The Journal of Experimental Biology 202, 1909–1915 (1999)
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JEB1976

FUNCTIONAL COMPLEMENTATION OF THE *malvolio* MUTATION IN THE TASTE PATHWAY OF *DROSOPHILA MELANOGASTER* BY THE HUMAN NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 1 (Nramp-1)

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Accepted 20 April; published on WWW 22 June 1999

Summary

The *malvolio* (*mvl*) gene of *Drosophila melanogaster* encodes a protein with a high degree of homology to natural resistance-associated macrophage proteins (Nramps). This family of integral membrane proteins, many of which appear to function as cation transporters, is remarkably conserved in several phylogenetically distinct species. In *Drosophila melanogaster*, the protein Mvl is expressed in macrophages and in differentiated neurons; loss-of-function mutations lead to defects in gustatory behaviour. The human Nramp-1 protein was expressed in *Drosophila melanogaster* using the hsp70 promoter. Overexpression in normal animals does not lead to any alterations in their behaviour or physiology. In mutants, however, ubiquitous expression of human Nramp-1 can totally rescue the taste defect. This finding that Nramp-1 can complement the taste defect in *mvl* mutants provides a potent means of exploiting behavioural genetics to dissect the function of Nramp-1 and to identify other molecules involved with this transport system.

Key words: natural resistance-associated macrophage protein, Nramp-1, taste behaviour, *malvolio*, *Drosophila melanogaster*, feeding preference assay.

Introduction

The response of a fly to chemical stimuli involves the correct functioning of sensory as well as motor elements within gustatory pathways. Hence, taste behaviour can serve as a sensitive index of brain function, allowing subtle defects within functioning circuits to be detected. The feeding preference test measures the response of flies to sugars and salts and has been used to isolate mutations in more than a dozen genes affecting taste perception (Tanimura et al., 1982; Arora et al., 1987; Siddiqi et al., 1989; Rodrigues et al., 1991; VijayRaghavan et al., 1992; Inamdar et al., 1993). Most of the genes that have been analysed encode molecules that play rather general roles in the development or function of the nervous system; their apparently specific effect on gustatory behaviour results from hypomorphic alleles that allow adult viability (Murugasu-Oei et al., 1996).

Adults of the *malvolio* (*mvl*) of *Drosophila melanogaster* strain show a reduced acceptance of sucrose, trehalose and fructose and an increased acceptance of low concentrations of sodium chloride (Rodrigues et al., 1995). Electrophysiological recordings from the labellar taste neurons demonstrated that the mutation does not affect the peripheral level of stimulus detection. It was proposed that Mvl plays a role in the integrative synapses between sensory neurons and postsynaptic partners in the suboesophageal ganglion. This was supported by the demonstration that *mvl* is expressed in differentiated neurons of both the peripheral and central nervous systems. *mvl* encodes a molecule that belongs to a family of integral membrane proteins defined by natural resistance-associated macrophage proteins (Nramps) (Cellier et al., 1995). Members of this family, which have been identified in yeast, mycobacteria, plants, nematodes, insects and mammals, all possess very similar hydropathy profiles and are typified by the presence of 10 transmembrane domains (Cellier et al., 1995, 1996).

The first mammalian Nramp (Nramp-1) was identified through analysis of inbred strains of mice (Bcg^R and Bcg^S) with altered sensitivity to infection by mycobacterial species (Gros et al., 1981; Vidal et al., 1993). Nramp-1 is expressed in the late endocytic compartment in macrophages and has been shown to be recruited to phagosomal membranes (Gruenheid et al., 1997). While Nramp-1 is macrophage-specific, the highly homologous protein Nramp-2 (78% identity) is more generally expressed (Gruenheid et al., 1995). A large body of evidence favours the hypothesis that Nramp-2 is a transporter for divalent cations (Vulpe and Gitschier, 1997; Fleming et al., 1997, 1998; Supek et al., 1997; Orgad et al., 1998). Nramp-2

can functionally complement the defect of the plasma-membrane-associated transporter (SMF1) in yeast *Sacchromyces cerevisiae* mutants (Supek et al., 1996; Pinner et al., 1997). Recent genetic evidence has shown that mutations in Nramp-2 were causative of the iron-deficiency syndromes seen in the *mk* mouse and the *Belgrade* rat. Both rodent models show defects in Fe^{2+} uptake in the intestine and into reticulocytes (Vulpe and Gitschier, 1997; Fleming et al., 1997, 1998).

It is not yet clear whether Nramp-1, like Nramp-2, transports metal ions, and the mechanism by which the killing of parasites is mediated is still controversial. However, the linkage between susceptibility to tuberculosis and leprosy in human populations and polymorphisms in the Nramp-1 gene make it crucial to study the mechanism of action of this putative transporter (Bellamy et al., 1998; Abel et al., 1998). The *Drosophila* Mvl protein shows a very high degree of similarity with the Nramps and, like Nramp-1, is also associated with macrophages (Rodrigues et al., 1995; Cellier et al., 1995). The fly can therefore serve as a very important experimental system in which to study the mechanisms of Nramp-1 action and the systems with which it participates. The effect of hypomorphic mutations in the *mvl* gene on macrophage function has not been investigated in *Drosophila*. In addition to macrophages, however, Mvl expression has been demonstrated in differentiated neurons of the central and peripheral nervous systems, and loss-of-function allelic mutations lead to defects in taste behaviour. Interestingly, these aberrant behaviour patterns could be completely suppressed when flies were grown on media supplemented with Fe^{2+} or Mn^{2+} for a minimum of 2 h before testing (Orgad et al., 1998). This striking result suggests that Mvl also participates in the transport of certain divalent cations that are essential for the proper functioning of the taste circuits.

This finding allows us to test whether Nramp-1 can also function as a metal transporter. We expressed human Nramp-1 cDNA in mvl^{97f} under the control of the hsp70 promoter. Constitutive expression of Nramp-1 could functionally complement the taste defect in mutant flies. This finding therefore provides an *in vivo* assay system for human Nramp-1 function, allowing an analysis of the properties of this molecule which has direct relevance to human infectious disease.

Materials and methods

All standard molecular biology procedures were performed according to the method described by Sambrook et al. (1989). Protocols for *Drosophila melanogaster* were as described by Ashburner (1989).

Drosophila *stocks*

The Canton-S strain of *Drosophila melanogaster* was used as the wild type, except where specifically mentioned. The mvl^{97f} strain carries a P(w^+ lacZ) insertion in the *malvolio* (*mvl*) gene and results in a partially dominant taste defect (Rodrigues et al., 1995). The strain *Cy*/*Sp*; (Δ2-3 ry^+)*ry Sb*/TM6-*Ubx* contained a constitutively expressing transposase insertion at 99B (Robertson et al., 1988). The attached-X stock C(1)DX *y w f* allows the segregation of the X chromosome of males to their sons. Details of strains and markers are available in the review by Lindsley and Zimm (1992).

Generation of hs-Nramp-1 trangenic flies

The full-length cDNA of human Nramp-1 was cloned into pBluescript SK (M13-) (P. Gros, unpublished results). The complete coding sequence was released by digestion with *Kpn*I and *Eco*RI and subcloned into pCaSpeR-hs in frame with the hsp70 promoter. The recombinant vector *P(w^+* hs-Nramp-1*)* was microinjected with Pπwc into yw^{1118} embryos following standard protocols (Ashburner, 1989). Germline transformants were selected by monitoring the w^+ marker in subsequent generations. One transgenic line (hs-Nramp-1) was obtained in which the mutation mapped on the third chromosome and allowed homozygous viability.

Mobilisation of the P(w^+ *hs-Nramp-1*) *transposon*

To mobilise the insertion onto the X chromosome, *y w*; hs-Nramp-1/hs-Nramp-1 females were crossed to *CyO*/*Sp*; (Δ2-3 ry^+) *Sb*/TM2-*Ubx* males. Jumpstarter males [*y w*; P(hs-Nramp-1)/(Δ2-3 ry^+) *Sb*] from among the progeny were crossed to C(1)DX *y w f*; *CyO*/+ virgin females. Progeny were screened for males with w^+ eye colour; single males were mated with C(1)DX; *y w f* virgin females and set up as lines. Insertions on the X chromosome were recognised by the segregation of the w^+ marker in the progeny of these individual lines; in such cases, only the males would be w^+. Two of these lines (designated hs-Nramp-1 line 1 and hs-Nramp-1 line 4) were analysed further.

Feeding preference test

The feeding preference test described by Tanimura et al. (1982) was carried out with some modifications (Balakrishnan and Rodrigues, 1991). Alternate wells of a 6×10 µl plate were filled with 1 % agar containing the stimulus. The remainder of the wells contained 0.2 % Carmoisine Red in agar. Control experiments established that the food dye did not interfere with the test. Flies were starved in humid conditions for 18 h prior to testing. Approximately 100 flies were introduced into each test plate and left for 1 h in the dark. Following the test, flies were immobilised by cooling and scored by visual inspection for colour in their abdomen. The acceptance response was calculated from the percentage of flies in the population with an uncoloured abdomen. Means and standard deviations of each data point were obtained from a minimum of 10 independent tests.

Excision of the P element

To generate revertants, *y w*; P(hs-NRAMP-1) males were mated with C(1)DX *y w f*; *CyO*/+; (Δ2-3 ry^+)*Sb*/+ virgin females. Jumpstarter males of genotype *y w* P(hs-NRAMP-1); (Δ2-3 ry^+)*Sb*/+ were crossed to C(1)DX *y w f* virgin females.

Progeny males were scored for the eye colour marker. White-eyed males were crossed to C(1)DX *y w f*; *mvl/mvl* virgin females and bred as lines. In subsequent generations, *y w* (Pex); *mvl/mvl* flies were selected and tested in feeding preference assays. Eleven such lines were analysed in detail.

Southern blot analysis

Genomic DNA was prepared from adult flies, and Southern hybridisation was carried out as described by Sambrook et al. (1989). DNA was transferred to nylon membranes and hybridised with labelled probes. An 800 base pair (bp) internal fragment of Nramp-1 cDNA was used as the probe. Probes were labelled with [^{32}P]dATP by random priming reactions.

In situ *hybridization to RNA in whole-mount embryos*

Localisation of mRNA to embryos was carried out as described by Ashburner (1989). Nramp cDNA was labelled with digoxygenin-dUTP using random-priming reactions. Embryos of different ages were collected on yeast agar plates, fixed with 4% paraformaldehyde and washed in phosphate-buffered saline containing 0.1% Triton X-100 (PBT). They were treated with proteinase K for 3 min and hybridized for 16 h with digoxygenin-labelled probes. The embryos were washed extensively in PBT, and hybridised probe was detected using anti-digoxygenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim). The enzymatic reaction was visualised using Nitroblue Tetrazolium and X-phosphate. Stained embryos were dehydrated through increasing concentrations of ethanol and mounted in DPX.

Results

The high degree of homology between Mvl and Nramp-1 suggested the attractive possibility that *Drosophila* genetics could be used to analyse the structure and function of the human Nramp-1 protein. As a first step towards this goal, we created transgenic flies carrying the complete human Nramp-1 cDNA cloned downstream from an hsp70 promoter. Adult flies bearing viable hypomorphic alleles of *mvl* demonstrate a lowered sensitivity to sucrose and trehalose as measured in feeding preference assays (Rodrigues et al., 1995). Using this assay, we tested whether human Nramp-1 could functionally complement for Mvl in the neural circuits underlying taste behaviour.

Transgenic flies in which human Nramp-1 was ubiquitously expressed showed normal development and gustatory behaviour

The complete human Nramp-1 coding sequence was subcloned downstream from and under the control of the hsp70 promoter and microinjected into *Drosophila* embryos of the *y* w^{1118} strain. We obtained a single transformant from this experiment, and the insertion P(w^+ hs-Nramp-1) (hereafter called hs-Nramp-1) was located on the third chromosome. Since insertions of ectopic genes often result in positional effects, we carried out a remobilisation experiment to generate strains bearing insertions at different positions on the genome. To facilitate testing for rescue of the behavioural defect in *mvl*, we biased our selections for insertions on the X chromosome.

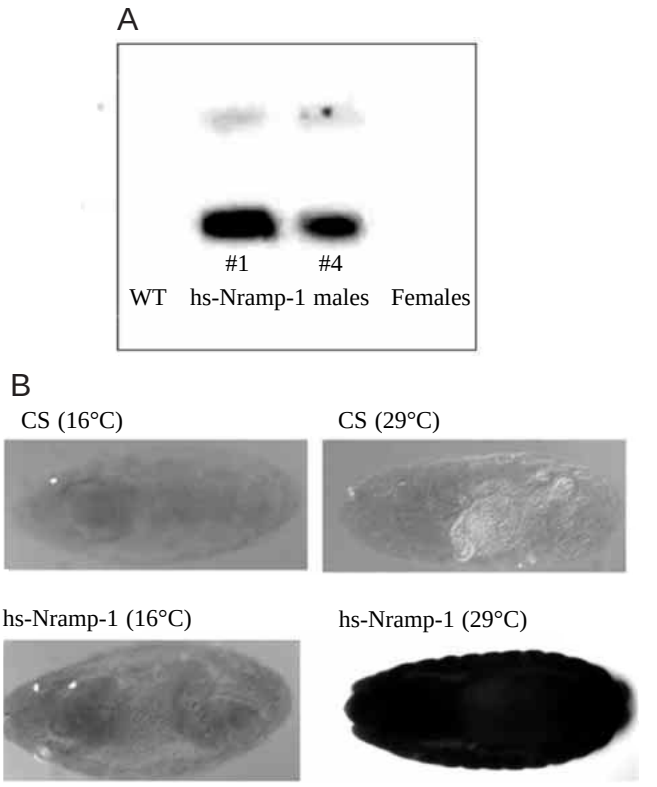

Fig. 1. (A) Southern analysis of the transgenic lines. Genomic DNA was probed with a 800 bp probe from the human Nramp-1 gene at high stringency. The wild-type (WT) flies showed no cross-hybridising bands under these conditions. Males from hs-Nramp-1 lines 1 and 4 showed bands of 800 bp. The females showed no hybridisation. (B) RNA *in situ* hybridization of Nramp-1 in embryos. The 800 bp genomic probe was labelled with digoxygenin-dUTP and used to probe embryos from wild-type and hs-Nramp-1 embryos. Hybridization was visualised using alkaline-phosphatase-coupled anti-digoxygenin antibodies. There was no hybridization to wild-type Canton-S (CS) embryos or to hs-Nramp-1 embryos raised at 16 °C. Embryos collected at 29 °C showed strong ubiquitous expression of human Nramp.

We selected X chromosome insertions using the attached-X [C(1)DX *y w f*] strain (Lindsley and Zimm, 1992). This strain allows the segregation of paternal X chromosomes to male progeny; hence, only males will carry the hs-Nramp-1 transgene. Female progeny will be C(1)DX, *y w f* and will not carry the transgene. We selected such two independent lines (lines 1 and 4) for further analysis. Southern analysis of genomic DNA from transformant flies probed with an 800 bp internal fragment of human Nramp-1 verified the presence of the hs-Nramp-1 transgene in male but not in female genomes (Fig. 1A).

Transcription of the Nramp-1 gene in the transformants was tested by RNA *in situ* hybridization. Embryos were collected from hs-Nramp-1 mvl^{97f} and Canton-S parents at 16 °C and

29 °C; the transcription of the transgene was monitored by hybrization with digoxygenin-labelled Nramp-1 probes (Fig. 1B). There was no native expression of Nramp-1, nor any cross-hybridizing gene, in wild-type embryos at any temperature of growth. The hs-Nramp-1 strain showed ubiquitous expression of the transgene when reared at 29 °C but not when reared at 16 °C. These data show that the human Nramp-1 gene could be transcribed in *Drosophila* under regulation of the hsp70 promoter.

We examined transgenic flies reared at 25 °C and 27 °C for any apparent morphological changes as well as their behavioural responses to 1 mmol l^{-1} sucrose. The ratio of males to females [hs-Nramp1 males:C(1)DX, *y w f* females] of the transgenic strain was comparable with that in normal flies [+ males:C(1)DX, *ywf* females], indicating that ubiquitous expression of hs-Nramp-1 did not result in lethality. The hsp70 promoter is leaky when flies are reared at 25 °C but, to induce higher protein levels, we heat-pulsed embryos, larvae and pupae at 37 °C for 1 h at different times during development and examined the flies under the light microscope. No defects were observed. Flies reared at 25 °C and 27 °C were tested in the feeding preference assay, and these showed responses comparable with those of the wild-type strains (see below).

Human Nramp-1 can substitute for Mvl in the taste pathway of Drosophila

To test for functional complementation of the taste phenotype, we generated hs-Nramp-1; mvl^{97f}/mvl^{97f} males and maintained them with C(1)DX; *y w f*; mvl^{97f}/mvl^{97f} females. Male progeny in such a strain are homozygous for the mvl^{97f} mutation as well as carrying hs-Nramp-1. The females serve as controls and are genotypically mvl^{97f}/mvl^{97f}.

Flies were reared at different temperatures and tested in the feeding preference assay for their responses to sucrose. When adults are given a choice between agar containing 1 mmol l^{-1} sucrose and control agar, more than 90 % of normal flies preferentially eat from the sucrose-containing wells. In mvl^{97f}/mvl^{97f} mutants, however, only approximately 30 % of flies preferentially choose sucrose, irrespective of their temperature of rearing. Induction of transgene expression by rearing Nramp-1; mvl^{97f}/mvl^{97f} flies at either 25 °C or 27 °C resulted in a higher acceptance of sucrose in the feeding preference assay ($P<0.001$; Fig. 2). The hsp70 promoter is not active at 16 °C, and flies reared at this temperature did not show any alteration in behavioural response compared with mvl^{97f}/mvl^{97f} mutants. At 25 °C, a low level of hsp70 activity is observed which is increased at 27 °C (data not shown). Transgenic mvl^{97f} flies showed a slightly greater acceptance of sucrose when reared at 27 °C than at 25 °C ($P=0.1$). We were unable to test the effects of increasing Nramp-1 levels further since rearing flies at 29 °C or subjecting them to pulses of 37 °C affects the behavioural responses of wild-type controls themselves (V. Rodrigues, unpublished results).

In mvl^{97f} females, hs-Nramp1 showed a dose-dependent rescue of the taste defect (Fig. 3). Mutant flies carrying one copy of hs-Nramp-1 (Nramp-1/+; *mvl/mvl*) showed a 50 % acceptance response to 1 mmol l^{-1} sucrose (compared with 40 % in the absence of the transgene), while those with two copies showed a 65 % response (Fig. 3).

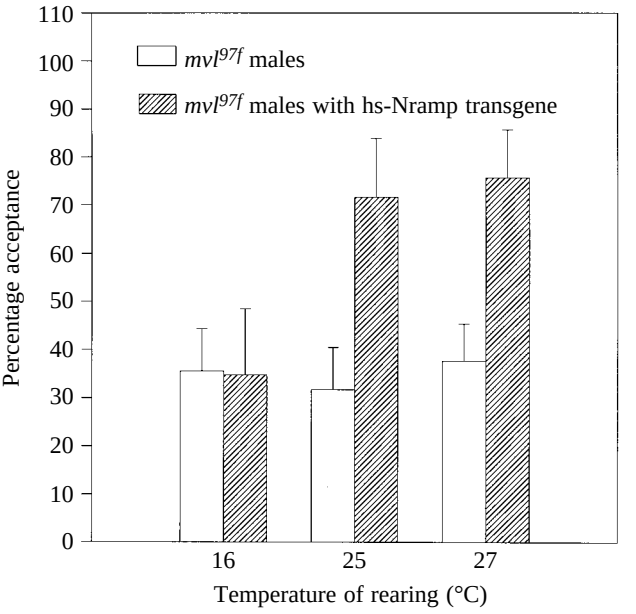

Fig. 2. Behavioural responses of mvl^{97f} and hs-Nramp-1; mvl^{97f} males to 1 mmol l^{-1} sucrose in the feeding preference assay. Flies were reared at 16 °C, 25 °C and 27 °C and tested in the feeding preference test. The number of flies that fed only from the stimulus-containing wells (uncoloured wells) was estimated. The number of such flies was used to compute the percentage acceptance. The columns represent the means and standard deviations of a minimum of 10 independent experiments.

An independent hs-Nramp-1 line (line 4) showed results comparable with those of line 1. These results demonstrate that ubiquitous expression of human Nramp-1 can rescue the taste defect of *mvl* mutants. The observation that two independent insertions can rescue the behavioural defect argues against the possibility that insertion itself led to mutation of genes leading to second-site modification of the *mvl* phenotype. However, this possibility could be ruled out more directly by generating lines in which the transposon was excised by transposase activity.

Excision of P(w^+ hs-Nramp-1) reverted the rescue of the taste defect in mvl *mutants*

To generate strains in which P(w^+ hs-Nramp-1) was excised, we crossed line 4 to a strain carrying transposase activity. The presence of the transposon could be monitored by following the w^+ eye marker. Excision of P elements has been shown to occur by a 'cut-and-paste' mechanism leaving sequences of DNA of varying length within the gene (Engels, 1989). Hence, if the locus into which the P element was located itself acted as second-site suppressor, some of the excision lines would continue to show this effect. We tested 11 excision lines for their ability to rescue the *mvl* mutant phenotype when reared at 27 °C, and no such lines were found. A representative line is shown in Fig. 4.

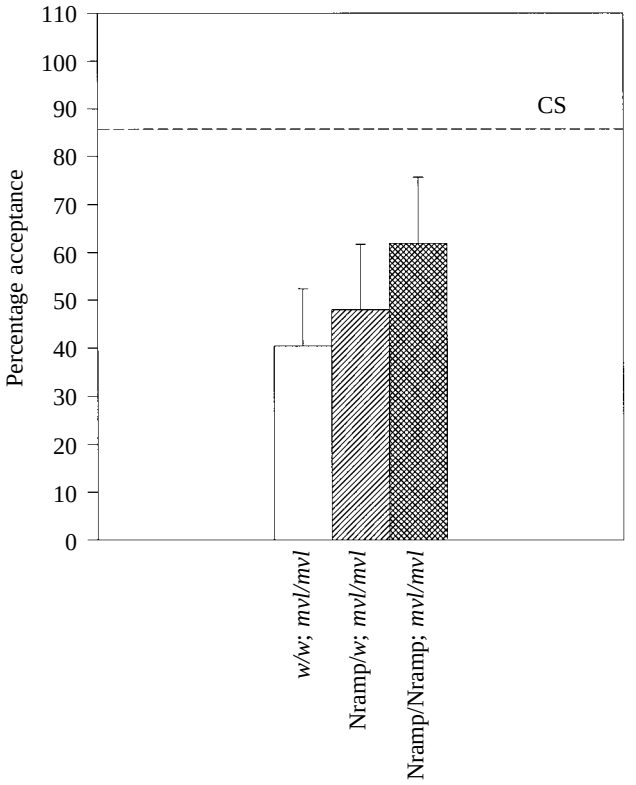

Fig. 3. Effect of dosage of the hs-Nramp-1 transgene on the feeding preference response of females to 1 mmol l^{-1} sucrose. All flies were reared at 25 °C and tested in the feeding preference assay. Each column represents the mean and standard deviation of more than 10 independent experiments. The percentage acceptance is closer to that of the wild type (dotted line labelled CS) when there are two copies of the hs-Nramp-1 transgene. There is a small but significant difference in the response of flies with one and two copies of the transgene (P<0.05).

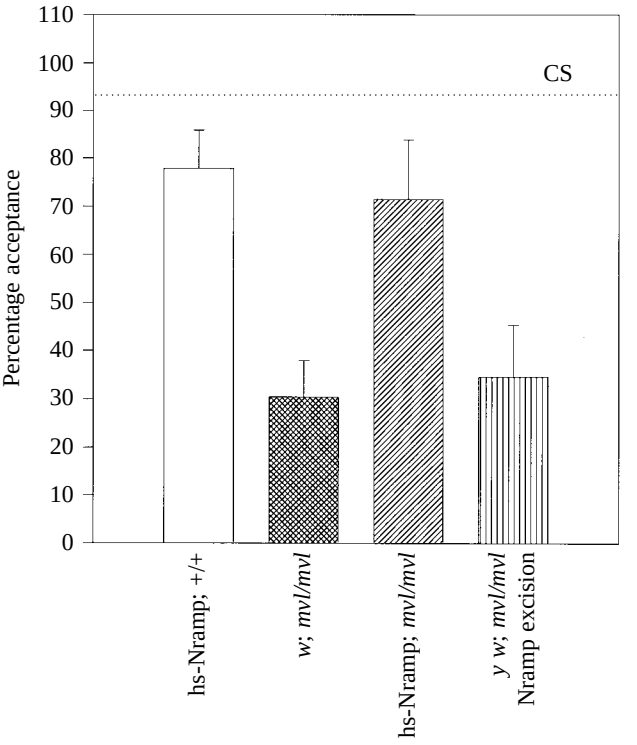

Fig. 4. Lines in which $P(w^+$ *hs-Nramp-1*) was excised failed to rescue the *mvl* phenotype. The phenotype of the excision chromosome (*y w* Pex; *mvl/mvl*) is compared with that of the hs-Nramp-1; *mvl/mvl* strain. The presence of hs-Nramp-1 in the wild-type strain (hs-Nramp-1; +/+) does not affect the behavioural response of flies to 1 mmol l^{-1} sucrose (P>0.1).

These data together provide compelling evidence that the ubiquitous expression of human Nramp-1 can complement for Mvl in the taste pathway of *Drosophila melanogaster*.

Discussion

We have shown that expression of human Nramp-1 under control of the heat-shock promoter can rescue the taste defect in mvl^{97f} mutant flies. Previous molecular analysis has demonstrated that the insertion of $P(w^+)$ in mvl^{97f} strain is located 313 bp 5′ to the translation initiation site in the transcribed but untranslated region of the transcription unit (Rodrigues et al., 1995). This observation, together with northern analysis and genetic data, suggests that P element insertion results in a partial loss of *mvl* function. We favour the hypothesis that the Mvl protein is translated, but is present in smaller amounts than in the wild type. The *mvl* locus encodes a protein with a high degree of homology to a family of integral membrane proteins, many of which have been demonstrated to transport metal ions. It is therefore interesting that the behavioural defect in *mvl* mutants can be completely suppressed by supplementing growth media with 10 mmol l^{-1} $MnCl_2$ or $FeCl_2$ but not with $CaCl_2$ or $MgCl_2$ (Orgad et al., 1998). This implies that the transport function in *mvl* mutants is less efficient than in the wild type but can be compensated for by increasing the amount of Mn^{2+} or Fe^{2+} available in the external milieu.

Mvl is expressed in differentiated neurons, and loss of function leads to a reduction in the sensitivity of the gustatory circuits to stimuli. The experiments of Orgad et al. (1998) strongly suggest that the absence of specific divalent cations in cells is causative of the behavioural defects and that Mvl is involved in the transport of these ions (Supek et al., 1997; Orgad et al., 1998). The role of metal ions in regulating the sensitivity of gustatory circuits is not clear. One possibility is that Mn^{2+} and Fe^{2+} directly or indirectly modulate the effects of neurotransmitter receptors found at synapses between sensory neurons and their postsynaptic partners in taste circuits (Shuto et al., 1997). Alternatively, the effect of metal ions on neural function could result from an indirect effect of their role as cofactors for various metalloproteins. In mammalian macrophages, the role of Nramps is linked to the killing of parasites, which is presumably mediated through reactive oxygen or nitrogen species. It is conceivable that increased levels of these radicals induced by metalloproteins are also crucial for proper function of Nramps within neural circuits.

Mvl is also expressed in macrophages, although the effect of loss of function on these cells has not yet been unassayed. It is amazing that species separated by 540 million years of evolutionary history (flies and humans) show not only a conservation of molecular homologies but a similarity in the cell types in which these molecules are found. It is tempting to speculate that human and *Drosophila* phagocytic cell types have a common evolutionary history. Increasingly it is being shown that mechanisms detected in one system are not far removed from those occurring in another. The observation that human Nramp-1 can fully complement the defect in *mvl* mutants provides a unique opportunity for exploiting *Drosophila* genetics to study a transport mechanism in humans. This is of particular medical value since Nramp-1 polymorphisms have been shown to be involved in susceptibility to mycobacterial infection in natural populations (Abel et al., 1998; Bellamy et al., 1998). It therefore becomes relevant to understand the function of the transporter and to identify other molecules with which Nramp-1 collaborates to bring about its physiological effects. The fact that human Nramp-1 can function in the taste pathway of *Drosophila* provides a means of using the easily assayable taste behaviour of the fly as an *in vivo* model system for Nramp-1. Site-directed mutagenesis of the Nramp-1 coding region and subsequent transformation into *mvl* flies is an effective means of dissecting the structure–function relationship of this molecule. The isolation of second-site modifiers is a potent means of identifying other molecules in the transport mechanism in which human Nramp-1 functions.

We are grateful to all members of the Chia laboratory for their discussion and interest in this project. V.R. is grateful to the Institute of Molecular and Cell Biology for a Visiting Fellowship where part of this work was carried out.

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