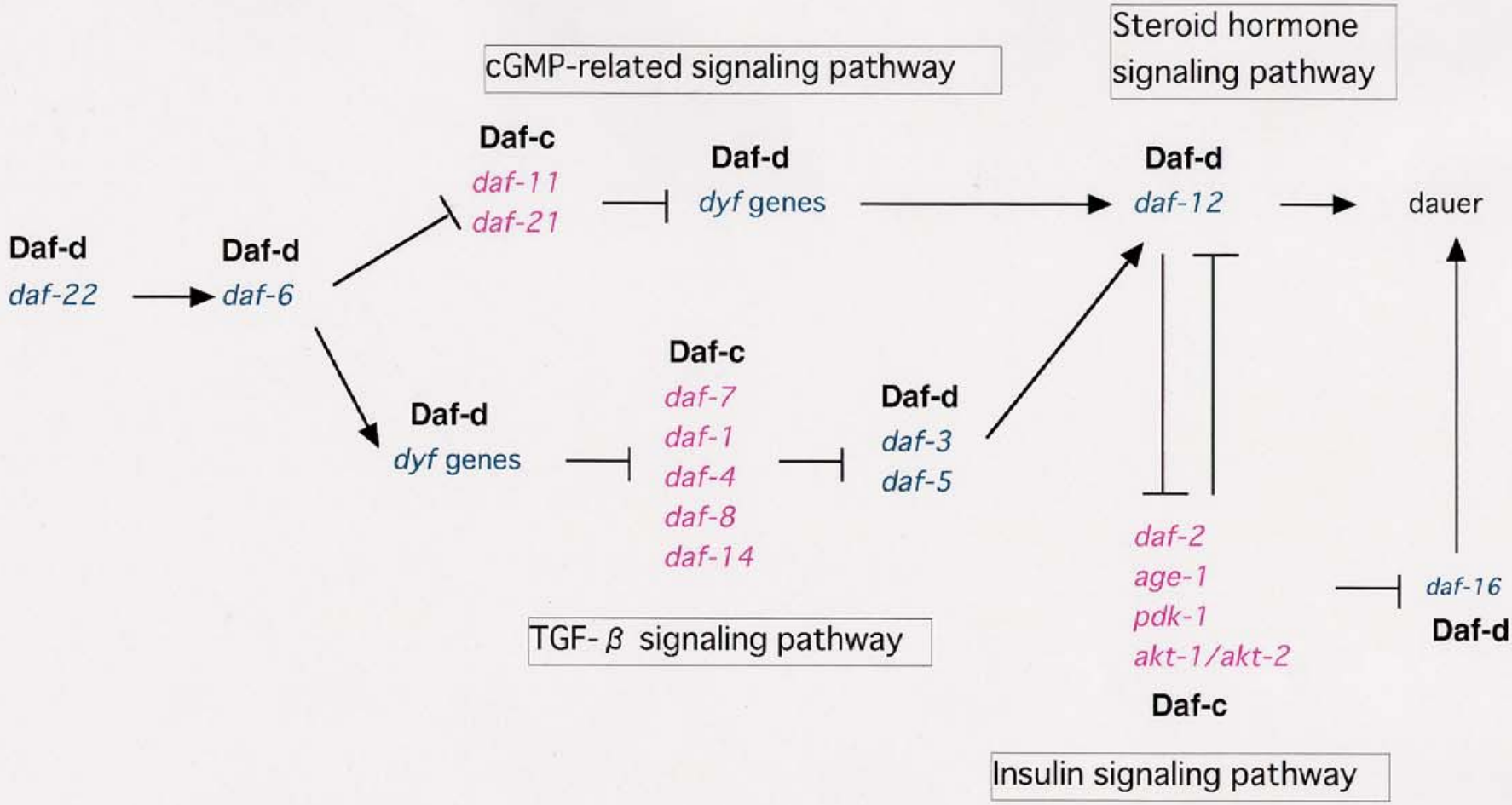

Fig. 2. Genetic pathway of dauer larva formation
Dyf genes, i.e., *daf-10*, *che-2*, *che-3*, *osm-1*, etc., show phenotypes in which amphid neurons fail to take up lipophilic fluorescent dyes (dye-filling abnormal). Daf-c genes are shown in pink, while Daf-d genes are shown in blue. Proteins encoded by these genes are listed in table 1.

Table 1. List of genes that affect dauer formation

Genes	Proteins	Reference
cGMP-related signaling pathway		
***daf-c* genes**		
daf-11	guanylate cyclase	Birnby et al., 2000
daf-21	Hsp90	Birnby et al., 2000
***daf-d* genes (*dyf* genes)**		
che-2	WD40 protein	Fujiwara et al., 1999
che-3	dynein heavy chain	Wicks et al., 2000
che-11	component of IFT complex A	Qin et al., 2001
che-13	component of IFT complex B	Haycraft et al., 2003
daf-10	component of IFT complex A	Qin et al., 2001
osm-1	component of IFT complex B	Cole et al., 1998
osm-3	kinesin	Shakir et al., 1993 Tabish et al., 1995
osm-5	component of IFT complex B	Haycraft et al., 2001 Qin et al., 2001
osm-6	component of IFT complex B	Collet et al., 1998 Cole et al., 1998
TGF-ß signaling pathway		
***daf-c* genes**		
daf-7	TGF-β	Ren et al., 1996
daf-1	TGF-β type I receptor	Georgi et al., 1990
daf-4	TGF-β type II receptor	Estevez et al., 1993
daf-8	smad	Estevez & Riddle, 1996
daf-14	smad	Inoue & Thomas, 2000
***daf-d* genes**		
daf-3	smad	Patterson et al., 1997
daf-5	Sno/Ski	da Graca et al., 2004
Insulin signaling pathway		
***daf-c* genes**		
daf-28	insulin	Li et al., 2003
daf-2	insulin receptor	Kimura et al., 1997
age-1	PI3 kinase	Morris et al., 1996
pdk-1	PDK1	Paradis et al., 1999
akt-1	Akt/PKB	Paradis & Ruvkun, 1998
akt-2	Akt/PKB	Paradis & Ruvkun, 1998
***daf-d* genes**		
daf-16	forkhead transcription factor	Ogg, et al, 1997; Lin et al., 1997
daf-18	PTEN	Ogg & Ruvkun, 1998
Hormone signaling pathway		
***daf-c* genes**		
daf-9	cytochrome P450	Jia et al., 2002
sdf-9	tyrosine phosphatase	Ohkura et al., 2003
***daf-d* gene**		
daf-12	nuclear hormone receptor	Antebi, et al., 2000
Upstream of cGMP-related and TGF-ß signaling pathways		
***daf-d* genes**		
daf-6 (Dyf)	patched-related protein	Shaham et al., 2002
daf-22	not identified (pheromone biosynthesis)	Golden & Riddle, 1985

cGMP-related signaling pathway acts in signal transduction of many sensory neurons including ASI and ASJ, in which it controls the expression of TGF-ß and/or insulin (Murakami et al., 2001; Li et al., 2003), but epistasis experiments showed that the cGMP-related signaling pathway is located also in parallel to the TGF-ß signaling pathway (Vowels & Thomas, 1992; Thomas et al., 1993). Recently, in addition of the 3 signaling pathways, a fourth signaling pathway, steroid hormone signaling pathway, is suggested (Jia et al., 2002; Ohkura et al., 2003; Mak & Ruvkun, 2004; Gerisch & Antebi 2004). The *daf-12* nuclear hormone receptor gene in the steroid hormone signaling pathway seems to be located downstream of all the other *daf-c* and *daf-d* genes. However, it remains to whether other genes in the steroid hormone signaling pathway is located downstream or in parallel to genes in other pathways.

In addition to mutations that show abnormal phenotypes in dauer larva formation by themselves, mutations that show the dauer-constitutive phenotype only in the background of another mutation have been discovered and called synthetic dauer-constitutive mutations. A great majority of them were isolated by other phenotypes, mostly those in neuronal functions, and later found to show this phenotype when double mutants of these mutations were constructed. For example, *aex-3*, *unc-3*, *unc-31* and *unc-64* single mutants produce no or few dauer larvae at 15 to 25°C, but the double mutants of *unc-64;unc-3*, *unc-64;unc-31*, *unc-31;aex-3* and *unc-31;unc-3* (but not *aex-3;unc-3*) produce many dauer larvae at 20 and 25°C, suggesting that these genes function in regulating dauer larva formation (Bargmann et al., 1990; Iwasaki et al., 1997; Ailion & Thomas, 2000). *aex-3* gene encodes a guanine nucleotide exchange factor for the *rab-3* GTPase required for intraneuronal transport (Iwasaki et al., 1997; Iwasaki & Toyonaga, 2000), and its mutants show abnormality in defecation behavior (Thomas,1990). *unc-3* gene encodes an O/E family transcription factor expressed in ASI neurons (Prasad et al., 1998), and its mutants show abnormality in locomotion (Brenner, 1974). *unc-64* gene encodes a protein showing sequence and structural similarities to mammalian syntaxin required for synaptic transmission, and its mutation results in uncoordinated locomotion (Brenner, 1974; Ogawa et al., 1998). *unc-31* gene encodes a homolog of CAPS (calcium activated protein for secretion) (Livingstone, Ph. D.

Thesis, Univ. of Cambridge, 1991, cited in Miller et al., 1996), which is required for the exocytosis of dense core vesicles, which contain neuropeptides and biogenic amines (Walent et al., 1992; Ann et al., 1997; Berwin et al., 1998). *unc-31* mutants show many phenotypes: slow locomotion, defective egg-laying, constitutive pharyngeal pumping, insensitive to prodding, dauer-constitutive when only ASI neurons are killed (Avery et al., 1993) and when they are cultured at very high temperature (27°C), at which *C. elegans* cannot reproduce (Ailion & Thomas, 2000). Although *daf-c* mutations have been saturated (Malone & Thomas, 1994), many synthetic Daf-c mutations remain to be identified.

To identify new genes regulating dauer formation and to discover new mechanisms, 44 synthetic dauer-constitutive mutants were isolated in the *unc-31(e169)* background, mapped and named *sdf* (**s**ynthetic abnormality in **d**auer **f**ormation) mutants (Norio Suzuki, Takeshi Ishihara and Isao Katsura, unpublished results). Of these mutants, the genes of *sdf-9* and *sdf-13* have been cloned. *sdf-9* gene encodes a protein tyrosine phosphatase-like molecule, is expressed in a pair of neuron-associated cells called XXXL/R, and regulates dauer larva formation through hormonal signaling (Ohkura et al., 2003). *sdf-13* encodes a homolog of the transcription factor Tbx2, is expressed in AWB, AWC, and ASJ neurons in amphids and in many pharyngeal neurons, and control olfactory adaptation in AWC and dauer larva formation in cells other than AWC (possibly ASJ) (Miyahara et al., 2004).

In this Doctor Thesis, I describe cloning, expression and functional analysis of another *sdf* gene, *sdf-14*, in which three mutant alleles, *ut151, ut153* and *ut155*, were isolated. It was found that *sdf-14* gene encodes the MRP1 (multidrug resistance-associated protein 1) homolog, that it acts in multiple tissues to regulate dauer formation, and that human MRP1 can substitute for SDF-14 in this function. The results suggest that dauer larva formation is regulated by export of unidentified substance from cells in multiple tissues.

Materials & Methods

Strains

Worms were grown by standard methods (Lewis & Fleming, 1995; Stiernagle T. 1999) except CB4856 strain, for which 3 % NGM agar plates were used. The strains used in this study were Bristol N2 strain as reference wild type, Hawaiian CB4856 strain as a polymorphic type of Bristol N2 for mapping, and the following mutants of N2 strain: *sdf-14/mrp-1 (ut151, ut153, ut155, pk89)* X, *unc-31(e169)* IV, *daf-1(m40)* IV, *daf-2(e1370)* III, *daf-5(e1386)* II, *daf-6(e1377)* X, *daf-11(m47)* V, *daf-14(m77)* IV, *daf-16(m26)* I, *daf-16(m27)* I, *daf-16(mu86)* I, *daf-22(m130)* II, *che-3(e1124)* I, *rol-1(e91)* II, *unc-3(e151)* X.

SNP mapping

The CB4856 strain, an isolate from a Hawaiian island, shows a high density of polymorphisms compared with the reference Bristol N2 strain (the wild type strain for *sdf* mutants). These polymorphisms were detected by restriction fragment length polymorphisms (RFLPs) or DNA sequence. The polymorphisms data were kindly provided by Stephen R. Wicks (Wicks et al., 2001).

For mapping, CB4856 males were crossed to *unc-31(e169);sdf-14(ut153)* double mutant hermaphrodites (Bristol N2 background). Heterozygous non-Unc F1 hermaphrodites were allowed to self-fertilize, and then F2 animals that showed the Unc-31 phenotype were picked and cultured separately at 25.5°C for the test of dauer larva formation. Then, genomic DNA was prepared from bulk of F3 self-progeny, and single nucleotide polymorphisms (SNPs) were detected by RFLPs or DNA sequencing. If necessary, homozygous animals were isolated from F3 plates, their dauer larva formation was checked, and their genomic DNA was prepared for the tests of SNPs. Because SNPs data provided by Dr. Wicks were insufficient to map *sdf-14* gene, I also found some SNPs by sequencing CB4856 genomic DNA, which are listed in Table 2A and B.

Table 2A. SNPs detectable by RFLPs

SNP marker	SNP	primer set	primer sequence	restriction enzyme	PCR size (bp)	N2 (bp)	CB4856 (bp)
Y35H6	G300T	Y35H6-1	gggctacggtagttgtggtg	*Taq* I	875	263, 160, 149,	306, 304, 149,
	T501A	Y35H6-2	ggaatcggcggaaaacactg			144, 64, 52, 43	64, 52
M02E1	T8684C	M02E1-21	gcataggcctccaaagtag	*Mnl* I	508	489, 19	435, 54, 19
		M02E1-22	gattagtcatgtcaggttaaccg				
F02G3	G2935A	F02G3-7	cgccctttgcgtgaattcgag	*Eco*T141 I	444	346, 98	444
		F02G3-8	gtgatcgaacccgccactgc	(Sty I)			
F25E2	C4055G	F25E2-5	ctctgactgattggggac	*Hph* I	991	991	684, 306
		F25E2-6	gagcttgcctgaaaccacag				
F35H12	additional T	F35H12-3	cctcatcactctgagtacgtg	*Dra* I	864	541,188,80,55	540,188,40,16
	at 17444/17445	F35H12-4	ggatccgattgatcgcagc				

Table 2B. SNPs detectable by DNA sequencing

SNP marker	SNP	PCR primer set	primer sequence	primer for sequencing
ZK1193	additional T	ZK1193-1	gttcctttcatgaattgtgg	ZK119-3R
	at 15174/15175	ZK119-3R	cacacaagtactccgcagg	
F52D1	A9752G	F52D1-7	gttccgtatattggtttctgg	F52D1-7
		F52D1-9R	gcctcgttacgtccgtgtg	
F52D1	deficient T	F52D1-8	cgctcagaaaatgtgacg	F52D1-8
	at 10538	F52D1-9R	gcctcgttacgtccgtgtg	
M6	deficient A	M6-3	cactgtcctgtcttttcg	M6-3
	at 12360	M6-4R	cagctacagtcgtctaatcc	
T19D7	repeated seqence	T19D7-1	ggatcaccgcttattaaggac	T19D7-2R
	between 11077-11084	T19D7-2R	ctgaaagctgacgaatttcccg	

Table 2A and B show SNPs found in this study. (**A**) PCR primer sets, restriction enzymes and length of RFLPs for detection of SNPs are shown. (**B**) PCR primer sets and sequencing primers for detection of SNPs are shown.

Rescue experiment of the *sdf-14* mutation for cloning

Transformants were obtained by microinjection of DNA into the germline (Mello et al., 1991). Cosmid clones of the *C. elegans* genomic DNA were kindly provided by A. Fraser and A. Coulson. The following PCR primer set was used to amplify F57C12.5 gene together with about 6 kb of upstream sequence and about 3 kb of downstream sequence: F57C12.5-3 primer TCC GTC ATT CTT TTC GTC TAC CTT TGA TGT C and F57C12.5-4 primer GCT GGA TGA TTT GCA CTT CGA GTA GTT GGC. The Cosmid clones and the PCR product were purified with ABI mini prep kit. Injection marker DNA (*gcy-10*::GFP) and test DNA were coinjected into *unc-31(e169);sdf-14(ut153)* animals at concentrations of 25-30 ng/μl, and 10 ng/μl (cosmids) or 5 ng/μl (PCR product), respectively. Dauer formation of transformants was assayed at 25.5°C. I first used *myo-3*::GFP as the injection marker, but it was inappropriate, because it rescued the dauer formation phenotype by itself. DNA sequencing of the three *sdf-14* mutant alleles (*ut151, ut153 and ut155*) was performed using primers shown in Table 3.

Rescue experiment of the *sdf-14* mutation with SDF-14 isoforms

The DNA constructs of b-, c- or e-type isoforms for transformation experiments were made from cDNA clones (yk46e1, yk494b1, yk831b09 and yk1067b9, which were kindly provided by Yuji Kohara). The cDNA sequences were confirmed by DNA sequencing. Then, the exon 3-19 region of *sdf-14*::GFP fusion gene (Fig. 3 as pSDF14::GFP) was replaced by the corresponding part of each cDNA, flanked by *Bst*E II and *Sap* I sites. The resulting isoform constructs (pSDF14b::GFP, pSDF14c::GFP and pSDF14e::GFP; Fig. 3) were injected together with the *gcy-10*::GFP injection marker into *unc-31(e169);sdf-14(ut153)* animals. The DNA concentrations were 25-30 ng/μl for the marker and 10 ng/μl for the isoform constructs. Dauer formation of the transformants was assayed at 25.5°C.

Table 3. Primers used for DNA sequencing of mrp-1/sdf-14 gene

primer	sequence
F57C12.4-2	CGACTGAATACGGTACATTTCTCGAGC
F57C12.5-7	GTCGAGAACAAGTAGACG
F57C12.5-8	GTGGTGAGCTGCGAACG
F57C12.5-9	GAACCAACCCAGTTAAGC
F57C12.5-10	GACACCCTTGACGTGGG
F57C12.5-11	ACCGACACCCTTGACCC
F57C12.5-12	GAGGGTTTGGGTGATCTG
F57C12.5-13	CGGTAAAACGAGGCGTG
F57C12.5-14	GTAAACAGCAGCTCCGGCG
F57C12.5-15	CTCAGTAGGCGCGTTCG
F57C12.5-15-1	GCGAGGGATGTCGGTGTC
F57C12.5-16	GTGCGCACGAGGCTAGGC
F57C12.5-17	TGACGTCAATGTCCTGG
F57C12.5-18	GACTACCGCCAGAACAG
F57C12.5-19	GCTATGCGACGCCTTTTGC
F57C12.5-20	TGTCCGATGGGTAGGAG
F57C12.5-21	CACTCTTGATCCGCATG
F57C12.5-22	TGCTCAGCGTAGTGCC
F57C12.5-23	GCTCGGAAATATGACATG
F57C12.5-24	CGAGAAGTACAATTTCAGC
F57C12.5-25	GGGAATCAACTTGTCCG
F57C12.5-26	CTTCCCACAATGGCAAC
F57C12.5-27	CACTTTCATCCCGTTCAGG
F57C12.5-28	GTCAAATGGAGCAGATG
F57C12.5-29	TGCACTAGTCAACACAG
F57C12.5-30-1	TTGAGGTGGTGTAGTCG
F57C12.5-30-2	GTCTGAAAACACCGAG
F57C12.5-30-3	GCACTGGAACTCTTTCTGAG
F57C12.5-31	TTCCACGCGCTGCGGTG
F57C12.5-32	AGGACTCCGACTTGGG
F57C12.5-33	GAGAGAAATAGCAGAAAAGC
F57C12.5-33-1	CGACAACGAGTAGGCAACAG
F57C12.5-34	ACCCATTTTGTTCTACG
F57C12.5-35	GCCGAACATCAATTTGACGG
F57C12.5-36	CTACGATATTACTGGCC
F57C12.5-36-1	CGTTCAACCTTCGTCAACTGC
F57C12.5-37	CCGGTAATTGACAATTGTC
F57C12.5-38	GAGACGTGTGGTAATGCTG
F57C12.5-38-1	GAAGCAGAGAAGAGGCAG
F57C12.5-39	CTTTCTCTACGCACACG
F57C12.5-40	AACGCGTTAAAGCCGACC

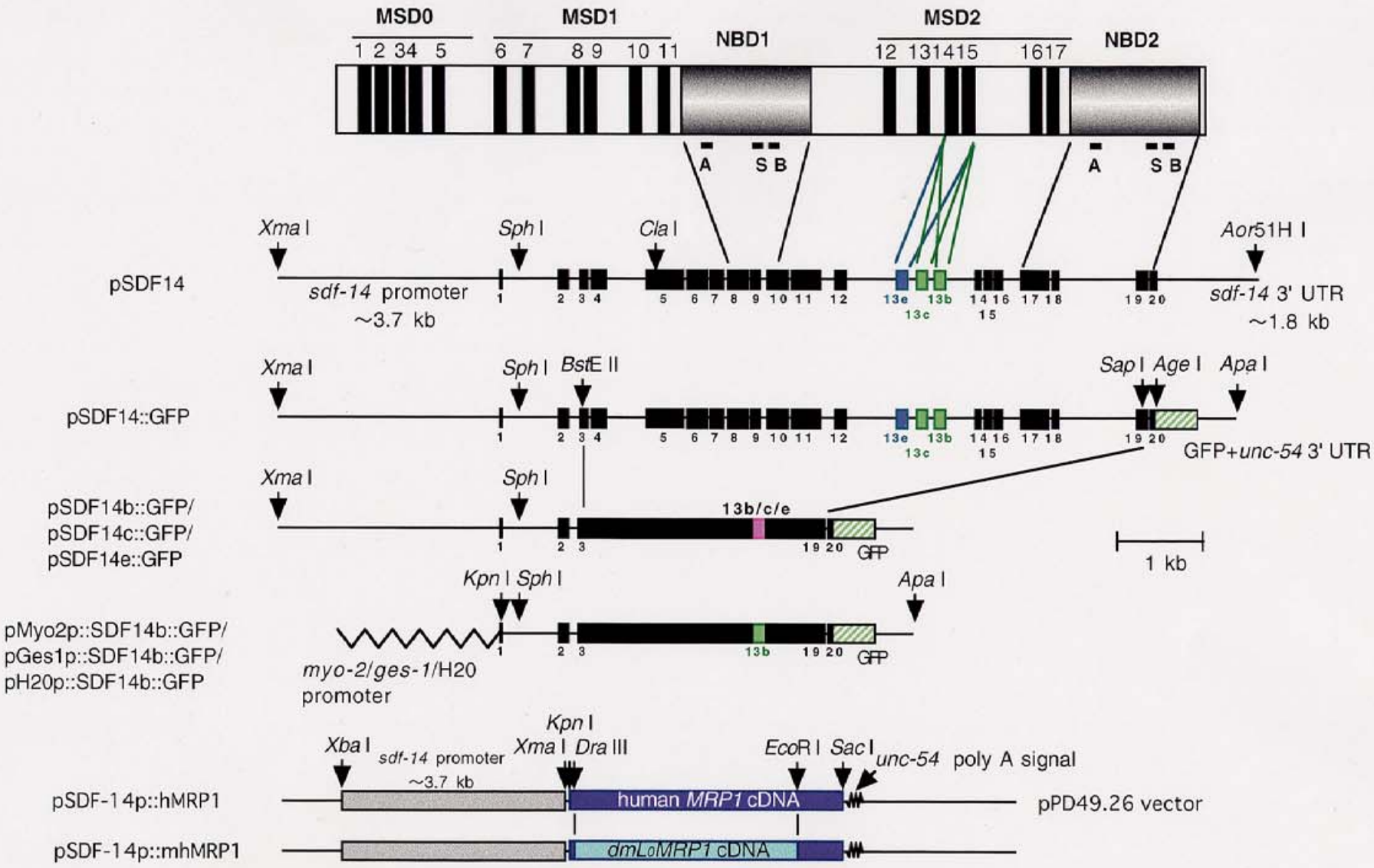

Fig. 3. Various DNA constructs used for microinjection

Rescue experiment by tissue specific expression with extrinsic promoters

An N-terminal coding region of *sdf-14* gene was amplified by PCR from pSDF14b::GFP using the following primer set containing the restriction enzyme site of *Kpn* I: KpnI/5up primer CGG GGT ACC AAT TAA GAA ATG TTC CCG TTA G and F57C12.5-36-1 primer CGT TCA ACC TTC GTC AAC TGC. The *Kpn* I-*Sph* I fragment from PCR product and the *Sph* I-*Apa* I fragment from pSDF14b::GFP were integrated together into *Kpn* I and *Apa* I sites of pPD30.69 (gift from A. Fire) for pharyngeal muscle expression vector containing *myo-2* promoter, ges-1 construct for intestinal expression containing *ges-1* promoter or H20 construct for neuronal expression containing H20 prpmoter, respectively (shown in Fig. 3 as pMyo2p::SDF14b::GFP, pGes1p::SDF14b::GFP and pH20p::SDF14b::GFP).

These SDF-14b::GFP tissue specific expression constructs were injected either separately or in combination into *unc-31(e169);sdf-14(ut153)* animals together with the *gcy-10*::GFP injection marker. The concentration of *gcy-10*::GFP was 25-30 ng/μl, and that of SDF-14b::GFP tissue specific expression constructs was 10 ng/μl. Dauer formation of the transformants was assayed at 25.5°C.

Rescue experiment of *che-3*, *daf-5* and *daf-6* phenotypes

For the rescue of the suppressions of dauer formation by *che-3*, *daf-5* and *daf-6* mutations, I used the cosmid F18C12, the cosmid W01G7, and a PCR product of wild type *daf-6* gene, respectively. The *daf-6* PCR product consisted of the *daf-6* coding region, about 3.1 kb of the upstream sequence, and about 1.2 kb of the downstream sequence. They were injected into *unc-31(e169);sdf-14(ut153)* animals at concentrations of 5 ng/μl for cosmids or 10 ng/μl for the PCR product, respectively, together with the *gcy-10*::GFP injection marker (25-30 ng/μl). Dauer formation of the transformants was assayed at appropriate temperature. PCR primers for *daf-6* were as follows. F31F6/20023FW primer CCA GCA ATC GGC AAT TGC CCC TTG TAC TG and F31F6/29933RV primer CAC CAA CTA TTC AAG AAC TTA CGC CAT GCG. The cosmids were kindly provided by A. Fraser and A. Coulson.

Dauer formation assay

Three to eight adult hermaphrodites were transferred onto ø35 mm plates and allowed to lay eggs for 3-8 hours at 15, 18, 20, 25.5 or 27°C. The parents were then removed, and the progeny were cultured at the same temperature. The numbers of dauer larvae and non-dauer animals (L4/adults) among the progeny were scored after 6-7 days at 15°C, 4-5 days at 18°C and 20°C, 3-4 days at 25.5°C and 3-5 days at 27°C. At 27°C, the growth rates were variable from animal to animal.

Dauer formation assay in the presence of MRP1 inhibitors or sodium arsenite

After the NGM agar medium for plates was cooled to 60°C, PAK-104P, A-GA, MK571 and sodium arsenite dissolved in DMSO, EtOH, H_2O and H_2O, respectively, were added to the medium at appropriate concentrations. Then, *E. coli* OP50 was grown as food on the plates, and dauer formation was assayed using these plates.

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Analysis of expression pattern

DNA constructs

To examine the expression pattern of *sdf-14* gene, I first made pSDF14 (Fig. 3) and then inserted GFP cDNA in frame at the end of the coding region of pSDF14 to make pSDF14::GFP (Fig. 3). pSDF14 was made as follows. The *Sph* I-*Cla* I fragment and *Cla* I-*Aor*51H I fragment, which corresponds to the C-terminal region of SDF-14, were subcloned from cosmid F57C12 into the *Sph* I-*Cla* I site of pPD22.11 (gift from A. Fire) and the *Hinc* II-*Cla* I site of pBluescript II KS(-), respectively. I amplified the *Xba* I-*Sph* I fragment, which corresponds to the N-terminal region, from wild type genomic DNA using F57C12.5-4 primer GCT GGA TGA TTT GCA CTT CGA GTA GTT GGC and F57C12.5-35 primer GCC GAA CAT CAA TTT GAC GG and subcloned it into *Xba* I-*Sph* I sites of pUC119. The DNA sequence of the coding region of the PCR product was confirmed by sequencing. After the *Xma* I-*Sph* I fragment was taken out from the *Xba* I-*Sph* I fragment of the pUC119 construct, the three subcloned fragments were

integrated into the *Hinc* II-*Xma* I (*Sma* I) site of pBluescript II KS(-) to make pSDF14. pSDF14::GFP was made as follows. The region containing GFP coding sequence and *unc-54* 3' UTR sequence was amplified from pPD95.79 (gift from A. Fire) by PCR using pPD95.75-*Age*I primer GAG GGT ACC GGT AGA AAA ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA G and AMP3 primer CTC AAC CAA GTC ATT CTG AGA ATA GTG. And then, *Age* I-*Apa* I fragment of the PCR product was inserted at the end of the *sdf-14* coding sequence of pSDF14 at which an *Age* I site was made in advance.

Microinjection

pSDF14::GFP and the *rol-6* marker DNA were coinjected into wild type animals at concentrations of 40 ng/μl and 50 ng/μl, respectively, and the expression pattern was observed. To check whether pSDF14::GFP was functional, pSDF14::GFP was injected into *unc-31(e169); sdf-14(ut153)* animals together with the *gcy-10*::GFP marker at concentrations of 10 ng/μl and 12 ng/μl, respectively. Dauer formation of the transformants was assayed at 25.5°C.

Human *MRP1* cDNA experiment

DNA constructs

The constructs of human *MRP1* cDNAs fused to the *C. elegans sdf-14* promoter were made by the following procedure. An *Xba* I site and an *Xma* I site were made at the 5' and 3' ends, respectively, of the DNA fragment of the *sdf-14* promoter region by PCR, and the fragment was inserted into the *Xba* I-*Xma* I site of pBluescript II KS(-) vector. Then, this *Xba* I-*Xma* I promoter fragment was subcloned into the *Xba* I-*Xma* I sites in MCSI (multi-cloning site I) of pPD49.26, a canonical expression vector (gift from A. Fire). The wild type human *MRP1* cDNA and the mutant human *MRP1* cDNA (*dmLoMRP1* cDNA) were kindly provided by Tatsuhiko Furukawa. Since the full length cDNA could not be subcloned into pBluescript II KS(-) for unknown reasons, the 5' half (*Sal* I-*Eco*R I fragment) and the 3' half (*Eco*R I-*Not* I fragment) of the wild type human *MRP1* cDNA were subcloned into the *Sal* I-*Eco*R I site and *Eco*R I-*Not* I site of pBluescript II KS(-), respectively. Then, a *Kpn* I site and a *Sac* I site were made upstream

of the initiation codon and downstream of the stop codon, respectively, by PCR. Finally, the *Kpn* I-*Eco*R I fragment (5' half) and *Eco*R I-*Sac* I fragment (3' half) of human *MRP1* cDNA were inserted into the *Kpn* I-*Sac* I sites in MCSII (multi-cloning site II) of pPD49.26, into which the *sdf-14* promoter had been inserted (pSDF14p::hMRP1 in Fig. 3). The *Dra* III-*Eco*R I region of pSDF14p::hMRP1 was replaced by that of *dmL0MRP1* cDNA (pSDF14p::mhMRP1 in Fig. 3).

The primer sequences used in the experiments above were as follows. For the addition of *Xba* I and *Xma* I sites at the ends of the *sdf-14* promoter fragment, -3707/XbaI-FW primer GCT CTA GAA TTA TAT CAC TTT TCG and -3707/XmaI-RV primer TCC CCC CGG GTT CTT AAT TGG CTC GGT TCG G. For introducing a *Kpn* I site upstream of the initiation codon of human *MRP1* cDNA, hmrp1/KpnI-1FW primer CGG GGT ACC AAT TAA GAA ATG GCG CTC CGG GGC TTC TG and hmrp1-121RV CCC ACA CGA GGA CCG TG, where the underlined sequences show those just upstream of the initiation codon of *sdf-14* gene. For introducing a *Sac* I site downstream of the stop codon of human *MRP1* cDNA, hmrp1/3881FW primer GCT GGT TCG GAT GTC ATC TG and hmrp1/SacI-4739RV primer GAT GCG GAG CTC TAT CAC ACC AAG CCG GCG TC.

Microinjection

pSDF14p::hMRP1 or pSDF14p::mhMRP1 was injected into *unc-31(e169);sdf-14(ut153)* animals together with the *gcy-10*::GFP marker at concentrations of 40 ng/μl or 100 ng/μl, and 25-30 ng/μl, respectively. Dauer formation of the transformants was assayed at 25.5°C.

Construction of double and triple mutants

I constructed double and triple mutants by using standard genetic methods, except that the existence of the mutations was confirmed directly by genomic PCR followed by restriction enzyme cut. Since *daf-22(m130)* has not been cloned, I used a nearby mutation *rol-1(e91)* as a counter marker. Several double mutants were found in the course of the construction of the triple mutants. The PCR primer sets and restriction enzymes used are shown in Table 4.

Table 4. Primer sets and restriction enzymes for the detection of mutations

strain	mutation		PCR primer set		Restriction enzyme	N2 (wild type)	mutant
	DNA	AA					
daf-1(m40)	G3005A	W170STOP	daf-1/m2950	GGATTTTGAATTTTCAAATTTTCAGGCGCCAGGCCCGCAGCAGCCATCCACG	*Bst*X I	179, 51	230
			daf-1/3180RV	CGTCGCCCATTGTAACCATCGG			
daf-2(e1370)	C28250T	P1462	daf-2/m28201FW	GTTTGGAGCTTCGGAGTTGTTCTCTATGAAATGGTTACACTCGGTGCTGAG	*Hinf* I	189, 49	238
			daf-2/28438RV	CTGGTGAAGCTTCAGCTGC			
daf-5(e1386)	G1901A	(splicing)	daf-5/1814	GTTTTCGGGGTCAAAAGGC	*Bsl* I	202, 85	287
			daf-5/2100RV	GTGCATGATGAAGTCGGTC			
daf-6(e1377)	G934A	W490STOP	daf-6/MfeI	CCCTTGATCTTTATTACTATATTTTCAGGATCTATGCCTGAGCTACAATT	*Mfe* I	181, 46	227
			daf-6/1110RV	GAGTCAACTTGGCGTCGCTC			
daf-11(m47)	C3249T	Q490STOP	daf-11/3177FW	CAACATGATAATCTTAACAG	*Tsp*509 I	192, 69	261
			daf-11/3437RV	ATCGATAGCACTTGCATG			
daf-14(m77)	C1110T	Q192STOP	daf-14/998	GGAGAACTTTGGCTAACTG	*Rsa* I	150	103, 47
			daf-14/m1147RV	CTGCTCCTTATCGAATATTTTCATTCTTTTTTGATCTCCTTTGTTGT			
daf-16(m26)	G605A	(splicing)	daf-16-m558FW	CATACTCCAGATGGAAGCAATTCTCATCAGACATCGTTTCCTTCTGA	*Dde* I	155, 44	199
			daf-16-756RV	GATGCTAAAATGTCAAGTCTTTCTG			
daf-16(m27)	G10521A	W342STOP	R13H8.1-1*	GAATCCACGGCGTACACGTG	*Nla* III	554, 283	837
			R13H8.1-5*	ATGTTACTAATATTTTAGGG			
daf-16(mu86)	deletion(-975～9972)		daf-16/-1263FW	GCCACCACGACGCAACACAC	-	1288	-
			daf-16/25RV	CCTGATCTACCAGCATCTCC			
daf-16(mu86)	deletion(-975～9972)		daf-16/-1263FW	GCCACCACGACGCAACACAC	-	11709	762
			daf-16/10446RV	GAGAGGTTCGATTGAGTTCG			
che-3(e1124)	C9954T	Q2233STOP	che-3-9789FW	GACCATTTGTACAGACTGCCG	*Bsa*X I	~150, ~30	215
			che-3-m10003RV	GCTAACTGGCTTTCTGGGTCATTCTGGAAGCAATGCTTCAGGAGCTG			
rol-1(e91)	C1096T	R80C	rol-1/871	GCTATGTTTTGTTAGTAAACGAC	*Bsl* I	231, 44	275
			rol-1/m1157RV	GGAGCAAATGGAGTTGTCTCATCATTCGAGTACTCGTAGCCCGCTTGAC			
mrp-1(pk89)	deletion(3901～7112)		F57C12.5-47FW	CTATTGCATACGGAGGAACAGG	-	3526	314
			F57C12.5-17	TGACGTCAATGTCCTGG			
mrp-1(pk89)	deletion(3901-7112)		F57C12.5-47FW	CTATTGCATACGGAGGAACAGG	-	207	-
			F57C12.5-26	CTTCCCACAATGGCAAC			

The Table shows PCR primer sets and restriction enzymes for the detection of mutations as well as restriction fragment lengths of wild type and mutant strains. Pink letters indicate that mutations introduced for making restriction enzyme recognition sites. Mutation sites of genes that had been cloned, *daf-1(m40)*, *daf-5(e1386)*, *daf-11(m47)* and *rol-1(e91)*, were identified by sequencing in this study. Refer to Kimura et al. (1997), Shaham et al. (2001), Inoue & Thomas (2000), Lin et al. (1997) and Wicks et al. (2000) for the mutation sites of *daf-2(1370)*, *daf-6(e1377)*, *daf-14(m77)*, *daf-16* mutations and *che-3(e1124)*, respectively. Asterisks show primers designed by Ohkura et al. (2003).

Dye filling assay

Dye filling assay was carried out with DiI (Molecular Probes) as described in Michael Koelle's C. elegans Protocols (http://info.med.yale.edu/mbb/koelle/protocol_list_page. html).

Results

1. *sdf-14* gene encodes a multidrug resistance-associated protein-1 (MRP-1)

To elucidate the role of *sdf-14* gene in the regulation of dauer larva formation, I cloned the gene by positional cloning. Mutations in *sdf-14* genes had been previously mapped near the left end of the X chromosome (Norio Suzuki, Takeshi Ishihara and Isao Katsura, unpublished results). In this study, the map position was determined more precisely by single nucleotide polymorphisms (SNPs) (Wicks et al, 2001). By using a total of 15 recombinants out of 376 animals tested, the *sdf-14(ut153)* mutation was mapped between the SNP markers M02E1 and F02G3 (Table 5). Then, the cosmid clones in this region were injected into the *unc-31(e169);sdf-14(ut153)* double mutant either separately or as combinations of two adjacent clones. Of all the injected clones, only the combination of F57C12 and F55H6 rescued the dauer formation phenotype of the double mutant, while neither the neighboring cosmid B0027 and nor the combination of CD11 and F57C12 rescued the phenotype (Fig. 4). These results suggested that *sdf-14* gene was identical to F57C12.5. To confirm this, I injected PCR-amplified F57C12.5 gene that contained about 6 kb of upstream sequence as well as about 3 kb of downstream sequence, and found that the dauer formation phenotype was rescued in all the transgenic animals obtained (Fig. 4.).

The *C. elegans* database WormBase (http://www.wormbase.org/) indicates that F57C12.5 encodes a multidrug resistance-associated protein-1 (MRP-1) belonging to the ABC (ATP-binding cassette) transporter superfamily, which is based on data by Broeks et al. (1996). I confirmed this conclusion by BLAST searches using the non-redundant protein sequence database at NCBI. The results showed that F57C12.5 was the most similar to human MRP1 among all the human proteins, and that human MRP1 was the most similar to F57C12.5 among all the *C. elegans* proteins. Hence I refer to SDF-14 as MRP-1.

The amino acid sequence of MRP-1/SDF-14 has about 60% homology to that of human

Table 5. SNP mapping

recombinant phenotype*1		269(25) non-d	142(43) non-d	G-84-1 non-d	G-1-1 non-d	36-2 non-d	G-76-1*3 non-d	8-1 dauer	23-1 dauer	32-5 dauer	1-5 dauer	36 dauer	32-1 dauer	39-6 dauer	G-10 dauer	G-2-1 dauer
SNP marker	map position															
Y35H6*2	-20.9	N2	CB													
M02E1*2	-20.2	N2	CB													
ZK1193*2	-20.0	CB	CB													
F52D1*2	-19.8	CB	CB													
F28C10	-19.7	CB	CB	CB	CB	CB	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2
M6*2	-19.59	CB	CB													
T19D7*2	-19.56	CB	CB													
F02G3*2	-19.52	CB	N2	CB			N2	N2								
F25E2*2	-19.49	CB	N2													
F35H12*2	-18.9	CB	N2	N2			N2	CB								
C52B11	-18.4			N2	CB		N2/CB	CB	N2	N2	N2	N2	N2	N2		
EGAP7	-17.0			N2	CB			CB	CB	CB	N2	N2	N2	N2		
F47G3	-15.6			N2/CB	N2	CB	N2/CB	CB	CB	CB	CB	CB	CB	CB	N2	N2
F11D5	-10.5			N2	N2								CB	CB	CB	N2
ZK470	-8.2			N2	N2								CB		CB	CB

The results of 269(25) and 142(43) recombinants suggest that *sdf-14* gene lies between M02E1 and F02G3.
N2 means that this region of genomic DNA was N2 type.
CB means that this region of genomic DNA was CB4856 type.
N2/CB means that this region of genomic DNA was heterozygous.
*1 non-d: no dauer formation, dauer: dauer formation
*2 SNPs identified in this research. Detailed descriptions of these SNPs are presented in Table 2A and 2B.
*3G-76-1 may be an exceptional recombinant, because it did not show dauer formation.

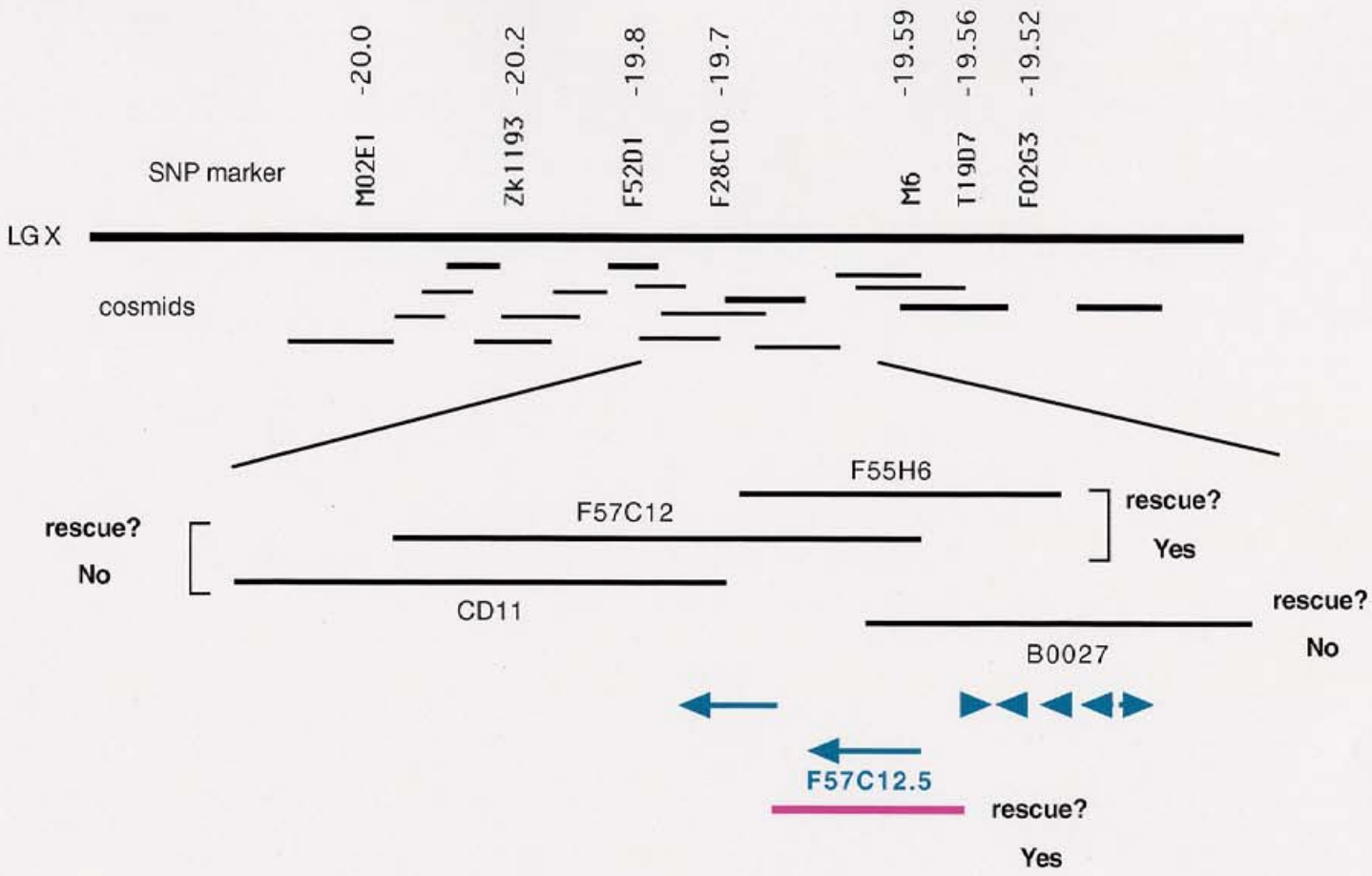

Fig. 4. Physical map of the region containing *sdf-14* gene

The dauer-constitutive phenotype of the *unc-31(e169);sdf-14(ut153)* double mutant was rescued by the combination of F55H6 and F57C12, or the PCR product of F57C12.5. Black lines indicate cosmids. Pink line indicat PCR product. Blue allows indicate predicted genes. SNP markers and its positions are shown on the top.

MRP1, mouse MRP1 and *S. cerevisiae* YCF1 (a homolog of human MRP1; Tusnady et al., 1997) (Table 6), and the homology extends all over the primary structure (Fig. 5). Like human MRP1, *C. elegans* MRP-1/SDF-14 seems to have three membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) (Fig. 6). It is not easy to predict the exact number and positions of transmembrane domains theoretically by using data analysis tools. However, since human MRP1 is shown to have 17 transmembrane domains by experiments (Bakos et al., 1996; Hipfner et al., 1997; Kast & Gros, 1997; Kast & Gros, 1998), I predict that *C. elegans* MRP-1/SDF-14 also has 17 transmembrane domains based on the sequence homology.

To identify the mutation sites of the three *mrp-1/sdf-14* alleles, I sequenced the genomic DNA of the three mutants, *ut151, ut153* and *ut155*. The mutations *ut151* and *ut155* were missense mutations in NBD1 (nucleotide binding domain 1) region (Table 7, Fig. 5 & Fig. 7), while *ut153* was a mutation at the acceptor splice site of the 4th intron (Table 7 & Fig. 7). All the mutations are located in the N-terminal region. Thus, all the splice variants (See the section "3. *C. elegans* MRP-1/SDF-14 has multiple isoforms" of Results) except d-type, which does not have the N-terminal part, should be affected by these mutations. Besides the three *mrp-1* mutants isolated in our laboratory, a deletion mutant *pk89* was isolated by Broeks et al. (1996) (Fig. 7). While *pk89* is a null allele by its DNA sequence, the splice acceptor mutation *ut153* showed a phenotype as strong as *pk89* (See the section 2. Phenotypes of *mrp-1/sdf-14* mutants) and is a candidate of a null allele. I therefore used *pk89* or *ut153* in the experiments below.

2. Phenotypes of *mrp-1/sdf-14* mutants

To investigate the functions of *mrp-1/sdf-14* gene, I studied the phenotypes of four mutant alleles in this gene; namely, *ut151, ut153* and *ut155*, point mutations isolated in our laboratory, and *pk89*, a deletion mutant isolated by Broeks et al. (1996). All the mutations showed strong dauer formation constitutive (Daf-c) phenotypes in the *unc-31(e169)* background

Table 6. Homology of MRP1 from various species

	Human MRP1	Mouse MRP1	*S. cerevisiae* YCF1
C. elegans MRP-1b	62 %	63 %	57 %
Human MRP1	-	94 %	60 %
Mouse MRP1	-	-	60 %

C. elegans MRP-1b (SwissProt accession number: Q95QE2), Human MRP1 (SwissProt accession number: P33527), Mouse MRP1 (SwissProt accession number: O35379), and *S. cerevisiae* YCF1 (SwissProt accession number: P39109) encode proteins consisting of 1534, 1531, 1528, and 1515 amino acid residues, respectively.


Worm 1 MFPLVRELVCGSDYELLETGWRNNSNIPQVTNCGQHTDFSTIPTLFLVIFSPILFYELYK 60
Human 1 -MALRGFCSADGSDPLWDWNVTWNTSNPDFTKCFQNTVLVWVPCFYLWACFPFYFLYLSR 59
Worm 61 SRNSYLRSFSAISLRIIFCCLLVVDLTATVIYDFYLLFTKSPLYNAIHFYGDLVQYAGFC 120
Human 60 HDRGYIQMTPLNKTKTALGFLLWIVCWADLFYSFWERSRGIFLAPVFLVSPTLLGIT-TL 118
Worm 121 LALVLTIACRNRGIITSGVITLYWLLVVVCGVPELRYYITGRLYKEYEIHSCRAALYVFA 180
Human 119 LATFLIQLERRKGVQSSGIMLTFWLVALVCALAILRSKIMTALKEDAQVDLFRDITFYVY 178
Worm 181 YVCSALELFLSCFADTP---SNGYIGKNSCPEYTASFLNQLTFQWFSGLAYLGNKKSLEK 237
Human 179 FSLLLIQLVLSCFSDRSPLFSETIHDPNPCPESSASFLSRITFWWITGLIVRGYRQPLEG 238
Worm 238 EDLWDLNERDKAENIIPSFIENLIPEVEGYRRKIKKNPEAAIPKN---------- 282
Human 239 SDLWSLNKEDTSEQVVPVLVKNWKKECAKTRKQPVKVVYSSKDPAQPKESSKVDANEEVE 298
Worm 283 ----------HPSILIPIFKTYKFTLLAGGCYKLMFDLLQFVAPELLRQLISFIEDKNQ 331
Human 299 ALIVKSPQKEWNPSLFKVLYKTFGPYFLMSFFFKAIHDLMMFSGPQILKLLIKFVNDTKA 358
Worm 332 PMWIGVSIALLMFLSSLLQSMILHQYFHEMFRLGMNIRSVLTSAVYTKTLNLSNEARKGK 391
Human 359 PDWQGYFYTVLLFVTACLQTLVLHQYFHICFVSGMRIKTAVIGAVYRKALVITNSARKSS 418
Worm 392 TTGAIVNLMSVDIQRIQDMTTFIMLFWSAPLQILLSLYFLWKLLGVSVLAGFVILILLIP 451
Human 419 TVGEIVNLMSVDAQRFMDLATYINMIWSAPLQVILALYLLWLNLGPSVLAGVAVMVLMVP 478
Worm 452 FNSFISVKMRNCQMEQMKFKDERIKMMSEILNGMKVLKLYSWEKSMEKMVLEVREKEIRV 511
Human 479 VNAVMAMKTKTYQVAHMKSKDNRIKLMNEILNGIKVLKLYAWELAFKDKVLAIRQEELKV 538
Worm 512 LKKLSYLNAATTLSWACAPFLVAVLTFGLYVLWDPENNVLTPQITFVALALFNILRFPLA 571
Human 539 LKKSAYLSAVGTFTWVCTPFLVALCTFAVYVTID-ENNILDAQTAFVSLALFNILRFPLN 597
Worm 572 VFAMVFSQAVQCSASNTRLKEFFAAEEMSPQTS----IAYGGTDSAIKMDGGSFAWGSKE 627
Human 598 ILPMVISSIVQASVSLKRLRIFLSHEELEPDSIERRPVKDGGGTNSITVRNATFTWARSD 657
ut151(T→I)
Walker A motif
Worm 628 EDRKLHDITFNIKRGQLVAIVGRVGSGKSSLLHALLGEMNKLSGSVQVNGSVAYVPQLAW 687
Human 658 PP-TLNGITFSIPEGALVAVVGQVGCGKSSLLSALLAEMDKVEGHVAIKGSVAYVPQQAW 716
ut155(D→N)
Linker peptide
Worm 688 IQNLSLRNNILFNRPYDAKLYQNVIENCALVQDLESLPAEDRTEIGEKGINLSGGQKQRV 747
Human 717 IQNDSLRENILFGCQLEEPYYRSVIQACALLPDLEILPSGDRTEIGEKGVNLSGGQKQRV 776
Walker B motif
Worm 748 SLARAVYQNAEIVLLDDPLSAVDSHVGKHIFENVISTATGCLGTKTRVLLTHGLTYLKHC 807
Human 777 SLARAVYSNADIYLFDDPLSAVDAHVGKHIFENVIG-PKGMLKNKTRILVTHSMSYLPQV 835
Worm 808 DQVIVLKDETISEMGTYQELMNSNGAFSEFLEEFLLEESKHKGRSVSFGEDSKEVNELLR 867
Human 836 DVIIVMSGGKISEMGSYQELLARDGAFAEFLRTYASTEQEQD---------- 877

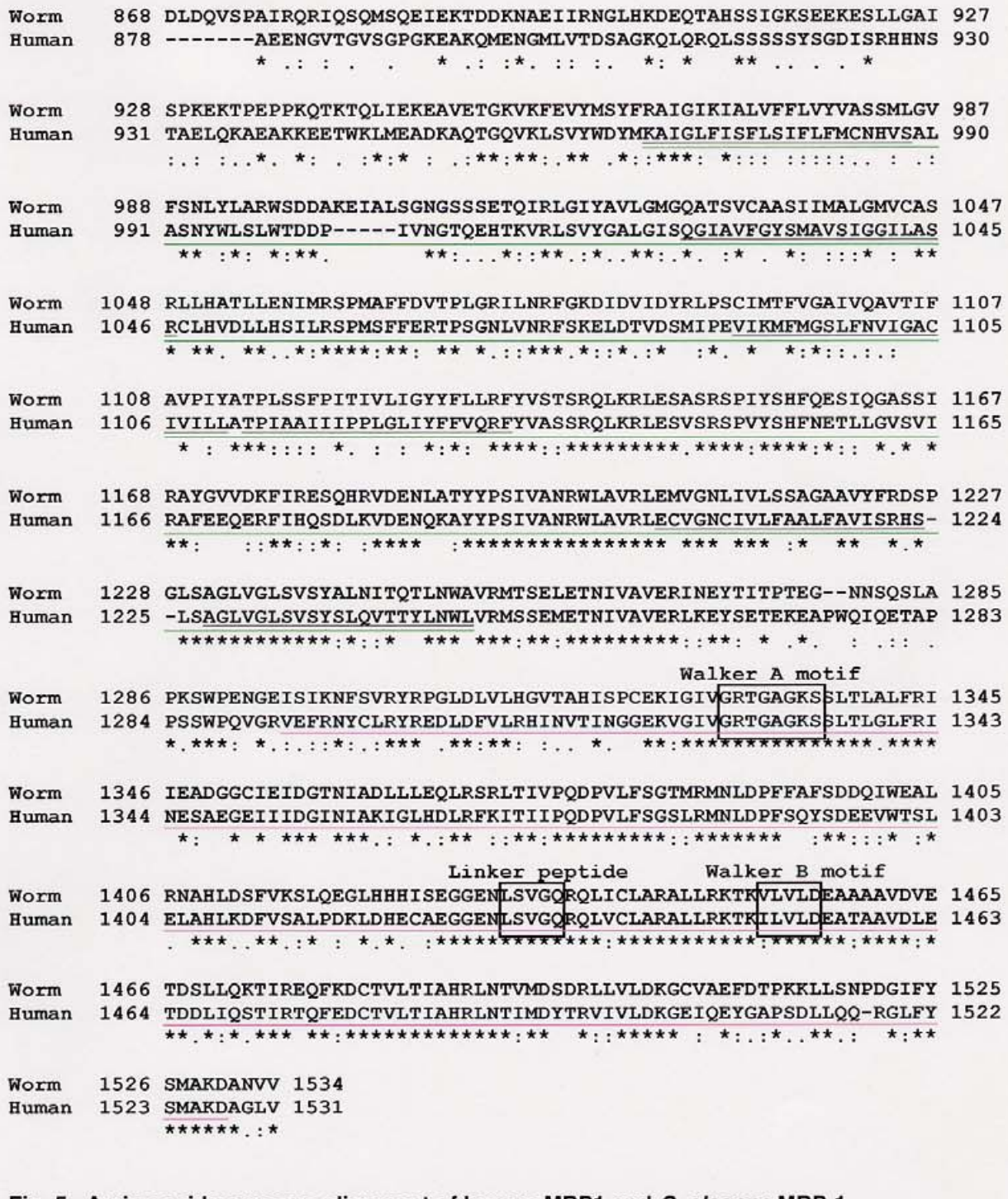

Fig. 5. Amino acid sequence alignment of human MRP1 and *C. elegans* MRP-1
The amino acid sequence of b-type isoform was used for *C. elegans* MRP-1 (SwissProt accession number: Q95QE2). For human MRP1 sequence and its predicted domains, I referred to SwissProt data base (accession number: P33527), and NCBI protein database or PROSITE domain search. Pink underlines show NBDs (nucleotide binding domains). Green underlines show MSDs (membrane spanning domains). Black underlines show TMDs (transmembrane domains). Arrows indicate mutation sites of *sdf-14* mutants. Alignment was carried out by ClustalW.

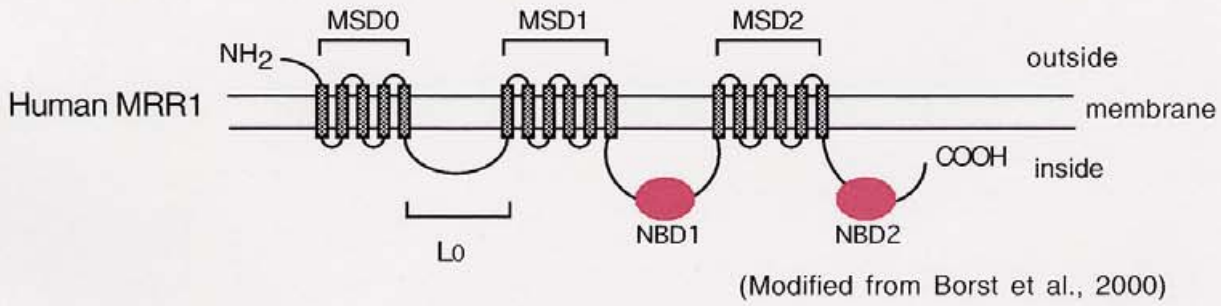

Fig. 6. Topology of human MRP1
Human MRP1 has three membrane spanning domains (MSDs), which consist of 17 transmembrane domains, and two nucleotide binding domains (NBDs). The cytoplasmic region between MSD0 and MSD1 is called L0 region.

Table 7. Mutations in *mrp-1/sdf-14* gene

allele	Nucleotide change	Amino acid change	
ut151	3922 ACC → ATC	T 636 I	
ut153	2468 AG → AA	—	(splice acceptor site)
ut155	4173 GAT → AAT	D 720 N	

Nucleotides changed are shown in pink letters.

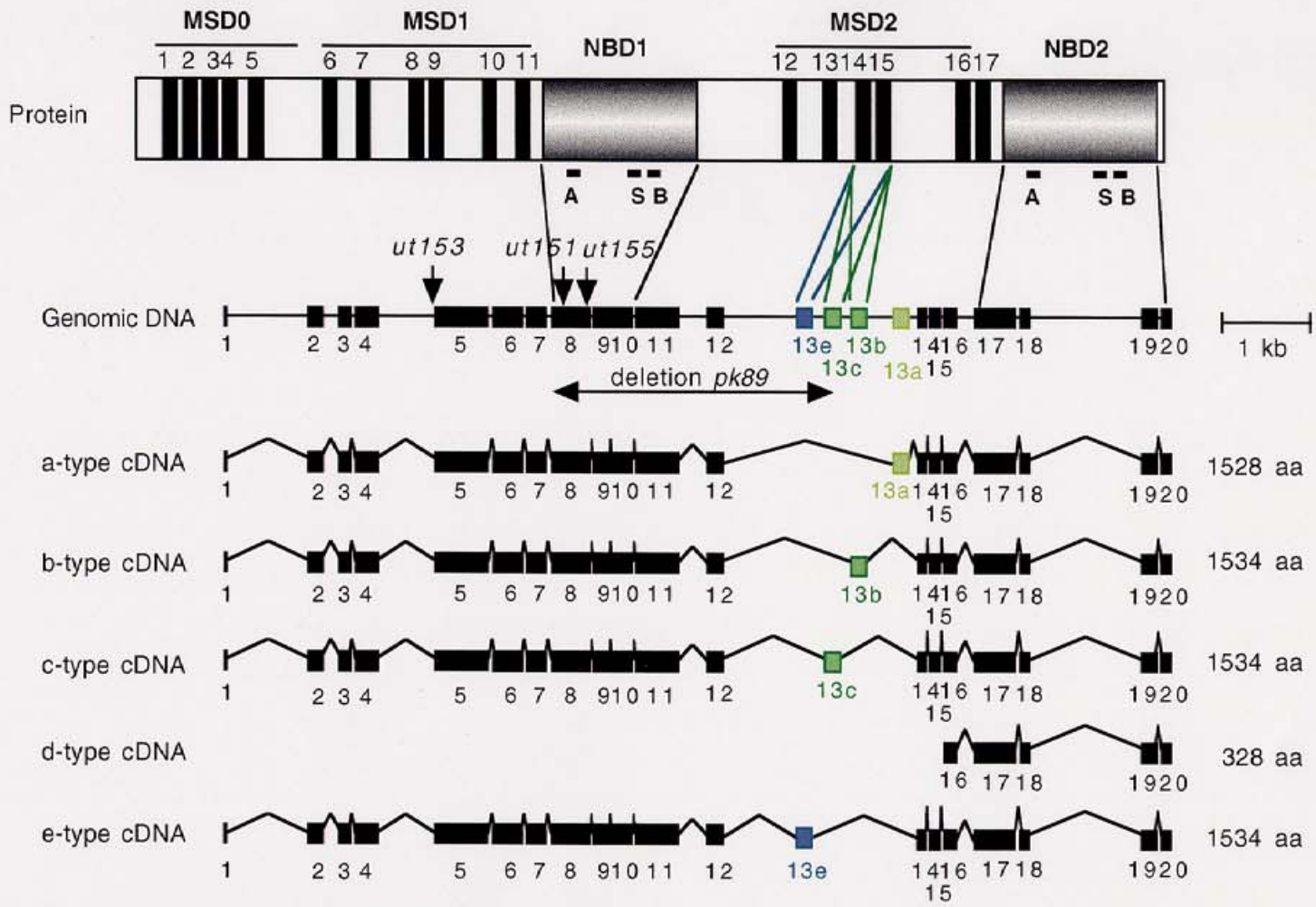

Fig. 7. Protein, gene and cDNA structures of *mrp-1/sdf-14*

Taking human MRP1 into consideration, *C. elegans* MRP-1 MSDs (membrane-spanning domains) were predicted by the SOSUI and TopPred programs, and its NBDs (nucleotide-binding domains) by the PROSITE program. Four isoforms, a-, b-, c-, and d-types are mentioned in Wormbase. I found a 5th isoform (e-type), but could not confirm the a-type isoform. A and B show the Walker A and B motifs for nucleotide-binding, while S shows the ABC transporter signature sequence (Linker peptide). Arrows indicate mutation sites of *ut151*, *ut153* and *ut155*, and deletion region of *pk89*.

at 25.5°C (Fig. 8A), but not at all in the wild type background at 25.5°C or even at 27°C (Fig. 8B).

It was reported that the *mrp-1(pk89)* mutant is sensitive to sodium arsenite and cadmium chloride (Broeks et al., 1996). I therefore tested the influence of these chemical compounds on the dauer formation of the *sdf-14(ut153)* mutant and the *unc-31(e169);sdf-14(ut153)* double mutant. The chemical compounds were added to the NGM agar medium for the plates with which dauer formation was assayed. The *sdf-14(ut153)* mutants did not form dauer larvae even in the presence of sodium arsenite at 27°C (Fig. 9A), but the dauer formation of *unc-31(e169);sdf-14(ut153)* was promoted with sodium arsenite (Fig. 9B). However, this effect was not specific to *sdf-14(ut153)*, because the dauer formation of the *unc-31(e169)* mutant was also promoted by the addition of sodium arsenite to the medium (Fig. 9C). Comparison of the dauer formation among these mutants are shown in Fig. 9D. The influence of cadmium chloride on the dauer formation was not tested, because animals exposed to this compound looked very unhealthy.

3. *C. elegans* MRP-1/SDF-14 has multiple isoforms

WormBase (*C. elegans* data base, http://www.wormbase.org/) suggested that *mrp-1/sdf-14 gene* produces 4 isoforms (a-, b-, c- and d-type), where a-, b- and c-types are different in exon 13, and d-type consists only of exons 16-20 (Fig. 7). I sequenced the exon 13 region of all the cDNA clones that contained this region (cDNA clones were kindly donated by Yuji Kohara). In total, 9 clones were sequenced.

The results showed that 4 clones were b-type, 2 were c-type, and 2 were a new type, which I will call e-type (Table 8). The remaining 1 clone, a partial-length cDNA, had the a-type sequence in exon 13, but retained intron 13, which caused a frame shift that results in a stop codon in exon 14 . One of the e-type clones, yk831b09 which contains a full length transcribed region, was sequenced completely. Although Broeks et al. (1996) reported that yk46e1 was a-

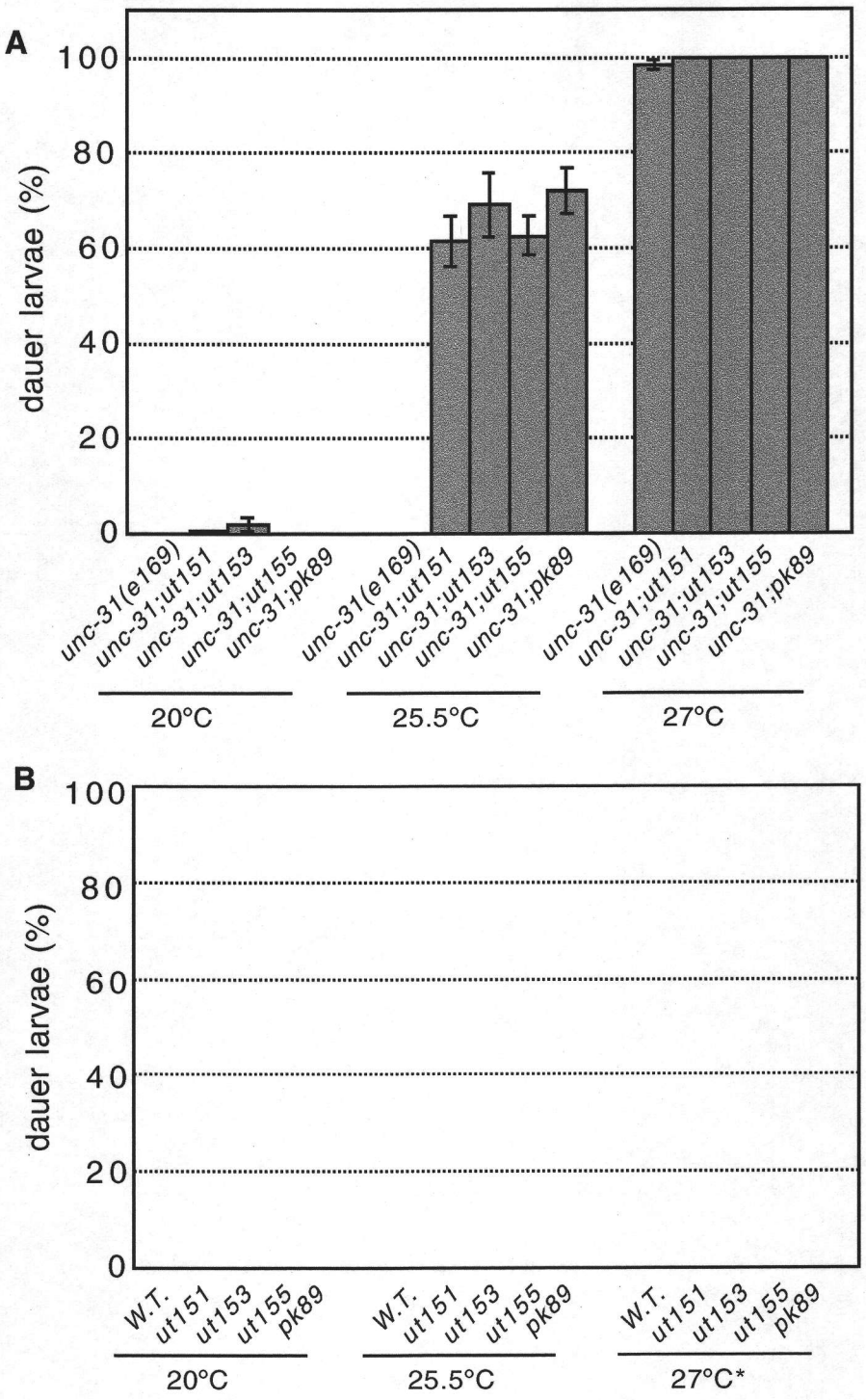

Fig. 8. Dauer formation of *unc-31(e169);sdf-14* double mutants and *sdf-14* single mutants.

(**A**) All the *unc-31(e169);sdf-14* double mutants formed dauer larvae at high percentages at 25.5°C, while the *unc-31(e169)* single mutant formed dauer larvae only at 27°C and not at 25.5°C. (**B**) None of the *mrp-1/sdf-14* mutants formed dauer larvae at 20, 25.5 or 27°C. The means of 3 plates are shown (33-199 animals/plate) in (A) and (B). The error bars indicate standard errors

*In other experiments, *sdf-14* mutants formed dauer larvae at low percentages at 27°C, similar to the level of wild type animals. The variability of dauer formation of at 27°C has been reported (Ailion and Thomas, 2000).

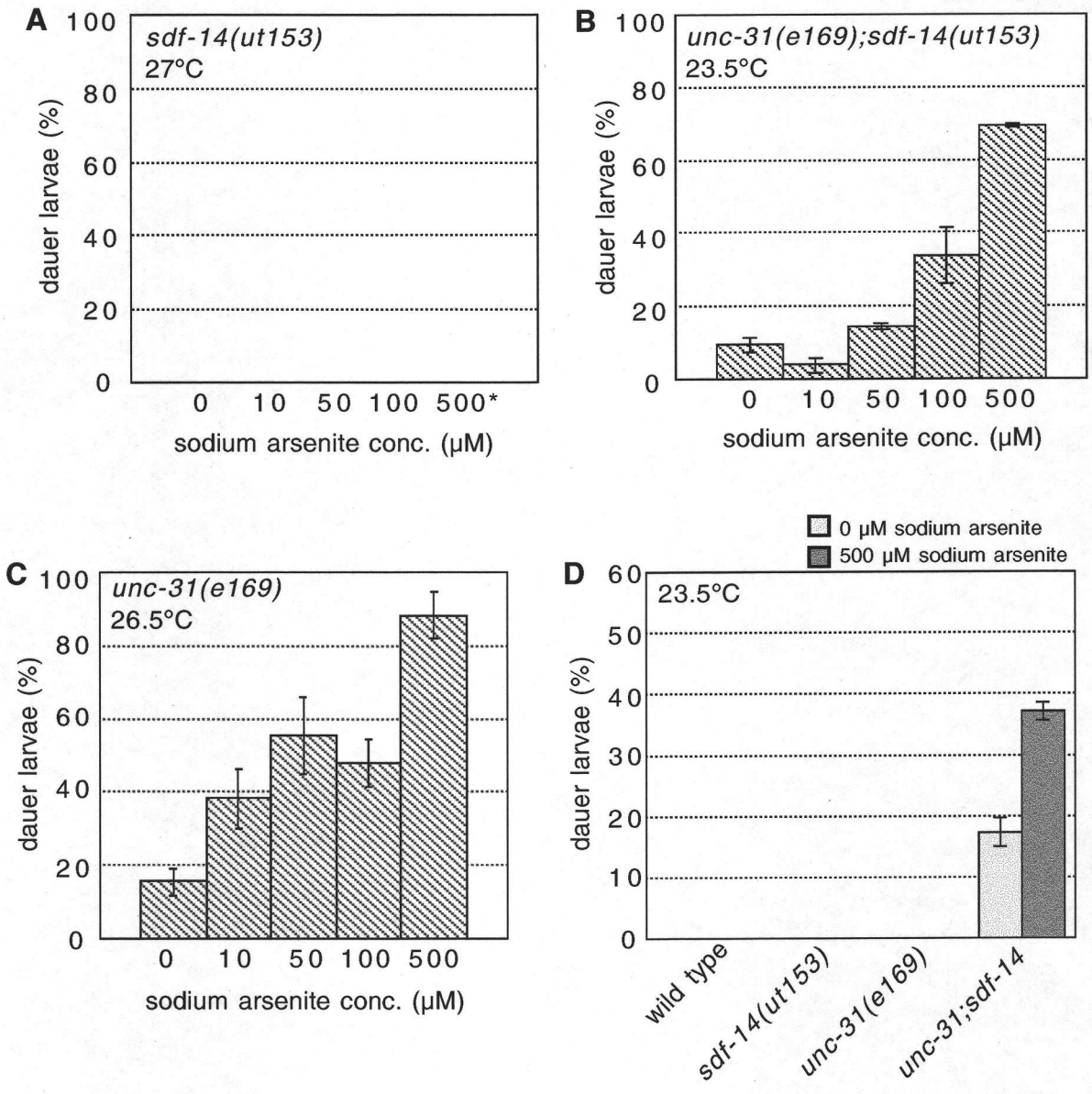

Fig. 9. Influence of sodium arsenite on the dauer formation

The dauer formation of both the *unc-31(e169);sdf-14 (ut153)* double mutant and the *unc-31(e169)* mutant increased with increasing concentrations of sodium arsenite, although *sdf-14(ut153)* single mutant did not form dauer larvae at any concentration of sodium arsenite. (**A**) the dauer formation of the *sdf-14 (ut153)* mutant at 27°C. (**B**) the dauer formation of the *unc-31(e169);sdf-14 (ut153)* mutant at 23.5°C . (**C**) the dauer formation of the *unc-31(e169)* mutant at 26.5°C. (**D**) Comparison of the dauer formation among these mutants. The means of 3 plates are shown (53-229 animals/plate). The error bars indicate standard errors.

*The growth rate of the *sdf-14(ut153)* mutant was reduced at 500μM sodium arsenite.

Table 8. Classification of cDNA clones

Transcript	cDNA clone
a-type	none
b-type	yk15b10, yk131e9, yk1067b9, yk892h9
c-type	yk46e1, yk1289f1
e-type*1	yk494b1, yk831b09
abnormal*2	yk1240d1

*1 The e-type transcript was newly found in this study.
*2 This transcript has the a-type exon13 sequence, but contains a stop codon in exon14 and retains intron 13.

type, its sequence, as determined in this study, was c-type. Thus, I could not confirm the presence of the a-type cDNA except the one retaining intron 13.

To know if there are functional differences among these isoforms, I made DNA constructs shown in Fig. 3 as pSDF14b::GFP, pSDF14c::GFP and pSDF14e::GFP, injected each of them into *unc-31(e169);sdf-14(ut153)* double mutant animals, and examined the transformants for dauer formation. I neglected a-type, which could not be identified, and d-type, which consists only of exons 16-20. The dauer formation phenotype of the *unc-31(e169);sdf-14(ut153)* double mutant was rescued by the b- and c-type constructs, but not by the e-type construct (Fig. 10).

4. Expression pattern of MRP-1/SDF-14

To know the expression pattern of MRP-1/SDF-14, I observed animals that express a GFP fusion gene (construct shown in Fig. 3 as pSDF14::GFP). This construct was functional and rescued the dauer formation abnormality of *unc-31(e169);sdf-14(ut153)* (Fig. 11A). Previously, Broeks et al. (1996) reported that MRP-1/SDF-14 is expressed in the pharynx, pharynx-intestinal valve cells, anterior intestinal cells, intestinal-rectum valve cells and epithelial cells of the vulva. In addition, I found in this study that MRP-1/SDF-14 was weakly expressed in neurons, all intestinal cells and hypodermal seam cells, and that it was localized in cell membrane (Fig. 11B-E). The expression of *C. elegans* MRP-1/SDF-14 in many tissues is similar to that of human MRP1, which is expressed ubiquitously (Flens et al., 1996).

To learn in which tissue MRP-1/SDF-14 acts for the regulation of dauer larva formation, I examined the rescue of *unc-31(e169);sdf-14(ut153)* dauer formation by expressing MRP-1/SDF-14 in various tissues (constructs shown in Fig. 3 as pMyo2p::SDF14b::GFP, pGes1p::SDF14b::GFP and pH20p::SDF14b::GFP). Expression of MRP-1/SDF-14 in only one tissue (neurons, intestinal cells or pharyngeal muscles) rescued the dauer formation abnormality only weakly. In contrast, the dauer formation abnormality was almost completely rescued in

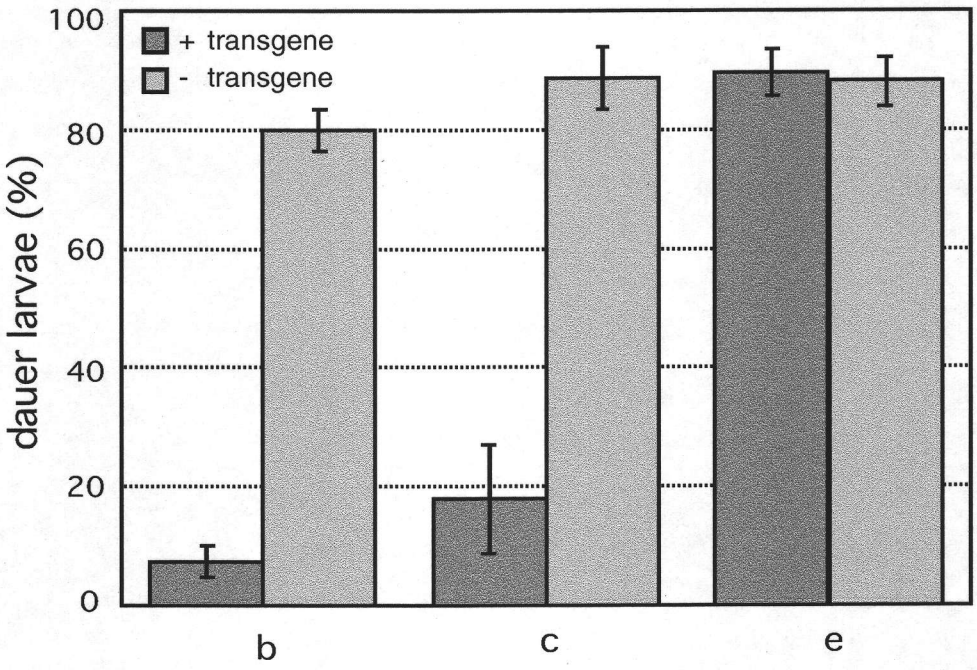

Fig. 10. The b- and c-type isoforms but not the e-type isoform can rescue of the Daf-c phenotype of *unc-31(e169);sdf-14(ut153).*

The means of multiple lines are shown (2 lines of b-type, 8 lines of c-type, and 4 lines of e-type). For each line, the assays were carried out with 13-128 animals/plate at 25.5°C. The error bars indicate standard errors.

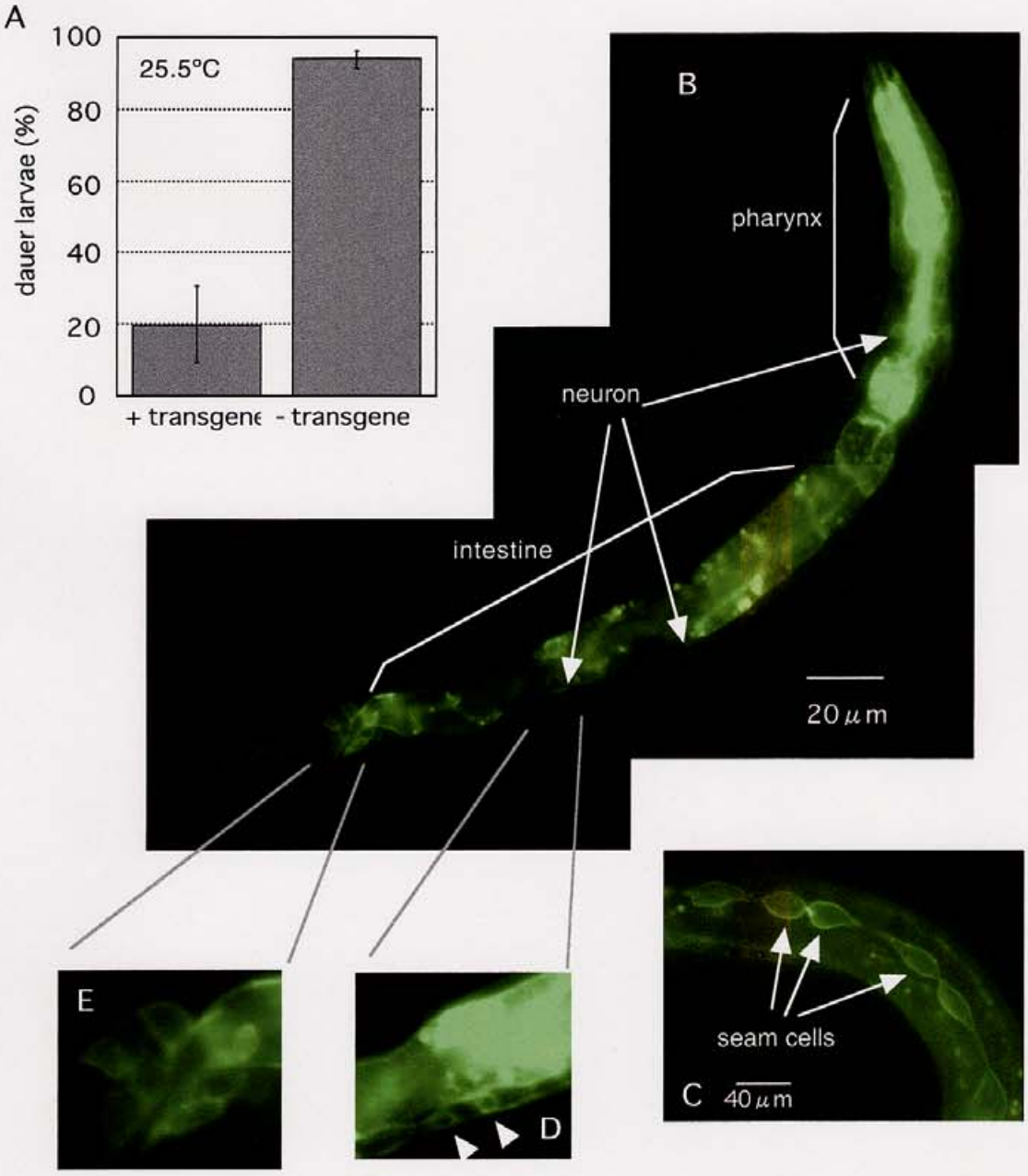

Fig. 11. Expression pattern of a functional *mrp-1*::GFP fusion gene.
(**A**) Dauer larva formation of *unc-31(e169);sdf-14(ut153)* was rescued by introducing an *mrp-1*::GFP fusion gene. The error bars indicate standard errors. (**B**) MRP-1 was expressed in various tissues (L1 larva). (**C**) MRP-1 was expressed also in seam cells (L2 or L3 Larva).(**D**) Neuronal expression. Neurons are indicated by arrowheads (**E**) Localization of GFP at cell membrane. Expression patterns were observed in wild type animals. The GFP fusion gene was coinjected with the *rol-6* dominant marker.

animals that expressed MRP-1/SDF-14 in 2 or 3 tissues (Fig. 12). The results show that there is no specific tissue in which MRP-1 functions to prevent dauer formation, and that MRP-1 molecules in neurons, intestinal cells and pharyngeal muscles act additively for this function.

5. Rescue experiments using human *MRP1* cDNA

If human MRP1 can substitute *C. elegans* MRP-1 for a function *in vivo*, it will provide useful information on the mechanism of the function, because human MRP1 has been extensively characterized. I therefore examined whether the expression of human MRP1 can rescue the dauer formation abnormality of *unc-31(e169);sdf-14(ut153)*. The human *MRP1* cDNA, kindly donated by Tatsuhiko Furukawa, was connected to the *C. elegans sdf-14/mrp-1* promoter (Fig. 3 as pSDF14p::hMRP1) and introduced into *unc-31(e169);sdf-14(ut153)* double mutant animals by microinjection. In total, 9 lines of transformants were obtained, of which 5 lines seemed to show partially rescued phenotypes in the dauer formation abnormality. Three of these lines, whose rescue were superior to others, were checked again for the rescue. The results showed that the rescue of dauer formation abnormality was almost complete in one line (Fig. 13A). Namely, human MRP1 can substitute for *C. elegans* MRP-1/SDF-14 in the regulation of dauer larva formation.

The mutant human *MRP1* cDNA, *dmLoMRP1* cDNA, also kindly donated by Tatsuhiko Furukawa, has several amino acid mutations in the Lo region (Fig. 6) and can transport neither leukotriene C_4 (LTC_4) nor 17ß-estradiol 17-(ß-D-glucuronide) ($E_2$17ßG) (Noguchi, Ren and Furukawa, unpublished data). I examined whether this cDNA can rescue the dauer formation abnormality of *unc-31(e169);sdf-14(ut153)* (construct shown in Fig. 3 as pSDF14p::mhMRP1). Although as many as 19 lines were examined, the dauer formation abnormality of *unc-31(e169);sdf-14(ut153)* was not rescued at all in any of them (Fig. 13B). Thus, the amino acid residues of human MRP1 essential for its function as a transporter in human cells are also

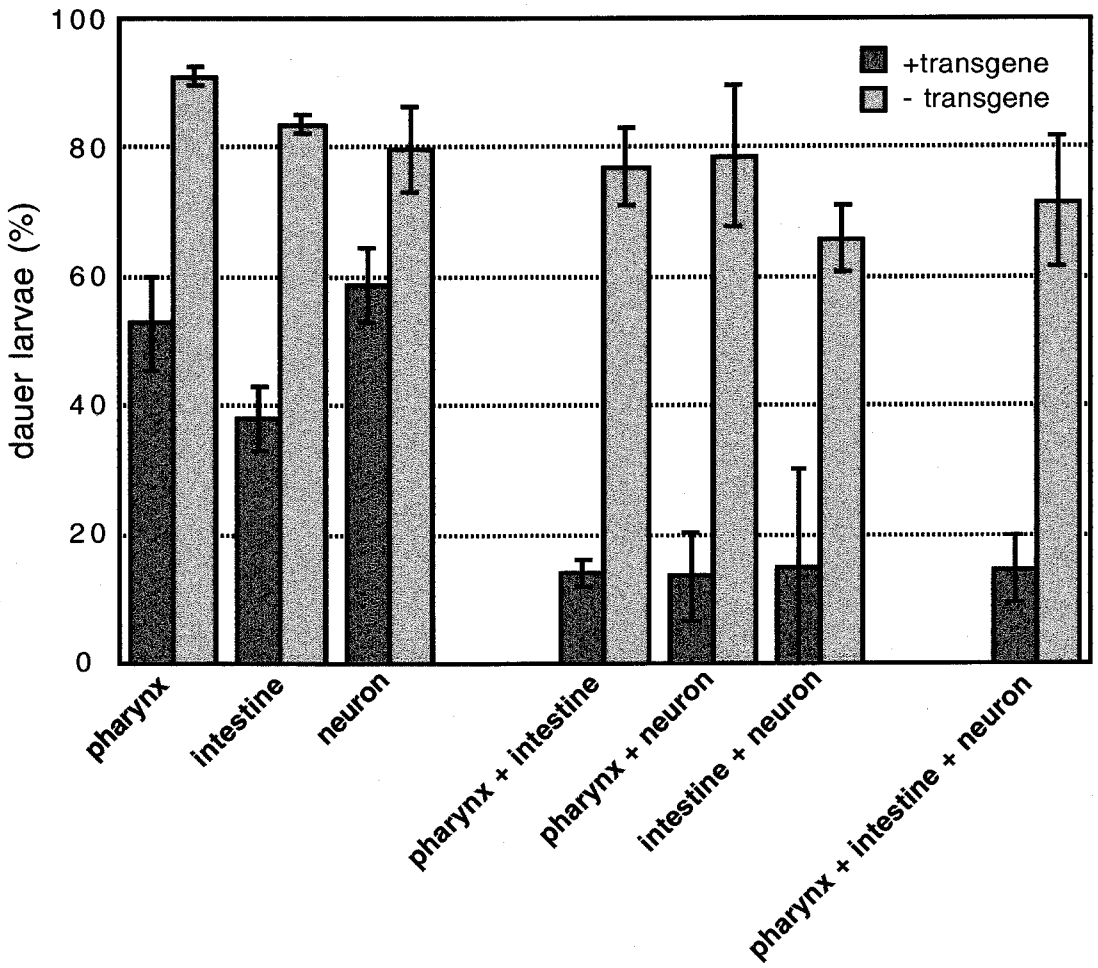

Fig. 12. Rescue of dauer larva formation in the *unc-31(e169);sdf-14(ut153)* mutant by the expression of SDF-14/MRP-1 in various tissues

mrp-1::GFP fusion constructs driven by promoters for expression in various tissues (*myo-2* promoter for pharyngeal muscles, *ges-1* promoter for intestinal cells, and H20 promoter for neurons) were introduced either separately or in combination into *unc-31(e169);sdf-14(ut153)* double mutant animals, and the dauer larva formation of the transformants was examined at 25.5°C. The results show that multiple tissue expression is necessary to rescue the abnormality in dauer formation efficiently. The means of 2-8 lines are shown (19-92 animals/line). The error bars indicate standard errors.

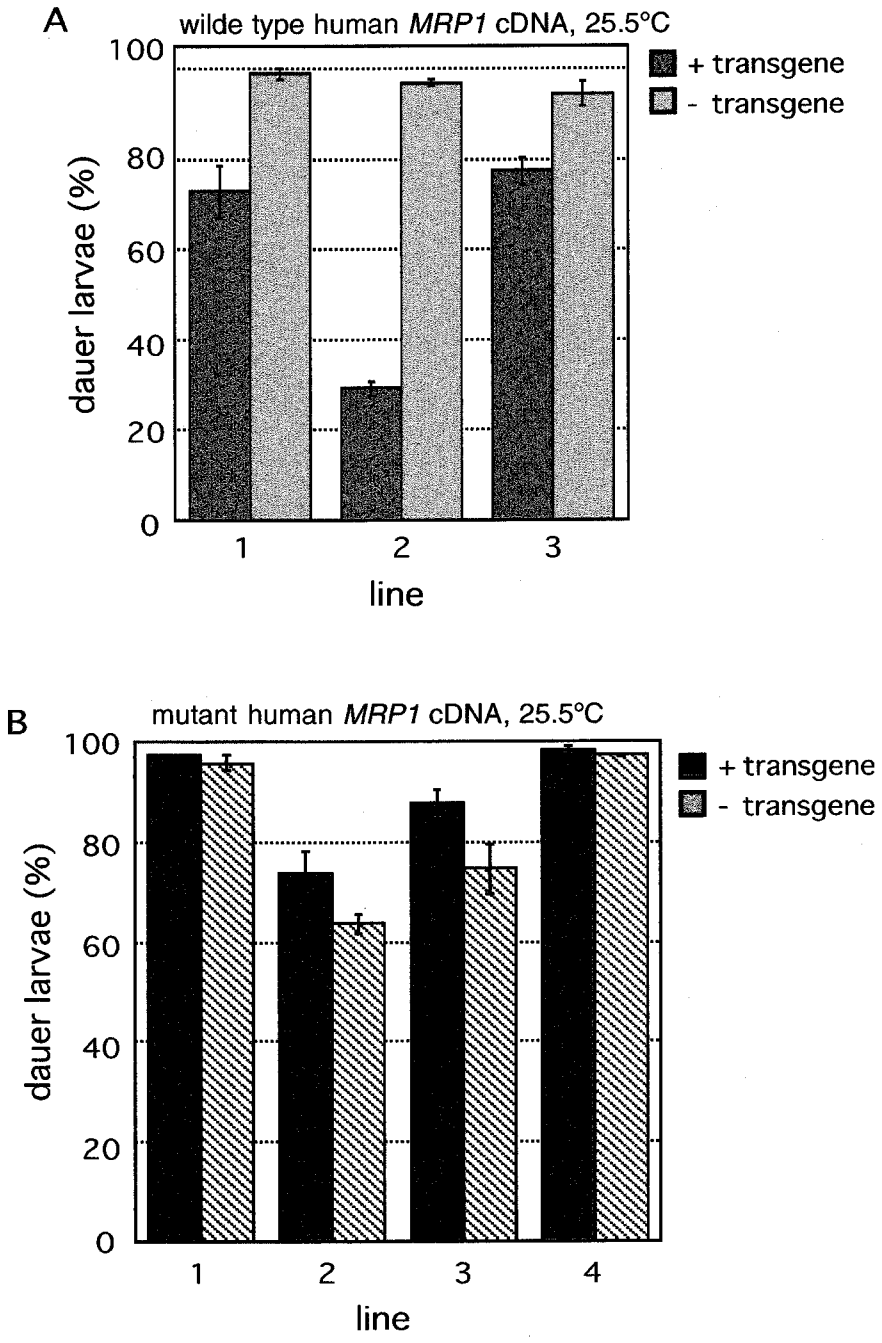

Fig. 13. Dauer larva formation in the transformants of the wild type human *MRP1* cDNA and a mutant human *MRP1* cDNA

(**A**) *unc-31(e169);sdf-14(ut153)* double mutant carrying an extrachromosomal array of the wild type human *MRP1* cDNA driven by the *sdf-14* promoter. In total, 9 lines of transformants were obtained, of which one lines showed almost complete rescue of the dauer formation (line No. 2) The results of 3 lines of the 9 transformants are presented. The means of 3 plates (42-107 animals/plate) are shown. The error bars indicate standard errors. (**B**) *unc-31(e169);sdf-14(ut153)* double mutant carrying an extrachromosomal array of a mutant human *MRP1* cDNA driven by the *sdf-14* promoter. In total, 19 lines of transformants were obtained, and the results of four of them are presented. The means of 3 plates (58-122 animals/plate) are shown. The error bars indicate standard errors.

essential in the regulation of dauer larva formation in *C. elegans*.

The effects of human MRP1 inhibitors on the dauer formation of rescued lines were investigated. If the inhibitors can suppress the transport activity of human MRP1 or *C. elegans* MRP-1/SDF-14, the percentage of dauer larvae should increase. I used three inhibitors, PAK-104P (a pyridine analog) (Shudo et al., 1990), agosrerol A (AG-A) isolated from a marine sponge (Aoki et al., 1998), and MK571 (an anionic quinoline derivative) (Jones et al., 1989), each of which was added to the NGM agar medium for the plates with which dauer formation was assayed. The results showed that only PAK-104P had effect on the dauer formation of a line expressing human MRP1 but not on the dauer formation of a line expressing *C. elegans* MRP-1/SDF-14 (Fig. 14A & Fig. 15A). The other inhibitors had no detectable effect on a line expressing either human MRP1 or *C. elegans* MRP-1/SDF-14 (Fig. 14B, C, & Fig. 15B, C). Those experiments confirmed that the transporter activity of human MRP1 is important in its function of dauer larva regulation in *C. elegans*.

6. Position of *mrp-1/sdf-14* gene in the genetic pathways of dauer formation

Dauer larva formation is controlled by four pathways: the *daf-11* cGMP-related signaling pathway, the *daf-7* TGF-β signaling pathway, the *daf-2* insulin signaling pathway, and the *daf-9* steroid hormone signaling pathway (Vowels & Thomas, 1992; Thomas et al., 1993; Gottlieb & Ruvkun, 1994; Malone et al., 1996; Riddle & Albert, 1997; Jia et al., 2002; Ohkura et al., 2003). In this section, I will discuss the position of *mrp-1/sdf-14* gene in the genetic pathways of dauer formation. Besides the possibility that *mrp-1/sdf-14* gene acs in one of the four known signaling pathways, I also considered the following two possibilities: *mrp-1/sdf-14* gene may act in an unknown pathway, and *mrp-1/sdf-14* mutations may have indirect influence on many known pathways.

In general, the position of a *daf-c* (dauer-constitutive) gene in the dauer regulatory

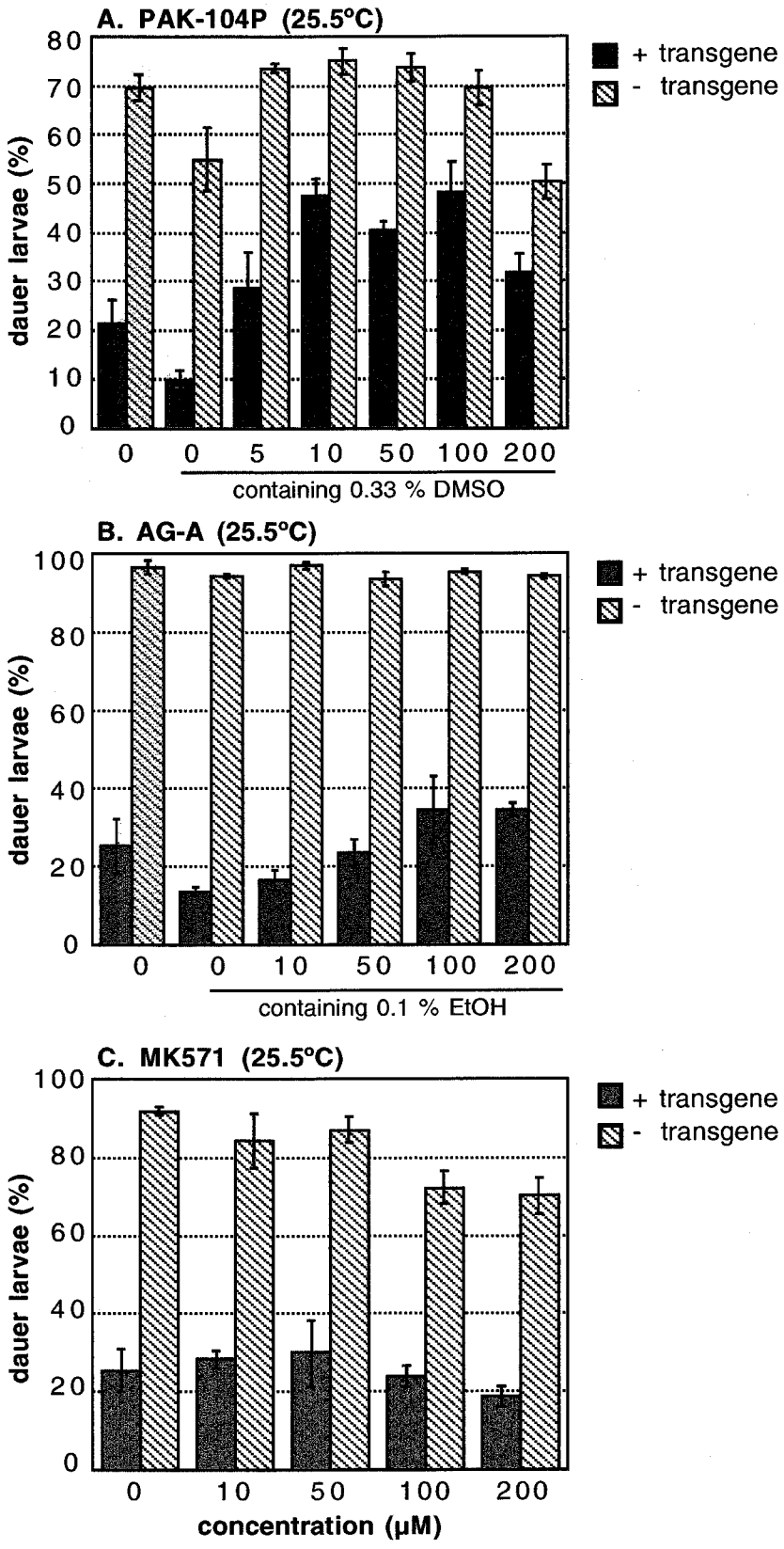

Fig. 14. The effect of human MRP1 inhibitors on the dauer larva formation of a *C. elegans* strain carrying human *MRP1* gene

The effect of human MRP1 inhibitors was examined for the dauer formation of the *unc-31(e169);sdf-14(ut153)* mutant animals carrying an *sdf-14* promoter::human *MRP1* cDNA transgene as an extrachromosomal array. PAK-104P partially supressed dauer larva formation (**A**), but AG-A and MK571 did not supress it (**B** and **C**). The means of 3 plates are shown (81-227 animals/plate). The error bars indicate standard errors.

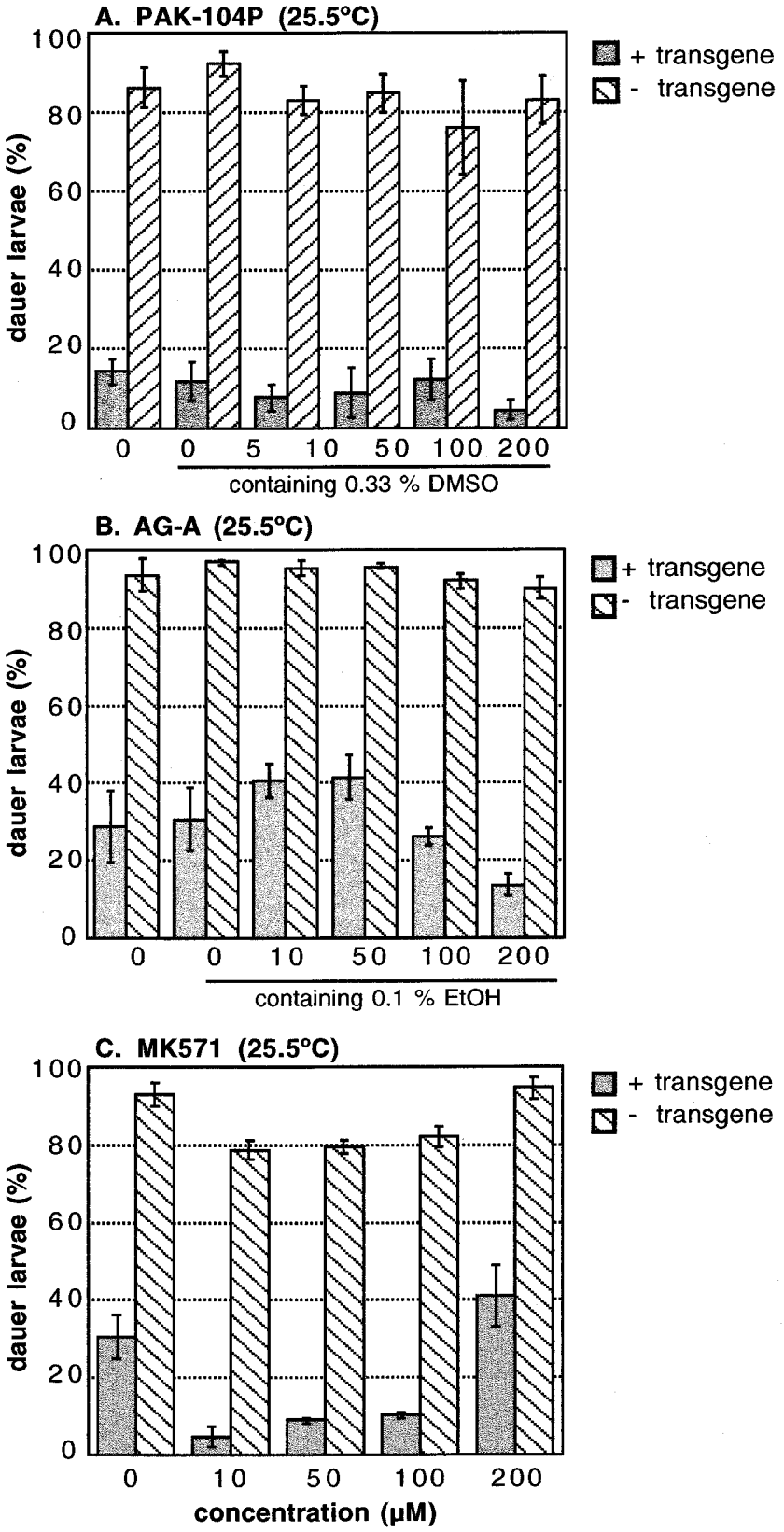

Fig. 15. The effect of human MRP1 inhibitors on the dauer larva formation of a *C. elegans* strain carrying *C. elegans mrp-1* gene

The effect of human MRP1 inhibitors were examined for the dauer formation of *unc-31(e169);sdf-14(ut153)* mutant animals carrying *C. elegans mrp-1* transgene as an extrachromosomal array. All the inhibitors, PAK-104P, AG-A and MK571, did not supress the dauer formation (**A, B** and **C**).The means of 3 plates are shown (68-292 animals/plate). The error bars indicate standard errors.

pathways can be estimated by epistasis between a mutation in this gene and a *daf-d* (dauer-defective) mutation in a known pathway. If the *daf-c;daf-d* double mutant shows a Daf-d phenotype, the *daf-c* gene is located upstream of the *daf-d* gene. In contrast, if the *daf-c;daf-d* double mutant shows a Daf-c phenotype, the *daf-c* gene is located downstream of or parallel to the *daf-d* gene. In the experiments below, I modified this method and used *x;mrp-1* double mutants as *daf-c* mutants, because *mrp-1/sdf-14* mutations did not show Daf-c phenotypes by themselves. As the "x" mutations, I used low-penetrance *daf-c* mutations in known dauer regulatory pathways, while as the *daf-d* mutations, I used those which do not suppress the Daf-c phenotype of "*x*" in order to know suppression of *mrp-1/sdf-14* and not "*x*".

I also used another method for estimating the pathway in which *mrp-1/sdf-14* gene acts. It is known that all the *daf-c* mutants in the cGMP-related and TGF-β signaling pathways show incompletely penetrant Daf-c phenotypes at low temperature and highly penetrant Daf-c phenotypes at high temperature. Daf-c double mutants between the cGMP-related signaling pathway and the TGF-β signaling pathway form nearly 100% dauer larvae even at low temperature, but all double mutants between genes within the same pathway remain incompletely penetrant for the Daf-c phenotype at low temperature (Thomas et al., 1993). Therefore, if *mrp-1/sdf-14* gene belongs to the cGMP-related signaling pathway, *mrp-1/sdf-14* mutations will cause strong enhancement of the Daf-c phenotype of the TGF-β signaling pathway but not that of the cGMP-related signaling pathway. In contrast, if *mrp-1/sdf-14* gene belongs to the TGF-β signaling pathway, *mrp-1/sdf-14* mutations will cause strong enhancement of the Daf-c phenotype of the cGMP-related signaling pathway but not that of the TGF-β signaling pathway. I first examined these possibilities in section 6-1.

6-1. *mrp-1/sdf-14* acts neither in the cGMP-related signaling pathway nor in the TGF-β signaling pathway

I used *daf-11(m47)* as a *daf-c* mutation in the cGMP-related signaling pathway and *daf-*

1(m40) as a *daf-c* mutation in the TGF-β signaling pathway in the construction of double mutants with *mrp-1(pk89)*. DNA sequencing of this study predicted that these mutations are null alleles: *daf-11(m47)* is a nonsense mutation at Q490, which is predicted to truncate DAF-11 before the functional domains, while *daf-1(m40)* is a nonsense mutation at W170, which is predicted to truncate DAF-1 before the protein kinase domain (Table 4). The results of dauer formation assays indicated that neither *daf-11(m47);mrp-1(pk89)* nor *daf-1(m40);mrp-1(pk89)* showed a remarkable increase of dauer formation (Fig. 16). Hence, *mrp-1/sdf-14* gene seems to be located neither in the cGMP-related signaling pathway nor in the TGF-β signaling pathway.

To confirm this prediction, I constructed the *daf-2(e1370);mrp-1(pk89)* double mutant. *daf-2* gene acts in the insulin signaling pathway and the *e1370* allele shows a temperature-sensitive Daf-c phenotype, which is not suppressed by *daf-d* mutations in the cGMP-related signaling pathway or the TGF-β signaling pathway (Vowels & Thomas, 1992). If the *mrp-1(pk89)* mutation enhances the Daf-c phenotype of the *daf-2(e1370)* mutant at low temperature and if *mrp-1/sdf-14* gene is positioned either in the cGMP-related signaling pathway or in the TGF-β signaling pathway, the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* double mutant will be suppressed by *daf-d* mutations in the cGMP-related or TGF-β signaling pathway as well as those in the insulin signaling pathway.

The Daf-c phenotype of *daf-2(e1370)* was strongly enhanced by *mrp-1(pk89)* at 20°C (Fig. 16). The Daf-c phenotype of the *daf-2(e1370);mrp-1(pk89)* double mutant was suppressed by a *daf-d* mutation of the insulin signaling pathway, *daf-16(mu86),* as expected (Fig. 17), but not by a *daf-d* mutation of the cGMP-related signaling pathway, *che-3(e1124),* or the TGF-β signaling pathway, *daf-5(e1386)* (Fig. 18 & 19). Therefore, I conclude that *mrp-1/sdf-14* gene is located neither in the cGMP-related signaling pathway nor in the TGF-β signaling pathway. Details of these experiments are described in 6-1a and 6-1b below.

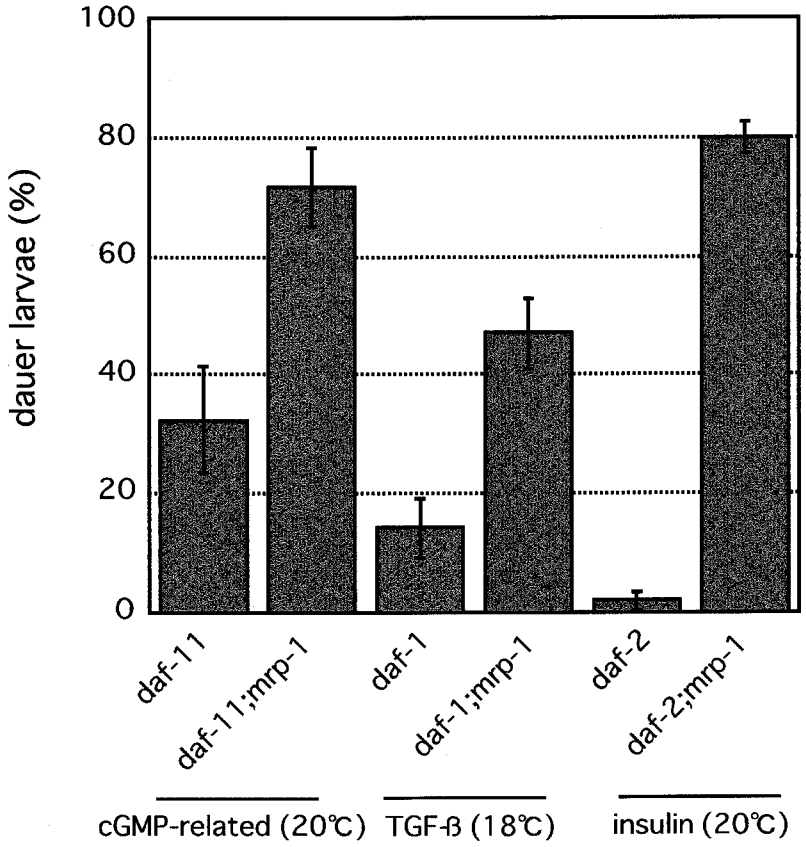

Fig. 16. The enhancement of the dauer larva formation of various *daf-c* mutants by *mrp-1(pk89)*

The *mrp-1(pk89)* mutation remarkably promoted the dauer formation of the *daf-2(e1370ts)* mutant. *daf-11(m47)* is a *daf-c* mutation in the cGMP-related signaling pathway. *daf-1(m40)* is a *daf-c* mutation in the TGF-β signaling pathway. *daf-2(e1370)* is a *daf-c* mutation in the insulin signaling pathway. The means of 3 plates are shown (41-119 animals/plate). The error bars indicate standard errors.

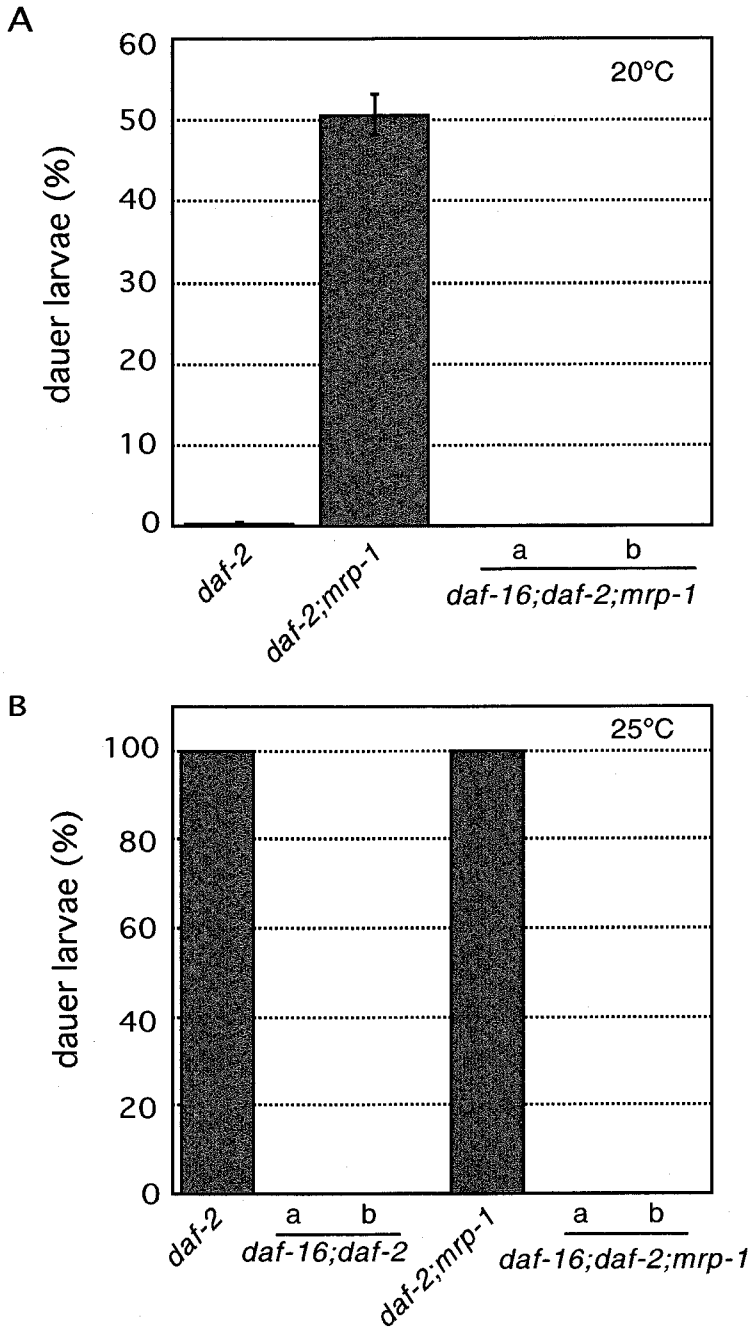

Fig. 17. The Daf-c phenotype of the *daf-2(e1370);mrp-1(pk89)* double mutant was completely suppressed by a *daf-d* mutation in the insulin signaling pathway, *daf-16(mu86).*

(**A**) *mrp-1(pk89)* enhanced dauer formation of *daf-2(e1370)*, a *daf-c* mutation in the insulin signaling pathway, while the Daf-c phenotype of *daf-2;mrp-1* was suppressed by *daf-16(m86)*, a *daf-d* mutation in the insulin signaling pathway. The experiments were carried out at 20°C. (**B**) The Daf-c phenotype of the *daf-2;mrp-1* double mutant was suppressed by *daf-16* also at 25°C. The means of 3 plates are shown. Data of 78-189 animals/plate in A and 38-165 animals/plate in B. The error bars indicate standard errors of mean. **a** and **b** correspond to different lines of multiple mutants.

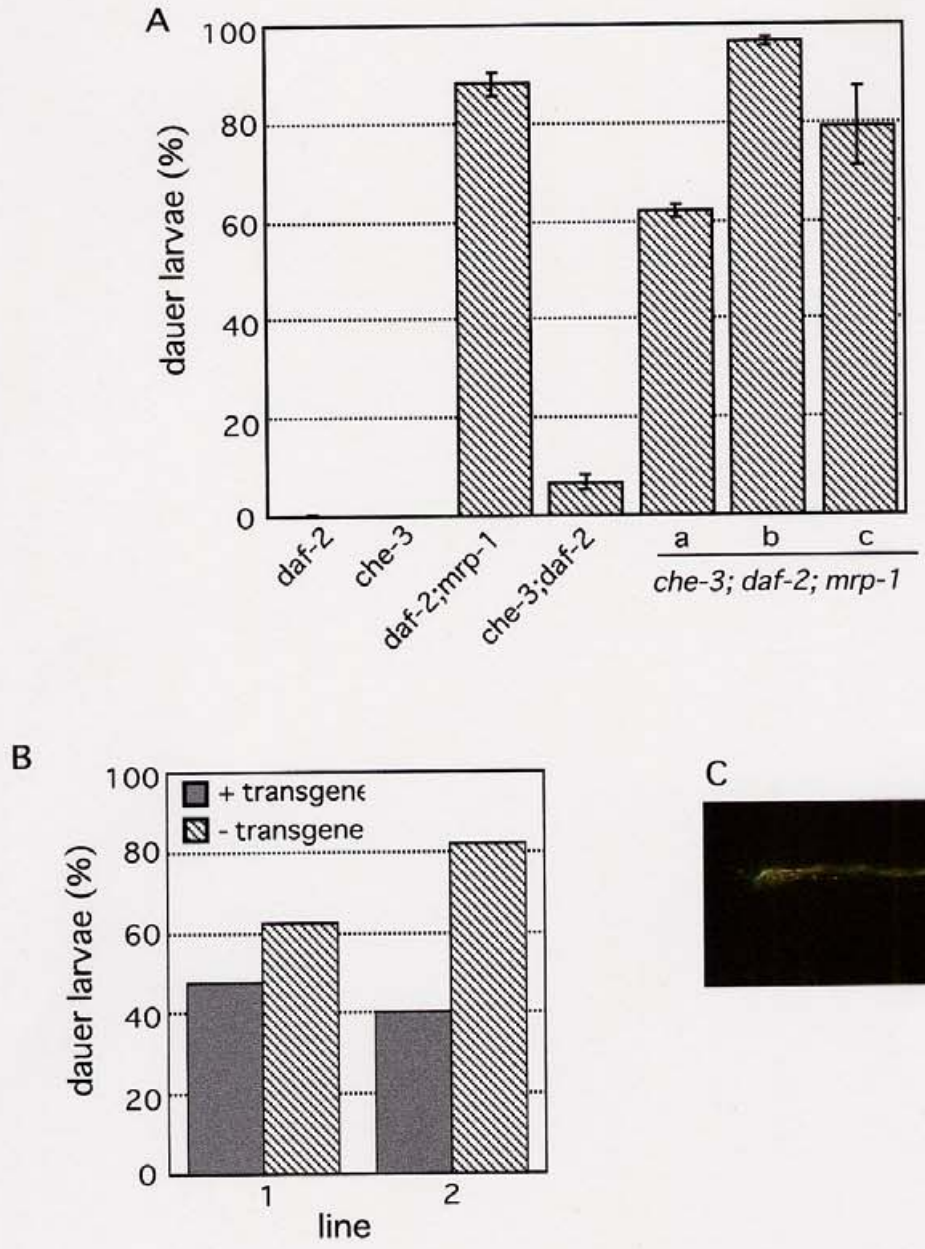

Fig. 18. The influence of the *che-3(e1124)* mutation on the dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant

(**A**) Dauer formation of three independent lines of the triple mutant *che-3(e1124);daf-2(e1370);mrp-1(pk89)*. The Daf-c phenotype of the *daf-2(e1370);mrp-1(pk89)* mutant was not suppressed by the *che-3(e1124)* mutation in line b, but looked as if partially suppressed by *che-3(e1124)* in lines a and c. The means of 3 plates are shown (40-160 animals/plate). The error bars indicate standard errors. The assays were performed at 20°C. (**B**) Introduction of the wild type *che-3* gene did not rescue the partial suppression of the dauer formation in *che-3(e1124);daf-2;(e1370);mrp-1(pk89)*. The data are based on the results of 29 animals in line 1 and 41 animals in line 2. The assays were performed at 20°C. (**C**) The dye-filling of the transformant line 1 in Fig. B, showing that the dye-filling defective phenotype of *che-3(e1124)* was rescued, namely, the transformation was successful. The orange fluorescence shows dye filling, while the green fluorescence is GFP used as the marker of transformation.

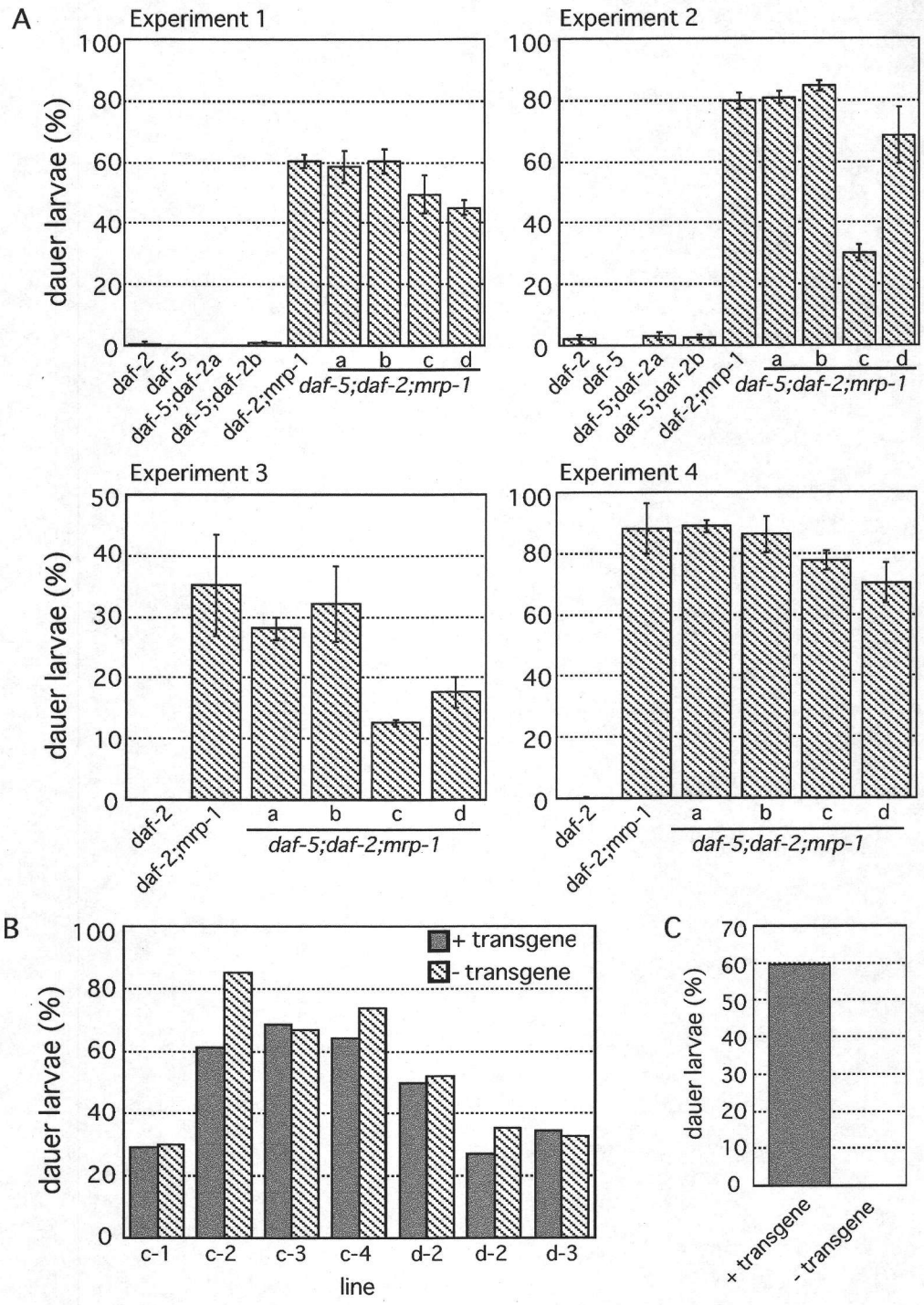

Fig. 19. The influence of the *daf-5(e1386)* mutation on the dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant

(**A**) Dauer formation of the *daf-5(e1386);daf-2(e1370);mrp-1(pk89)* triple mutant at 20°C. Four series of experiments were carried out, using four lines of the triple mutant. The percentages of dauer larvae changed considerably depending on the series of experiments, probably due to uncontrollable conditions such as slight changes in the temperature, conditions of bacteria (food), etc. However, the results show much less fluctuation within each series of the experiments. The means of 3 plates are shown (59-160 animals/plate). The error bars indicate standard errors. (**B**) Suppression of the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* by *daf-5(e1386)* was not rescued by the wild type *daf-5* transgene. The assays were performed at 20°C. Data from plates containing 45-242 animals. (**C**) A positive control of the transgene. The suppression of the dauer formation of *unc-3(e151)* at 25.5°C by *daf-5 (e1386)* (Ailion & Thomas, 2000) was overcome by the wild type *daf-5* transgene. Data of 103 animals on one plate.

6-1a. cGMP-related signaling pathway

I chose *che-3(e1124)* as the *daf-d* mutation for the epistasis test. It is already known that the *che-3(e1124)* mutation suppresses all the *daf-c* mutations in the cGMP-related signaling pathway (*daf-11* and *daf-21*), but not *daf-2(e1370)* (Vowels & Thomas, 1992). Hence, the suppression of the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* by *che-3(e1124)* depends on whether the *che-3(e1124)* mutation can suppress the effect of the *mrp-1(pk89)* mutation, and if not, it strongly suggests that *mrp-1/sdf-14* gene does not act in the cGMP-related signaling pathway.

The results showed that the phenotype depends on the lines of the triple mutant *che-3(e1124);daf-2(e1370);mrp-1(pk89)*. Namely, I made three lines of *che-3(e1124);daf-2(e1370);mrp-1(pk89)*, of which one line produced slightly more dauer larvae than *daf-2(e1370);mrp-1(pk89)* (i.e., no suppression), while the other two lines produced a little fewer dauer larvae (Fig. 18A). The latter cases, however, are not due to suppression by the *che-3(e1124)* mutation but probably by a side mutation, because introduction of the wild type *che-3* gene into one of the two lines did not rescue the effect of the *che-3(e1124)* mutation (Fig. 18B).

Strictly speaking, it may be possible that the triple mutant *che-3(e1124);daf-2(e1370);mrp-1(pk89)* produces many dauer larvae under the conditions in which *che-3(e1124)* suppresses the effect of *mrp-1(pk89)*, if *che-3(e1124)* enhances the Daf-c phenotype of *daf-2(e1370)* to a large extent. However, this is unlikely because the double mutant *che-3(e1124);daf-2(e1370)* produces only a few dauer larvae (Fig. 18A). Considering all these arguments, I conclude that *mrp-1/sdf-14* gene does not act in the cGMP-related signaling pathway.

6-1b. TGF-β signaling pathway

I chose *daf-5(e1386)* as the *daf-d* mutation for the epistasis test. The *daf-5(e1386)* mutation suppresses all the *daf-c* mutations in the TGF-β signaling pathway, but not *daf-2(e1370)* (Vowels & Thomas, 1992). Hence, the suppression of the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* by *daf-5(e1386)* depends on whether the *daf-5(e1386)* mutation can

suppress the effect of the *mrp-1(pk89)* mutation, and if not, it is strongly suggests that *mrp-1/sdf-14* gene does not act in the TGF-β signaling pathway.

The results showed that the phenotype depends on the lines of the triple mutant *daf-5(e1386);daf-2(e1370);mrp-1(pk89)*. Of the four lines of *daf-5(e1386);daf-2(e1370);mrp-1(pk89)*, two lines (a and b) showed no suppression by *daf-5(e1386)*, while the other two lines occasionally produced a little fewer dauer larvae than *daf-2(e1370);mrp-1(pk89)* (Fig. 19A). However, the latter cases are probably due to suppression by a side mutation, because introduction of the wild type *daf-5* gene into the two lines did not rescue the effect of the *daf-5(e1386)* mutation (Fig. 19B).

Since the *daf-5(e1386)* mutation does not enhance the dauer formation of *daf-2(e1370)* (Fig. 19A), these arguments indicate that *mrp-1/sdf-14* does not act in the TGF-β signaling pathway.

6-2. Effect of *daf-6* and *daf-22* mutations on the synthetic dauer-constitutive phenotype of *mrp-1/sdf-14*

On the basis of similar experiments I conclude that the effect of the *mrp-1(pk89)* mutation on dauer formation is not suppressed by the *daf-6(e1377)* mutation, which is located upstream of the TGF-β and cGMP-related signaling pathways (Vowels & Thomas, 1992). Although the triple mutant *daf-6(e1377);daf-2(e1370);mrp-1(pk89)* produced smaller percentages of dauer larvae than the double mutant *daf-2(e1370);mrp-1(pk89)* (Fig. 20A), this effect was due to a side mutation, because it was not rescued by the wild type *daf-6* transgene (Fig. 20B).

I also tested whether the dauer formation of *daf-2(e1370);mrp-1(pk89)* is suppressed by the *daf-22(m130)* mutation, which is required for the production of dauer-inducing pheromone (Golden & Riddle, 1985) and which is located most upstream in the whole dauer regulatory pathway (Vowels & Thomas, 1992). The triple mutant of *daf-22(m130);daf-2(e1370);mrp-1(pk89)* produced smaller percentages of dauer larvae than the double mutant of *daf-*

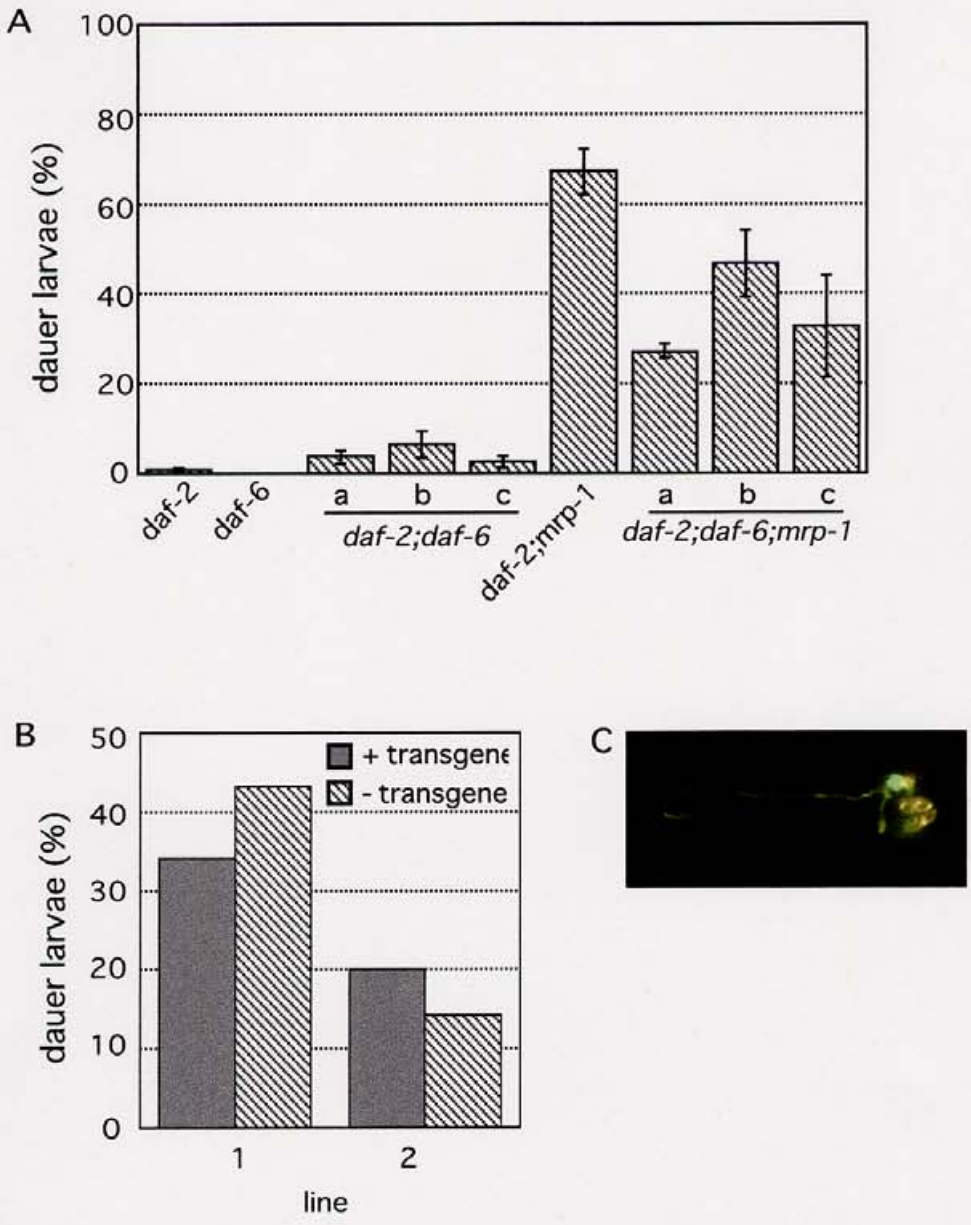

Fig. 20. The influence of the *daf-6(e1377)* mutation on the dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant

The assay was performed at 20°C. (**A**) The dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant was partially suppressed by *daf-6(e1377)*. The means of 3 plates are shown (62-186 animals/plate). The error bars indicate standard errors. (**B**) Transformation by the wild type *daf-6* gene did not cancel the partial suppression of the dauer formation of *daf-2(e1370);mrp-1(pk89)* by *daf-6(e1377)* (line a). Data of 80 animals for line 1 and 126 animals for line 2. (**C**) Normal dye filling of line 1, showing that the wild type *daf-6* transgene can rescue the *daf-6(e1377)* mutant phenotype (Fig. B). The orange fluorescence shows dye filling, while the green fluorescence is GFP, used as the marker for the transformation.

2(e1370);mrp-1(pk89) (Fig. 21). But I could not determine whether this effect was due to the *daf-22(m130)* mutation or a side mutation by using the wild type *daf-22* transgene, because *daf-22* gene has not been cloned. However, I think it is very unlikely that the *daf-22(m130)* mutation suppresses the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* for the following reasons. First, if the *daf-22(m130)* mutation suppresses it, *che-3(e1124)*, *daf-5(e1386)*, and *daf-6(e1377)*, which are located downstream of *daf-22(m130)*, should also suppress it. Second, if *mrp-1(pk89)* is located upstream of *daf-22(m130)* in the dauer regulatory pathway, the effect of the *mrp-1(pk89)* mutation must be the increase of dauer-inducing pheromone. But it is known that the increase of dauer pheromone cannot enhance the dauer formation of the *daf-2(e1370)* mutant (Golden & Riddle, 1984).

6-3. Does *mrp-1/sdf-14* act in the insulin signaling pathway?

The Daf-c phenotype of *daf-1(m40)*, a mutation in the TGF-β signaling pathway, is not suppressed by *daf-d* mutations in the insulin signaling pathway, e.g., *daf-16(m26)*, at 25°C (Vowels & Thomas, 1992). Furthermore, the Daf-c phenotype of *daf-1(m40)* is enhanced by the *mrp-1(pk89)* mutation (Fig. 16). I therefore planned to determine whether *mrp-1/sdf-14* acts in the insulin signaling pathway, by testing the suppression of the Daf-c phenotype of the double mutant *daf-1(m40);mrp-1(pk89)* by *daf-16(m26)*. However, I found that this test does not work, because the Daf-c phenotype of *daf-1(m40)* was suppressed by *daf-16(m26)* at low temperature (Fig. 21A), contrary to the results at 25°C. I also found that another *daf-c* mutation in the TGF-β signaling pathway, *daf-14(m77)*, is also partially suppressed by *daf-16(m27)* at low temperature (Fig. 21B). Furthermore, it was reported that the Daf-c phenotype of mutants in the cGMP-related signaling pathway is also partially suppressed by *daf-16* (Vowels & Thomas, 1992). Thus, I could not find any good method for testing this issue.

6-4. Does *mrp-1/sdf-14* act in the steroid hormone signaling pathway?

The steroid hormone signaling pathway is positioned most downstream among four

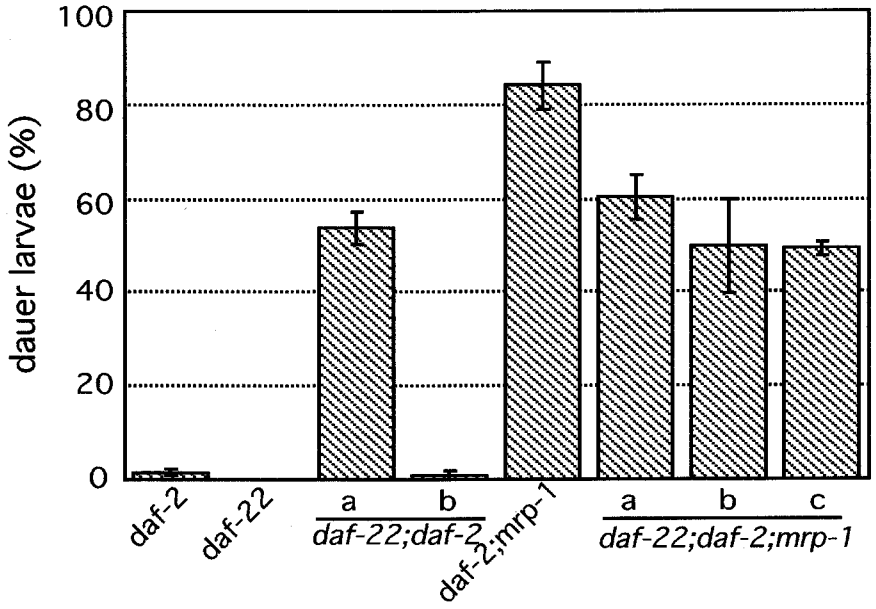

Fig. 21. The influence of the *daf-22(m130)* mutation on the dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant

The assay was performed at 20°C. The dauer formation of the *daf-2(e1370);mrp-1(pk89)* mutant looked partially suppressed by *daf-22(m130)*. The means of 3 plates are shown (52-121 animals/plate). The error bars indicate standard errors.

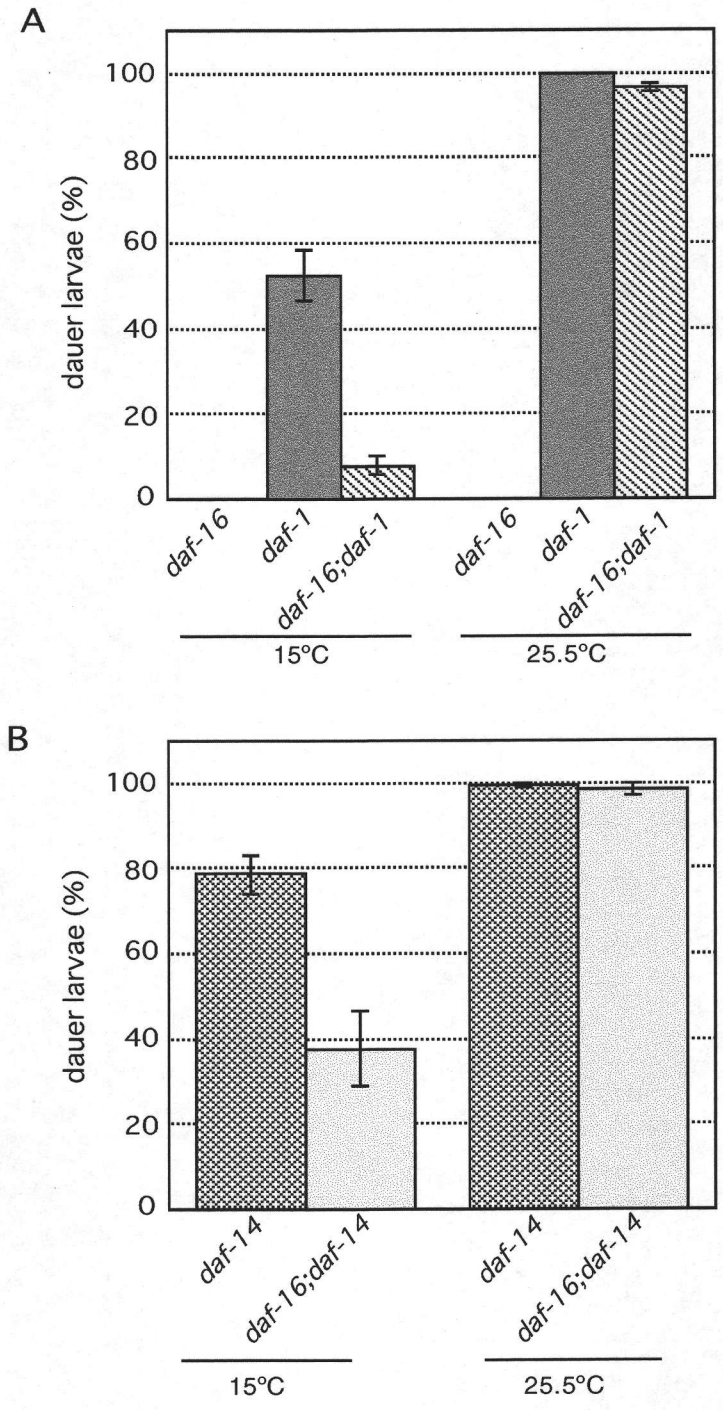

Fig. 22. The Daf-c phenotype of the TGF-β signaling pathway was suppressed by *daf-d* mutations in the insulin signaling pathway at low temperature.
(**A**) As reported (Vowels & Thomas, 1992), the Daf-c phenotype of *daf-1(m40)* was not suppressed by *daf-16(m26)* at 25.5°C. However, it was suppressed at 15°C, which was found for the first time in this study. (**B**) Also, the Daf-c phenotype of another *daf-c* mutant in the TGF-β signaling pathway, *daf-14(m77)*, was suppressed by *daf-16(m27)* at 15°C, although it was not suppressed at 25.5°C. The means of 3 plates are shown. Data of 46-221 animals/plate in A and 16-176 animals/plate in B. The error bars indicate standard errors of mean.

signaling pathways, and its *daf-d* mutations (loss-of-function mutations in *daf-12* gene) suppress *daf-c* mutations in all the four pathways. Therefore, it cannot be examined by epistasis tests whether *mrp-1/sdf-14* gene acts in the steroid hormone signaling pathway. Neither could I find other evidence showing that *mrp-1/sdf-14* acts in this pathway. It is known that *daf-c* mutants in this pathway (*daf-9, daf-12(gf),* and *sdf-9*) produce dauer-like larvae whose pharynx is pumping and thicker than that of normal dauer larvae (Ohkura et al., 2003), but I could not find conditions in which *mrp-1/sdf-14* mutants produce dauer-like larvae.

In conclusion, *mrp-1/sdf-14* gene acts neither in the cGMP-related signaling pathway nor in the TGF-β signaling pathway. It may act in the insulin signaling pathway or in the steroid hormone signaling pathway. Alternatively, *mrp-1/sdf-14* gene may act in an unknown pathway, or *mrp-1/sdf-14* mutations may have indirect influence on many pathways.

Discussion

1. *sdf-14* encodes a homologue of MRP1, a member of the ABC transporter superfamily

sdf-14 mutations were isolated as those showing a dauer formation-constitutive phenotype in the *unc-31(e169)* background. In this study, I identified *sdf-14* gene by positional cloning, and found that it encodes a multidrug resistance-associated protein-1 (MRP-1), a member of the ABC transporter superfamily. *sdf-14* gene was identical to *mrp-1* gene, which was studied by Broeks et al. (1996) as a homologue of mammalian MRP1. However, the involvement of *mrp-1/sdf-14* gene in the regulation of dauer larva formation was discovered for the first time in this study. Namely, this study suggests that dauer larva formation is controlled by the export of unidentified, possibly toxic, substance from cells, based on the present knowledge on MRP1 and ABC transporters, which is described below.

ABC transporters are found in all kingdoms, and probably the largest superfamily of proteins (Higgins, 1992; Saurin et el. 1999; Dassa & Bouige, 2001). There are as many as 60 ABC transporters in *C. elegans* (Sheps et al., 2004), in contrast with 50 in humans (Dean et al., 2001), 56 in *D. melanogaster* (Dean et al., 2001), 129 in *Arabidopsis* (Sanchez-Fernandez et al., 2001), 29 in *S. cerevisiae* (Decottignies & Goffeau, 1997) and 90 in *E. coli* (Dassa et al., 1999). ABC transporters are involved in the import or export of a wide variety of substrates, while those for import are found only in prokaryotes and those for export in both prokaryotes and eukaryotes (Saurin et el., 1999; Dassa & Bouige, 2001). The function of the importers is to provide essential nutrients to bacteria, and the function of the exporters is the extrusion of noxious substrates, the secretion of extracellular toxins and the targeting of membrane components (Dassa & Bouige, 2001).

A typical structure of ABC transporters consists of four domains, two membrane spanning domains (MSD) and two nucleotide binding domains (NBD). In prokaryotic importers,

these four domains are usually encoded by independent polypeptides (two NBD proteins and two MSD proteins). In contrast, in exporters, generally MSD is fused to NBD in a variety of combinations; $(MSD\text{-}NBD)^2$, $(NBD\text{-}MSD)^2$, (MSD-NBD-MSD-NBD), (NBD-MSD-NBD-MSD), etc. (Higgins, 1992; Croop, 1998; Dassa & Bouige, 2001). There are 3 consensus motifs in NBDs; Walker A motif (P-loop, GX(4)GK[S/T], where X can be varied), Walker B motif (h(4)D, where h is a hydrophobic residue) and Linker peptide (LSGGQ) (Walker et al., 1982; Saraste et al., 1990; Croop, 1998; Schneider & Hunke, 1998; Saurin et al., 1999). Walker A and B motifs are commonly found in many ATPases and Linker peptide is unique to the ABC transporter family. NBDs bind to and hydrolyze ATP, and directly couple the energy of hydrolysis to the transport process (Higgins, 1992; Schneider & Hunke, 1998).

In humans, the ABC transporter family consists of 7 subfamilies; subfamily A-G (Dean et al., 2001), of which MRP1 belongs to the subfamily C. The human *MRP1/ABCC1* gene was first identified as a gene similar to P-glycoprotein 1/multidrug resistance protein 1 (*MDR1/PGY1/ABCB1*) (Cole et al., 1992). MDR1 and MRP1 contribute to multidrug resistance of various cancer cell lines. Multidrug resistance, by which tumor cells become resistant to multiple structurally and functionally unrelated drugs, is due to the extrusion of drug compounds from inside cells to outside. MDR1 transports unmodified positively charged drugs, whereas MRP1 cannot. MRP1 transports organic anions and nonanionic compounds conjugated by glutathione, glucuronide, or sulfate, and also cotransports nonanionic compounds with glutathione without conjugation (Borst et al., 2000; Russel et al., 2002; Leonard et al., 2003; Morikawa et al., 2001; Ueda, 2001). Human MRP1 can transport more than 30 compounds, most of which are unnecessary compounds (conjugates, xenobiotics and detoxification products) for the organism except for leukotriene C_4 (LTC_4) (Renes et al., 2000). LTC_4, a glutathione conjugated organic anion that acts as an inflammatory mediator, is the best known substrate of human MRP1.

The structures of human MDR1 and MRP1 are shown in Fig. 23. The human MRP1 has an additional MSD in the N-terminal part (MSD0), and encodes a protein with 17

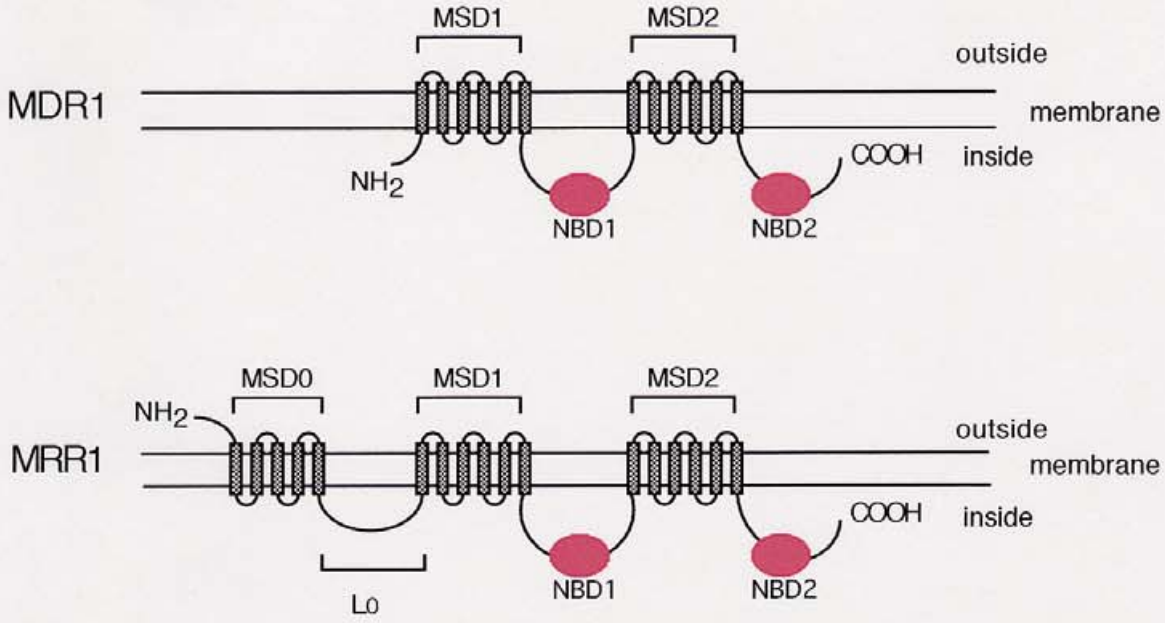

Fig. 23. Putative membrane topology of two ABC transporters involved in multidrug resistance
(Top) The MDR1/P-glycoprotein. (bottom) The multidrug resistance-associated protein, MRP1. (Modified from Borst et al., 2000)

transmembrane domains (Bakos et al., 1996; Hipfner et al., 1997; Kast C & Gros P, 1997; Kast C & Gros P, 1998). The intracellular linker domain (Lo), which connects MSD0 to MSD1, is required for transport function, but the MSD0 is not (Bakos et al., 1998). It was reported that a glutathione analogue binds to the Lo region (Karwatsky et al., 2003). *C. elegans* MRP-1/SDF-14 has homology to human MRP1 throughout the amino acid sequence, including MSDs, NBDs, Walker A and B motifs, and ABC transporter signature sequence (Linker peptide) (Fig. 5).

2. *C. elegans* MRP-1/SDF-14 resembles human MRP1 in its molecular function

This study revealed that human MRP1 can substitute for *C. elegans* MRP-1/SDF-14 in the regulation of dauer larva formation. I tested this possibility, partly because human MRP1 can act in distantly related species: it can rescue the cadmium transport abnormality of the mutant of *S. cerevisiae* YCF1, a human MRP1 homologue (Tommasini et al., 1996). The replaceability of MRP-1/SDF-14 with human MRP1 strongly suggests that MRP-1/SDF-14 acts as an exporter like human MRP1.

The function of MRP-1/SDF-14 as an exporter was supported by two additional experiments. First, expression of the mutant MRP1 (dmLoMRP1), which is defective in the transport of LTC_4 and $E_2$17ßG, did not suppress the dauer constitutive phenotype of *unc-31(e169);sdf-14(ut153)*. Second, the suppression of dauer formation by the wild type human MRP1 was inhibited by PAK-104P, which inhibits the binding of substrates to human MRP1 (Sumizawa et al., 1997). The effect of PAK-104P was specific to human MRP1, because it did not inhibit the suppression of dauer formation of *unc-31(e169);sdf-14(ut153)* by *mrp-1/sdf-14* transgene. The latter result rejects the possibility that PAK-104P may act on other ABC transporters in *C. elegans*, although it is known that PAK-104P has effects also on human MDR1 (Sumizawa et al., 1997). I tested two other substrate binding inhibitors, AG-A and

MK571 (Ren et al., 2001; Chen et al., 2001; Leier et a;., 1994; Jedlitschky et al., 1994; Gekeler et al., 1995), but these inhibitors showed no clear effect on the dauer formation of the *unc-31(e169);sdf-14(ut153)* mutant carrying human MRP1 transgene. This is not surprising, because both AG-A and MK571 are weaker inhibitors than PAK-104P (Chen et al., 2001). Because the effects of the inhibitors are examined by using cell culture systems, the failure in detecting the effect of the latter two inhibitors may be due to poorer penetrance into the animal than into cultured cells, which may be applicable to *C. elegans* MRP-1/SDF-14 case.

Furthermore, by assuming similarity between MRP-1/SDF-14 and human MRP1, some results of this study may be interpreted in the light of known structure-function relationship of human MRP1. First, the two *mrp-1/sdf-14* missense mutations *ut151* and *ut155*, which show phenotypes nearly as strong as the null allele *pk89*, were located in NBD 1. Although *ut151* mutation site was low sequence conservation in comparison to *ut155* mutation site which is highly conserved (Fig. 24), this is consistent with the functional importance of NBD1 as an ATP-binding site in human MRP1. The two NBDs of human MRP1 possess different properties: NBD1 binds ATP with high affinity, whereas NBD2 is hydrolytically more active and binds ADP with high affinity (Hou et al., 2000; Gao et al., 2000). NBD1 mutations markedly decrease transport activity but not completely (Hou et al., 2000; Gao et al., 2000; Payen et al., 2003). Alternatively, the abnormality of *ut151* and *ut155* may be due to poor expression, because mutations in the NBD1 of *S. cerevisiae* YCF1 show defects in the expression in addition to the function (Falcon-Perez et al., 1999)

Second, the MRP-1/SDF-14 isoforms, which are different in exon 13, may be produced to increase the variety of substrate specificity. In *C. elegans* MRP-1/SDF-14, exon 13 roughly corresponds to the 14 th and 15 th transmembrane domains, which are important for substrate specificity in human and mouse MRP1 (Fig. 25) (Zhang et al., 2001A; Zhang et al., 2001B; Zhang et al., 2003). Of the *C. elegans* MRP-1/SDF-14 isoforms, b-type and c-type, but not the e-type, rescued the Daf-c phenotype of *unc-31(e169);sdf-14(ut153)*. This may be due to difference in substrate specificity between b/c-type and e-type, although it remains to be proved.

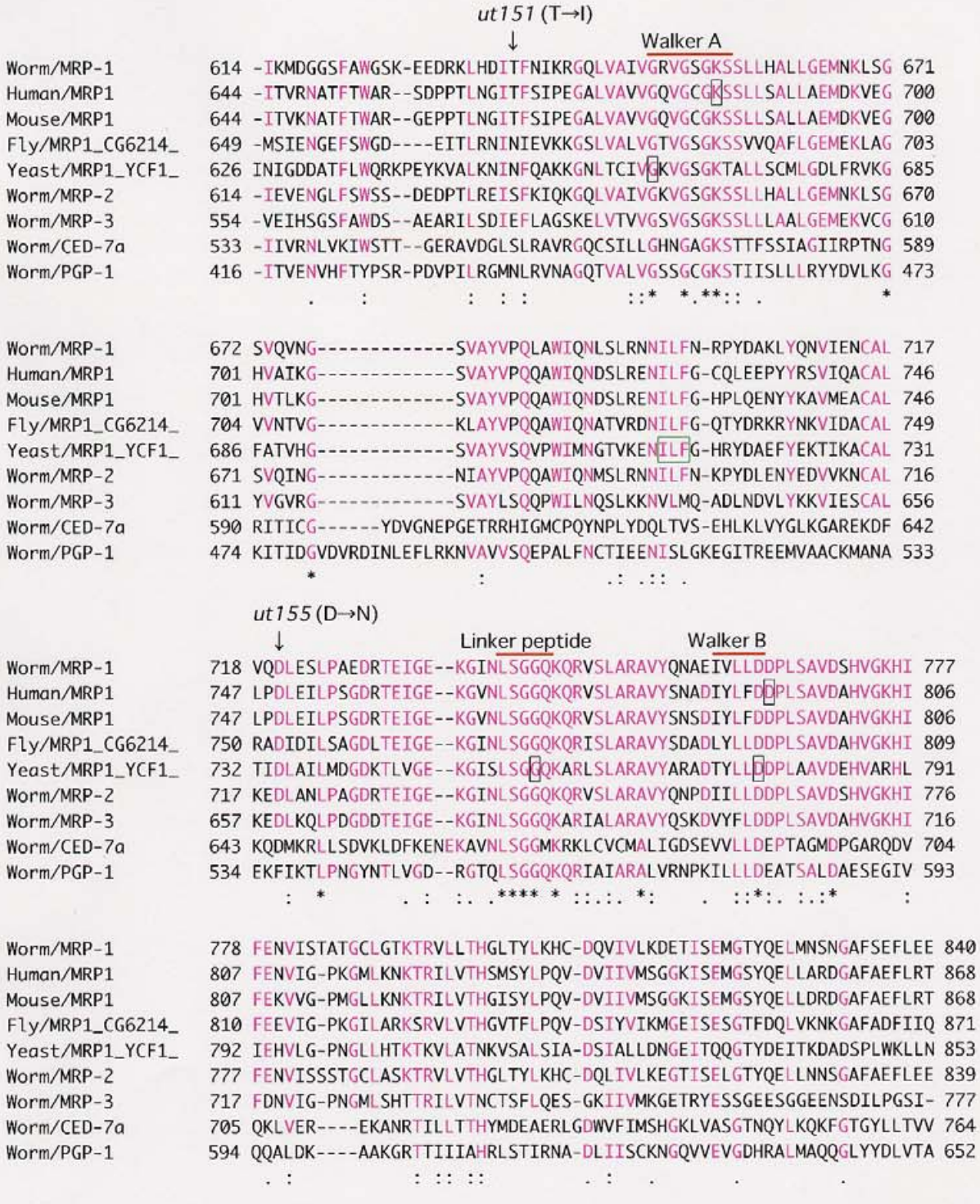

Fig. 24. Alignment of NBD1 of MRP-1/SDF-14 and related proteins from various organisms, in relation to the mutation sites of *ut151* and *ut155*.

The figure shows the alignment of the amino acid sequences of NBD1 (nucleotide-binding domain 1) of MRP1 from various species, other members of the *C. elegans* MRP subfamily (MRP-2 and MRP-3) and other subfamily members (CED-7a and PGP-1). NBD1 was predicted by PROSITE and the alignment was carried out by ClustalW. The arrows show mutation sites. Black boxed or green boxed residues are important in function or expression, respectively. Walker A, Walker B and Linker peptide are common motifs among ABC transporters. Relatively well conserved amino acids are shown by pink letters. SwissProt accession numbers: Q95QE2 (Worm/MRP-1b), P33527 (Human/MRP1), O35379 (Mouse/MRP1), P39109 (YCF1), P34358 (CED-7a), P34712 (PGP-1).TrEMBL accession numbers: Q8T9C5 (CG6214), Q19048 (MRP-2), Q20943 (MRP-3).

Fig. 25. Alignment of the amino acid sequences encoded by the exon 13s of *C. elegans* MRP-1 and corresponding sequences in human, mouse and *Drosophila* MRP1.

Pink letters show the polar and charged amino acids that are proved to be functionally important in human and mouse MRP1. Green letters show part of variable peptides encoded by the exon 8 of *D. melanogaster mrp1* gene. Two human MRP1 transmembrane domains, referred to NCBI protein data base, are underlined. The alignment was performed by ClustalW.

It is intriguing that *Drosophila* CG6214 gene, an MRP1 homologue, has 2 variant copies of exon 4 and 7 variant copies of exon 8 (Grailles et al., 2003), where exon 8 partially overlaps with the sequence encoding the 14 th and 15 th transmembrane domain (Fig. 25).

3. Possible mechanism of dauer larva regulation by *mrp-1/sdf-14*

The results of this study indicate that *C. elegans* MRP-1/SDF-14 is very similar to mammalian MRP1 not only in structure but also in function. Then, how does MRP-1/SDF-14 regulate dauer larva formation? This study partially, if not completely, answers to this question.

The epistasis experiments of this study showed that MRP-1/SDF-14 acts neither in the cGMP-related signaling pathway nor in the TGF-ß signaling pathway. It may act in the insulin signaling pathway or in the steroid hormone signaling pathway. Alternatively, it may act in an unknown pathway or may have indirect influence on many pathways.

The expression studies showed that a functional *sdf-14*::GFP fusion gene is expressed in the intestine, pharynx, some neurons, pharynx-intestinal valve cells, intestinal-rectum valve cells, epithelial cells of the vulva, and hypodermal seam cells. The experiments using extrinsic promoters showed that expression of *mrp-1/sdf-14* either in the intestine, pharyngeal muscles, or nervous system partially rescues the dauer-constitutive phenotype of *unc-31(e169);sdf-14(ut153)*, and that expression in two of the three tissues almost completely rescues the mutant phenotype. Thus, it seems that expression in multiple types of cells, possibly the total number or volume of cells that express *mrp-1/sdf-14*, is important for the function of MRP-1/SDF-14. This is in contrast to many other dauer-related genes, which act in neurons (Ren et al., 1996; Birnby et al., 2000; Gunther et al., 2000; Swoboda et al., 2000; Li et al., 2003) or in XXXL/R cells (Ohkura et al., 2003) for their function.

The results on sodium arsenite of this study provide a hint on the function of MRP-1/SDF-14 in dauer regulation. Sodium arsenite induced dauer formation of the *unc-31(e169)*

mutant, and the *mrp-1/sdf-14* mutation enhanced this effect. The role of the *mrp-1/sdf-14* mutation can be interpreted by reduced excretion of sodium arsenite or another unidentified substance that enhances dauer formation. The existence of the latter substance is suggested, because the *mrp-1/sdf-14* mutation enhanced dauer larva formation even in the absence of sodium arsenite in the *unc-31* background. Unlike sodium arsenite, the effect of this intrinsic dauer-inducing substance is too weak to be detected in the *unc-31* background, unless it accumulates in cells due to a mutation in *mrp-1/sdf-14* gene.

If there is such a weak natural dauer-inducing substance that is exported from cells by MRP-1/SDF-14, what can it be? I would like to present a hypothesis below. This study revealed that *mrp-1/sdf-14* mutations enhance the dauer formation of the *daf-c* mutant in the insulin signaling pathway, *daf-2(e1370),* to a greater extent than that in the cGMP-related signaling pathway, *daf-1(m40),* or in the TGF-ß signaling pathway, *daf-11(m47).* Since the *unc-31(e169)* mutation, the dauer formation of which is remarkably enhanced by *mrp-1/sdf-14* mutations, is also known to cause abnormality in the insulin signaling pathway (Ailion et al., 2000), the function of MRP-1/SDF-14 seems to be important for suppression of dauer formation through the insulin signaling pathway. In order to further support this hypothesis, I constructed the double mutants *unc-64(e246);mrp-1(pk89)* and *mrp-1(pk89) unc-3(e151),* where *unc-64(e246)* and *unc-3(e151)* affect the insulin signaling pathway and the TGF-ß signaling pathway, respectively, as (synthetic) dauer-constitutive mutations (Ailion et al., 2000). As expected, the *mrp-1/sdf-14* mutation enhanced the Daf-c phenotype of *unc-64(e246)* to a large extent, compared with that of *unc-3(e151)* (Fig. 26). Hence, one possibility is that the hypothetical dauer-inducing substance, which is transported by MRP-1/SDF-14, is produced in a larger quantity in the *daf-c* mutants of the insulin signaling pathway than in the *daf-c* mutants of the cGMP-related and TGF-ß signaling pathway or in wild type animals. Based on some published results on the *daf-2(e1370)* mutant, I would like to propose a candidate of the substrate of MRP-1/SDF14 for dauer larva regulation. The *daf-2(e1370)* mutant shows the phenotypes of longevity (Kenyon et al., 1993) and oxidative stress resistance (Honda & Honda, 1999) besides the Daf-c

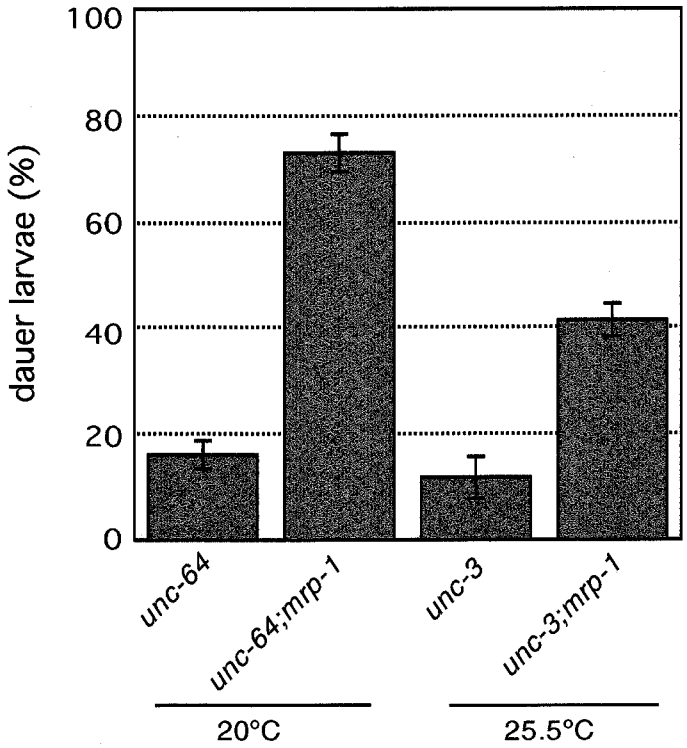

Fig. 26. The *mrp-1* mutation remarkably enhanced the Daf-c phenotype of *unc-64* mutant.
The Daf-c phenotype of the *unc-64(e246)* mutation was remarkably enhanced by the *mrp-1(pk89)* mutation, compared with that of the *unc-3(e151)* mutation. *unc-64* gene acts in the insulin signaling pathway, while *unc-3* gene probably acts in the TGF-β signaling pathway (Ailion et al., 2000). The means of the 3 plates are shown (82-185 animals/plate). The error bars indicate standard errors of mean.

phenotype. The oxidative stress resistant phenotype is related to the overexpression of *sod-3* gene, which encodes a Mn-superoxide dismutase (SOD) (Honda & Honda, 1999). SOD catalyzes the reaction $2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$, and H_2O_2 then oxidizes glutathione (GSH) with the help of glutathione peroxidase to make glutathione disulfide (GSSG), which is a substrate of human MRP1 (Leier et al., 1996) . Thus, it is likely that excess GSSG is made in the *daf-2(e1370)* mutant, and that its intracellular concentration becomes much higher, if MRP-1/SDF-14 cannot export it from the cytoplasm. I therefore speculate that GSSG may be a candidate of the natural dauer-inducing substance related to the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* at 20°C. It may also induce dauer larva formation in the *unc-31;mrp-1* mutants and the *unc-64;mrp-1* mutants, because the *unc-31* and *unc-64* mutations are known to cause abnormality in the insulin signaling pathway (Ailion et al., 2000).

In summary, this study revealed that an MRP1 homologue acts as an exporter to inhibit dauer larva formation in *C. elegans*. It is expected that further studies will identify the substrate of *C. elegans* MRP-1/SDF-14 that controls dauer larva regulation.

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