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Isolation, Characterization and Epistasis of Fluoride-Resistant Mutants of *Caenorhabditis elegans*

Isao Katsura,*,†,1 Kazunori Kondo,†,2 Tosikazu Amano,†,3 Takeshi Ishihara* and Minoru Kawakami*,‡

*DNA Research Center, National Institute of Genetics, Mishima 411, Japan, †Department of Biology, College of Arts and Sciences, University of Tokyo, Komaba, Tokyo 153, Japan and ‡Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

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ABSTRACT

We have isolated 13 fluoride-resistant mutants of the nematode *Caenorhabditis elegans*. All the mutations are recessive and mapped to five genes. Mutants in three of the genes (class 1 genes: *flr-1 X*, *flr-3 IV*, and *flr-4 X*) are resistant to 400 μg/ml NaF. Furthermore, they grow twice as slowly as and have smaller brood size than wild-type worms even in the absence of fluoride ion. In contrast, mutants in the other two genes (class 2 genes: *flr-2 V* and *flr-5 V*) are only partially resistant to 400 μg/ml NaF, and they have almost normal growth rates and brood sizes in the absence of fluoride ion. Studies on the phenotypes of double mutants showed that class 2 mutations are epistatic to class 1 mutations concerning growth rate and brood size but hypostatic with respect to fluoride resistance. We propose two models that can explain the epistasis. Since fluoride ion depletes calcium ion, inhibits some protein phosphatases and activates trimeric G-proteins, studies on these mutants may lead to discovery of a new signal transduction system that controls the growth of *C. elegans*.

ONE of the goals of molecular genetics is to elucidate complicated networks of biological molecular reactions by isolation and characterization of mutants in which one component is defective or altered. Isolation and characterization of drug-resistant mutants has been a particularly useful approach. In bacteria, mutants resistant to antibiotics have been useful in studies of protein synthesis, transcription, etc. The same is true for the nematode *Caenorhabditis elegans*. Mutants of *C. elegans* resistant to levamisole and trichlorfon (BRENNER 1974; LEWIS *et al.* 1980) have revealed genes and their functions concerning the neurotransmitter acetylcholine. Other drug-resistant mutants such as those resistant to α-amanitin (SAMFORD, GOLOMB and RIDDLE 1983), phorbol ester (TABUSE, NISHIWAKI and MIWA 1989), benzimidazole (DRISCOLL *et al.* 1989) and caffeine (HARTMAN 1987) have been isolated and characterized.

Although harmfulness of fluoride ion at low concentrations is an issue of active debate (MARSHALL 1990), it is evidently toxic to almost all living organisms at high concentrations. In acute poisoning of mammals it acts as an irritant in the stomach and causes emesis, diarrhea, muscular weakness and delays blood clotting, resulting in hemorrhages. As chronic poisoning it causes fluorosis, that is, faulty formation of teeth and change in the structure of bones (RADELEFF 1964). These effects suggest that fluoride ion interferes either with the metabolism of calcium ion or with a signal transducing system. Biochemical studies *in vitro* are in agreement with this suggestion. Fluoride removes calcium ion, since calcium fluoride is poorly soluble in water. It also fixes trimeric G-proteins to the active state (GILMAN 1984). Furthermore, fluoride ion is an inhibitor of some enzymes, such as some protein phosphatases (BALLOU and FISCHER 1986) and enolase (LOHMAN and MEYERHOF 1934). However, for all these studies the molecular action *in vivo* of fluoride ion remains to be clarified.

In this paper we report isolation and characterization of *C. elegans* mutants resistant to NaF. These mutants define five new genes (*flr-1* to *flr-5*). All mutations in three of the genes (*flr-1*, *flr-3* and *flr-4*) confer strong resistance to NaF; the mutants grow slowly both in the absence and presence of NaF. Conversely, *flr-2* and *flr-5* mutants are only weakly resistant to NaF and grow at almost normal rates in the absence of NaF. Interestingly, the latter mutations suppress the slow-growing phenotype but not the strong fluoride resistance of the former mutations. In fact, some of the weak mutations were isolated as suppressors of the slow-growing phenotype of the strong resistant mutations. Only subsequently were they found to have the phenotype of weak fluoride resistance after being separated from the latter mutations. We propose two models that can explain the relationship between class 1 and class 2 genes: one

[1] To whom correspondence should be addressed at: National Institute of Genetics (present address).
[2] Present address: Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192, Japan.
[3] Present address: Yoshizato MorphoMatrix Project, Research Development Corporation of Japan, 5-9-6 Tohkohdai, Tsukuba 326, Japan.

consisting of a pathway that confers fluoride sensitivity on *C. elegans* and the other consisting of a negatively regulated switch circuit for regulation of the growth rate. We hope studies on these mutants will elucidate a new signal transduction system that controls the growth of *C. elegans*.

MATERIALS AND METHODS

General techniques: General techniques for culturing and genetically studying *C. elegans* including compositions of culture media have been described (BRENNER 1974; WOOD 1988). NGM plates 6 cm in diameter were used for genetic experiments. For cultures of a large number of worms, we used 9 cm plates with a richer medium (the same as NGM plates except with 15 g/liter bacto peptone and 5 g/liter yeast extract), which we call PY plates in this paper. Before using the plates, *Escherichia coli* OP50 was spread and grown for more than 1 day at room temperature to feed worms. Since the lawns of *E. coli* became hard and thick on PY plates, they were used in a wet state by adding distilled water to the surface of the agar to soften the lawn. S basal medium was used to suspend worms. Alkaline hypochlorite treatment (HECHT *et al.* 1982) was employed to kill worms and to isolate embryos.

Strains: *C. elegans* var. Bristol strain N2 was used as the wild-type strain. The phenotypes of mutations used in this paper are Cat (abnormal catecholamine distribution), Che (abnormal chemotaxis), Daf (abnormal dauer formation), Dpy (dumpy), Flr (fluoride resistant), Let (lethal), Lin (abnormal cell lineage), Lon (long), Mut (mutator), Osm (osmotic avoidance defective), Tpa (TPA resistant) and Unc (uncoordinated movement). The mutations used in this study are listed below. LGI: *dpy-5(e61)*; LGII: *dpy-10(e128), mut-5(st701)*; LGIII: *dpy-18(e364)*; LGIV: *daf-1(m40), dpy-9(e12), flr-3(ut8, ut9, ut10), tpa-1(k501), lin-1(e1771), dpy-13(e184), mut-6(st702)*; LGV: *let-341(ut58ts), unc-46(e177), dpy-11(e224), flr-5(ut73), unc-23(e25), flr-2(ut5, ut71), sma-1(e30), cat-4(e1411), cat-6(e1861), osm-6(p811), che-11(e1810), che-12(e1812), unc-76(e911)*; LGX: *lon-2(e678), unc-9(e101), flr-1(ut1, ut2, ut4, ut6, ut11), unc-84(e1410), unc-3(e151), flr-4(ut3, ut7), unc-7(e5)*. Chromosomal aberrations: *ctDf1 V, sDf20 V, sDf26 V, sDf30 V, sDf35 V, mnDf4 X, mnDf19 X, mnDf20 X, mnDf21 X, mnDf43 X; mnDp1(X; V), mnDp9(X; I), mnDp25(X; I); eT1(III; V), nT1(IV; V)*. The chromosomal translocation *nT1* that we used was *nT1[unc(n754dom) let]*, which contained a dominant Unc mutation and a recessive Let mutation. The translocation *eT1* has a recessive Unc phenotype.

Isolation of fluoride-resistant (*flr*) mutants by selection on plates containing NaF: Cultures on PY plates of mutator strains (*mut-5* or *mut-6*; MORI, MOERMAN and WATERSTON 1988) or of EMS (ethyl methanesulfonate)-mutagenized worms (BRENNER 1974) containing many F_1 gravid adults were subjected to alkaline hypochlorite treatment to obtain embryos, which were then allowed to hatch in S basal medium at 20° overnight. About 100,000 newly hatched worms were put on a 9-cm NGM plate containing 400 μg/ml NaF, which had been seeded with *E. coli* OP50. Most worms did not grow and died within a few days. After 5–8 days at 20°, 5–50 progeny from EMS-mutagenized worms or 0–5 *mut* worms grew to adults on each plate. They were picked and tested for fertility and for fluoride resistance of the progeny. The percentage of sterile worms among the survivors varied from time to time, but most of the fertile survivors produced fluoride-resistant progeny. The fluoride-resistant (*flr*) mutants thus obtained were backcrossed five times to N2 males before they were used for further experiments. The *flr* mutants that were isolated from the mutator strains were crossed to a strain having a marker near the mutator (*dpy-10* for *mut-5* and *dpy-13* for *mut-6*) to remove the *mut* mutations before the backcross. If two or more mutants in the same gene were obtained from one culture, only one of them was kept to ensure independence. We used *ut11, ut5, ut9* and *ut7* as reference alleles of *flr-1, 2, 3* and *4*, respectively, in this study.

Mapping of fluoride-resistant mutants: Determination of linkage group, two- and three-factor crosses and complementation tests were performed according to standard methods (BRENNER 1974; WOOD 1988). Males homozygous (autosomal mutations) or hemizygous (*X*-linked mutations) for the *flr* mutations were obtained by heat shock (WOOD 1988) and by mating with N2 males, respectively. All of them were fertile and therefore used for genetic crosses.

Mutations in *flr-1, 3* and *4* were recognized by their phenotype of slow growth and smaller size rather than fluoride resistance during all the mapping procedures. When wild-type worms become adults, these *flr* mutants of the same age are small L3 larvae and much smaller than the wild-type adult worms, hence easily recognized. Complementation of *flr-1* and *flr-4* with deficiencies and duplications in the *X* chromosome was performed according to MENEELY and HERMAN (1979) and HERMAN, MADL and KARI (1979).

Two- and three-factor crosses for mapping *flr-2* and *flr-5* were scored by monitoring their ability to suppress the slow-growing phenotype of *flr-1* or *flr-3*. Complementation of *flr-2* or *flr-5* with deficiencies in LGV was performed as follows. Males of *flr-2; flr-1* or *flr-5; flr-1* were mated with hermaphrodites of *ctDf1/nT1[unc(n754dom) let] V* or *dpy-18/eT1 III; Df/eT1 V* (where *Df* stands for *sDf20, sDf26, sDf30* or *sDf35*). These crosses were chosen because of inefficient transfer of deficiency chromosomes through sperms and because of extremely poor growth of worms having a genotype of *+/nT1[unc(n754dom) let]; flr-1*. Twelve F_1 hermaphrodites were picked. Those F_1 worms having the deficiency (and not the translocation) in the heterozygous condition were identified by their non-Unc phenotype in the case of *ctDf1/nT1* and by absence of Unc worms (*eT1* homozygotes) and presence of Dpy worms among the F_2 progeny in the case of *dpy-18/eT1 III; Df/eT1 V*. Complementation between *flr-2* (or *flr-5*) and the deficiencies was judged by presence of slow-growing worms among the F_2 progeny. We found that *ctDf1* and *sDf35* delete *flr-2* and *flr-5*, respectively. Worms having the genotypes *flr-2/ctDf1; flr-1* and *flr-5/sDf35; flr-1* were found among the F_2 progeny by checking for the presence of dead eggs among the self-progeny and slow growth of male progeny produced by mating with N2 males.

Construction of the *flr* double and triple mutants: Since the phenotype of a *flr* mutation may be masked by another *flr* mutation, we used tightly linked markers (*unc-84, sma-1* or *unc-76, dpy-9, unc-7* and *unc-23* for *flr-1, flr-2, flr-3, flr-4* and *flr-5*, respectively) for construction of the *flr* double and triple mutants. For instance, we made the double mutant *flr-2 V; flr-1 X* by mating *unc-76 V; flr-1 X* hermaphrodites with *flr-2 V; unc-84 X* males at 15° and selecting for non-Unc F_2 hermaphrodites that did not segregate Unc worms at 25°. The temperature was chosen, because males hemizygous for *unc-84* are active in mating at 15°, but the Unc-84 phenotype is more easily discernible at 25°.

The *flr-5 flr-2* double mutants were made as follows. First, *flr-5 sma-1* and *unc-23 flr-2* were obtained as worms having phenotypes of Sma and Unc among the progeny of *flr-5/unc-23 sma-1* and *flr-2/unc-23 sma-1*, respectively. Then, *flr-5 flr-2* worms were obtained among the progeny of *flr-5 sma-1/unc-23 flr-2* as non-Sma, non-Unc worms that segregate neither Sma nor Unc worms.

Some of the class 1-class 2 double mutants were made

without counter-selection markers as follows, assuming that class 2 mutations suppress the slow-growing phenotype of class 1 mutations. Class 1 slow-growing hermaphrodites were mated with class 2 normal-growing males. The F_1 cross-progeny hermaphrodites were chosen by the normal-growing phenotype. Several slow-growing worms of the F_2 progeny were cloned. About two-thirds of them segregated normal-growing worms, which were considered to be the class 1-class 2 double mutants.

In some cases the genotypes of double and triple mutants were confirmed by the following methods. Presence of a class 1 mutation in double and triple mutants was checked by the slow growth of the F_1 cross progeny hermaphrodites in mating with the class 1 single-mutant males. Presence of a class 2 mutation in double and triple mutants containing at least one class 1 mutation was confirmed by absence of slow-growing worms among the F_2 progeny in mating with the class 2 single-mutant males. Presence of *flr-5* and *flr-2* in the *flr-5 flr-2* double mutants was shown by absence of slow-growing worms among the F_2 progeny in mating with the *flr-5;flr-1* and *flr-2;flr-1* double-mutant males, respectively.

Measurements of generation times and brood sizes: Brood sizes of the wild-type and the *flr* single, double and triple mutants were measured on NGM plates at 20° as follows. The worms were grown on NGM plates at 20° under good, nonstarving conditions. Five L4 larvae were transferred onto five fresh NGM plates seeded with *E. coli* OP50 (one worm per plate). The plates were incubated at 20° and checked after every 24 hr until the worms stopped laying fertilized eggs. The worms were transferred onto fresh plates if they had laid eggs within 24 hr. The numbers of the progeny were counted when they were grown to L3 larvae to young adults. We did not check whether each progeny worm could lay eggs. The average and standard deviation of the brood sizes of the five worms are shown in Table 4.

Generation times of the wild-type and the *flr* single, double and triple mutants were measured on NGM plates containing 0, 150 and 400 μg/ml NaF at 15, 20 and 25° as follows. The worms were grown on NGM plates at 20° under good, nonstarving conditions One to four young adult worms, depending on the estimated brood sizes, were transferred onto the plates for the measurements and incubated at the designated temperatures. After 24 hr the worms were removed from the plates, on which they had laid 20–200 fertilized eggs. This time was regarded as time 0. The plates were incubated at the same temperatures and checked after every 24 hr. The generation times were defined as the times when the progeny worms laid more than one fertilized eggs on the average. They are estimated to have errors of ± 1 day.

Isolation of revertants of *flr-1(ut11)* and *flr-3(ut9)*: To increase reversion frequency, we crossed *flr-1(ut11)* and *flr-3(ut9)* into a mutator genetic background. For this purpose *mut-6 IV* and *mut-5 II* hermaphrodites were mated with *dpy-13/+ IV; flr-1(ut11) X* males and *dpy-10/+ II; flr-3(ut9) IV* males, respectively. F_1 worms that produce both Dpy and non-Dpy worms were chosen. Worms having the genotypes of *mut-6; flr-1* and *mut-5; flr-3*, respectively, were obtained among the F_2 as non-Dpy slow-growing worms that did not segregate Dpy worms. Alternatively, reversion frequency was increased by treatment with EMS (ethyl methanesulfonate) (BRENNER 1974). The worms (mutator-containing or EMS-treated) for the isolation of revertants were cultured on PY plates. Cultures were continued until the F_1 progeny grew to gravid adults in the case of the EMS-treated worms. Then, the worms were harvested and treated with alkaline hypochlorite (HECHT *et al.* 1982) to isolate embryos, which were then allowed to hatch in S basal medium at 20° overnight to obtain L1 larvae. About 10,000 L1 larvae of the mutator strains or about 40,000 L1 larvae of the F_2 progeny of the EMS-treated worms were cultured on a 9-cm PY plate. After 3 days at 20° the slow-growing *flr* worms were at the stage of L3 larvae, and only growth-rate revertants had grown to gravid adults. At this time the worms were harvested and subjected to a second alkaline hypochlorite treatment, which killed worms but not embryos. The surviving embryos were put on NGM plates. After 3–4 days at 20°, worms that grew to adults were picked, and their progeny were tested for growth rate and for resistance to 400 μg/ml NaF. The revertants (fluoride-sensitive normal growers) and pseudo-revertants (fluoride-resistant normal growers) were backcrossed three times to the original *flr* mutants before further characterization.

Removal of the original *flr* mutation from the pseudo-revertants of *flr-1* and *flr-3*: Hermaphrodites of the pseudo-revertant *flr-3(ut9) IV; flr-2(ut71) V* were mated with *dpy-9(e12)/+ IV; unc-76(e911)/+ V* males and *flr-3/dpy-9; flr-2/unc-76* worms were selected among the cloned F_1 hermaphrodites by checking the phenotype of the F_2 progeny. Ten Dpy, non-Unc F_2 worms were cloned. Of these worms those segregating only Dpy non-Unc worms and no Dpy Unc worms were assigned the genotype *dpy-9; flr-2.* Then they were mated with *unc-76/+* males, and *dpy-9/+; flr-2/unc-76* worms were selected among the cloned F_1 hermaphrodites by checking the phenotype of the F_2 progeny. Non-Dpy, non-Unc worms among the F_2 progeny were cloned and those segregating neither Dpy nor Unc were assigned the genotype of *flr-2.* The assignment was confirmed by making the double mutant *flr-3(ut9); flr-2(ut71)* again and checking the growth rate.

The suppressor mutation *flr-5(ut73)* was separated from the original mutation *flr-1(ut11)* by mating *flr-5(ut73); flr-1(ut11)* with N2 males and selecting for F_2 weakly fluoride-resistant worms that do not produce slow-growing male progeny when mated with N2 males. The genotype of *flr-5(ut73)* was confirmed by making the double mutant *flr-5(ut73); flr-1(ut11)* again and checking its growth rate.

Tests of suppression of the slow-growing phenotype of *flr-1(ut11)* by various known mutations: To test the possibility of class 2 mutations being allelic to *cat-4(e1411)*, *cat-6(e1861)*, *osm-6(p811)*, *che-11(e1810)* or *che-12(e1812)*, we checked if the latter mutations suppress the slow-growing phenotype of *flr-1(ut11)* like class 2 mutations. Hermaphrodites of these mutants were mated with the *flr-1* males. Six thin, slow-growing worms were chosen among the F_2 progeny of each cross and cloned onto new plates. Since none of them, each of which has a 67% possibility of having the genotype of *cat-4/+; flr-1 etc.*, produced normal-growing worms, we concluded that none of the mutations suppress the slow-growing phenotype of *flr-1*.

We also tested if these mutations suppress the slow growth of *flr-1* if they are placed in *trans* to class 2 mutations, namely if *cat-4/flr-2; flr-1 etc.* are normal growers. Hermaphrodites of the mutants to be tested were mated with *flr-2(ut5); flr-1(ut11)* males and with *flr-5(ut73); flr-1(ut11)* males. Six thin, slow-growing worms were chosen among the F_2 progeny of each cross and cloned onto new plates. More than half of them produced normal-growing worms in all the cases of the crosses. In these cases the F_2 slow-growing worms should have the genotype of *cat-4/flr-2; flr-1, cat-4/flr-5; flr-1 etc.*, because *cat-4; flr-1 etc.* cannot produce normal growers as shown above and because *flr-2; flr-1* and *flr-5; flr-1* are normal growers. Hence we concluded that *cat-4/flr-2, cat-4/flr-5 etc.* do not suppress the slow growth of *flr-1*.

Miscellaneous: DNA fragments containing Tc1 that caused mutations *flr-1(ut11)* and *flr-3(ut9)*, respectively, were cloned by the transposon-tagging method. Details of

the experiments will be published elsewhere. See SAMBROOK, Fritsch and MANIATIS (1989) for DNA techniques including Southern blotting. Chemotaxis and osmotic avoidance were tested according to WARD (1973) and to CULOTTI and RUSSELL (1978), respectively. Dye-filling into amphids and phasmids was checked as described by HEDGECOCK et al. (1985), but using 10 μg/ml DiO (3, 3′-dioctadecyloxacarbocyanine perchlorate) instead of 4 mg/ml FITC (fluorescein isothiocyanate).

RESULTS

Effect of fluoride ion on wild-type *C. elegans*: Various amounts of NaF were put into the agar medium to test the effect on the wild-type *C. elegans*. No effect was observed, if the concentration of NaF was below 40 μg/ml (0.95 mM). However, at 150 μg/ml (3.6 mM) the growth of L1 larvae was markedly retarded, especially at 15°. When the agar medium contained 400 μg/ml (9.5 mM) NaF, worms of all stages stopped growing immediately except for embryos, which are protected by the highly impermeable egg shell. The worms eventually died within 1 or 2 days.

The effect of NaF in a liquid medium was essentially the same except that somewhat higher concentration of NaF was required for the same degree of toxicity: 34% and 93% of L1 worms were killed at concentrations of NaF of 400 μg/ml and 1500 μg/ml, respectively, in 72 hr. The killing effect was independent of the presence of *E. coli*, and killing of dauer larvae was not much different from that of L1 larvae. These results suggest that NaF can penetrate into worms through somewhere other than the alimentary canal. The toxic effect is due to fluoride and not to sodium ion. Sodium chloride, for instance, is nontoxic to *C. elegans* at these concentrations.

Isolation and characterization of fluoride-resistant mutants: Fluoride-resistant mutants of *C. elegans* were isolated by selecting for worms that can grow on plates containing 400 μg/ml NaF (see MATERIALS AND METHODS for details). Eleven mutants were kept and characterized further. All the mutations are recessive. They are grouped into two classes, class 1 and class 2, according to the phenotype.

Class 1 mutants consist of all but one of the 11 mutants. They are resistant to 400 μg/ml NaF and look different from wild-type worms even in the absence of fluoride ion. Namely, their larvae grow twice as slowly as wild-type larvae despite their almost normal speed of embryonic development. They also look thin and small (0.9 to 1.0 mm in adult length as compared with the wild-type adult length of 1.2 mm) and have small brood sizes. Since the slow-growing phenotype can be recognized easily, we used it for mapping and complementation tests. Complementation tests show that there are three genes for class 1 mutants, *flr-1*, *flr-3* and *flr-4* (Table 1). Mapping by two- and three-factor crosses and by complementation with deficiencies revealed the positions of the genes as follows: *flr-1* maps 0.4 unit to the right of *unc-9* (LGX); *flr-3* maps very close to *dpy-9*, *daf-1* and *tpa-1* (near the left end of LGIV); and *flr-4* maps 0.4 unit to the left of *unc-7* (LGX) (Tables 2, 3 and Figure 1). Heterozygotes *flr-4/mnDf19 X* and *flr-4/mnDf43 X* were made by mating *mnDp1(X;V)/+ V; Df X* hermaphrodites with *flr-4* males. They have essentially the same phenotype as *flr-4* homozygotes. They are slow-growing worms (generation time: about 10 days at 20°) with a small brood size (25 ± 25). Such heterozygotes were not made for *flr-1* or *flr-3*, because we could not obtain deficiencies covering these mutations.

TABLE 1

Genes, alleles and phenotypes of fluoride-resistant mutants

Gene	Allele	Mutagen	
Class 1 mutation: Resistant to 400 μg/ml NaF. Slow growth and small brood size even in the absence of NaF. Small and thin.			
flr-1 X	*ut1*	EMS	
	ut2	EMS	
	ut4	EMS	
	ut6	EMS	
	ut11	*mut-6*	Reference allele
flr-3 IV	*ut8*	EMS	
	ut9	*mut-5*	Reference allele
	ut10	*mut-5*	
flr-4 X	*ut3*	EMS	
	ut7	EMS	Reference allele
Class 2 mutation: Not completely resistant to 400 μg/ml NaF. Normal growth rate and brood size in the absence of NaF. Suppressor of the slow-growing phenotype of mutations in *flr-1*, *flr-3* and *flr-4*.			
flr-2 V	*ut5*	EMS	Reference allele
	ut71	EMS	
flr-5 V	*ut73*	EMS	Reference allele

Only one allele (*ut5*) belongs to class 2 among the 11 fluoride-resistant mutants isolated by selection with NaF. It is only weakly resistant to fluoride ion. Namely, only some of the newly hatched larvae can grow to adults in the presence of 400 μg/ml NaF. However, it grows faster than wild-type worms in the presence of 150 μg/ml NaF, especially at 15° (Table 4). Other properties are essentially the same as wild-type worms except that it is weakly dumpy. The dumpy phenotype is enhanced when the worms are starved. We were unable to separate the dumpy from fluoride-resistance phenotypes. The mutation complements all of *flr-1*, *flr-3* and *flr-4* alleles and maps on LGV, a different linkage group from those of *flr-1*, *flr-3* and *flr-4*. Therefore, we assigned it to a new gene, *flr-2*. However, detailed mapping of the mutation was difficult owing to the weak fluoride resistance. It became possible after we discovered that it suppresses the slow-growing phenotype of *flr-1*, *flr-3* and *flr-4* (see below).

Both class 1 and class 2 mutants are essentially the same as wild-type worms concerning the following properties: sensitivity to caffeine and to TPA, chemotaxis to NaCl and to lysine, osmotic avoidance from 5 M NaCl, dye-filling into amphids and phasmids, touch sensitivity and pharyngeal pumping rate. Males of all the *flr* mutants can mate with hermaphrodites. Although *flr-3* maps near *tpa-1*, it is not allelic to *tpa-1*, because worms having the genotype of *flr-3/tpa-1* are

TABLE 2

Two-factor crosses

Heterozygous parent	Segregants		Map distance
flr-1(ut11)X			
flr-1 unc-3/++	Wild type	1795	3.5%
	Flr Unc	479	
	Flr	35	
	Unc	45	
unc-9 flr-1/++	Wild type	1106	0.4%
	Flr Unc	326	
	Flr	4	
	Unc	2	
flr-1 unc-84/++	Wild type	1145	1.2%
	Flr Unc	315	
	Flr	9	
	Unc	9	
flr-2(ut71)V			
flr-3/flr-3;	non-Unc slow grower	336	2.0%
flr-2unc76/++	Unc slow grower	4	
	non-Unc normal grower	5	
	Unc normal grower	115	
flr-3(ut9)IV			
flr-3 dpy-13/++	Wild type	190	25%
	Dpy Flr	22	
	Dpy	33	
	Flr	27	
daf-1/flr-3	*flr-3/flr-3*	551	0%
	flr-3/daf-1 flr-3	0	
dpy-9/flr-3	*flr-3/flr-3*	388	0%
	flr-3/dpy-9 flr-3	0	
flr-3/tpa-1	*flr-3/flr-3*	357	0%
	flr-3/flr-3 tpa-1	0	
flr-4(ut7)X			
unc-3 flr-4/++	Wild type	361	1.9%
	Flr Unc	102	
	Flr	5	
	Unc	4	
flr-4 unc-7/++	Wild type	1166	0.4%
	Flr Unc	310	
	Flr	3	
	Unc	3	

The phenotype of *flr-1*, *flr-3* and *flr-4* was recognized by slow growth in the absence of fluoride ion, and that of *flr-2* by suppression of the slow-growing phenotype of *flr-3*.

normal growing, fluoride sensitive and TPA sensitive.

Small differences were sometimes found between wild-type worms and some or all of the *flr* mutants in the following properties. Hermaphrodites of *flr-1*, *flr-3* and *flr-4* commit suicide more often than wild-type hermaphrodites by climbing up the wall of plates. All the *flr* mutants are somewhat less active in spontaneous movement when they are placed on NGM plates without *E. coli*. Double mutants in *unc-3* and *flr-1*, *flr-3* or *flr-4* form dauer larvae more easily than the *unc-3* single mutant.

Suppression of the slow-growing phenotype of class 1 mutations by the class 2 mutation *flr-2(ut5)*: We constructed various double and triple mutants in the *flr* genes to test their interaction. Their phenotypes (Table 4) can be summarized as follows. (1) Double and triple mutants among class 1 mutations have essentially the same phenotype as the single mutants. (2) Double and triple mutants including the class 2 mutation *flr-2(ut5)* have a new, mixed phenotype. They are resistant to 400 μg/ml NaF, but they grow as fast as the class 2 mutant. Their brood sizes are larger than that of class 1 mutants. Namely, the class 2 mutation *flr-2(ut5)* is epistatic to class 1 mutations concerning growth rate and brood size, but hypostatic concerning the degree of fluoride resistance. In other words, the class 2 mutation suppresses the slow growth and small brood size but not strong fluoride resistance of class 1 mutations. The *flr-2(ut5)* mutation is recessive in the suppression as it is in fluoride resistance.

Isolation and mapping of revertants of *flr-1(ut11)* and *flr-3(ut9)*: Since mutants in *flr-1*, *flr-3* and *flr-4* grow much slower than wild-type worms, it was possible to isolate their revertants by selecting for worms that grow at normal rate. We have established a method of such selection and isolated normal-growing revertants from transposon-insertion mutants *flr-1(ut11)* and *flr-3(ut 9)*, which were isolated as spontaneous fluoride-resistant mutants from the mutator strains *mut-6* and *mut-5*, respectively.

The selection method (see MATERIALS AND METHODS for details) consists of two repeated treatments with alkaline hypochlorite, which kills worms but not embryos that are protected by highly impermeable egg shells. The first treatment followed by hatching in buffer solution yields a synchronized population of L1 larvae. When we culture them for three days at 20°, the slow-growing *flr* worms have not reached adulthood, but normal-growing revertants, if any, have grown to gravid adults. At this time the second alkaline hypochlorite treatment is performed to kill all except embryos of the revertants.

When we isolated spontaneous growth-rate revertants of the mutator-containing, slow-growing strains *mut-6(st702); flr-1(ut11)* and *mut-5(st701); flr-3(ut9)*, all the revertants were sensitive to NaF. Since we have cloned DNA fragments containing the Tc1 transposons that caused the *flr-1(ut11)* and *flr-3(ut9)* mutations (our unpublished data), we performed Southern blot analysis of genomic DNA from N2, the *flr* mutants and their revertants, using a DNA fragment flanking the Tc1 as a probe. The result showed that all the revertants lost the Tc1 that caused the original mutations (data not shown). Therefore, they are intragenic revertants.

When we used ethyl methanesulfonate to increase the reversion frequency, all the growth-rate revertants obtained were still strongly fluoride resistant. Two of them were kept and analyzed further. The secondary mutations were recessive in suppression of the slow-growing phenotype of *flr-1* or *flr-3*, because all the F_1 cross-progeny between hermaphrodites of the pseudo-revertants and males of the original *flr* mutants grow slowly. When the pseudo-revertants were mated with N2 males, about 3/16 of the F_2 worms grew slowly. This result shows that the secondary mutations are not linked to the original *flr* mutation. Complemen-

TABLE 3

Three-factor crosses

Heterozygous parent	Recombinant phenotype	Recombinant genotype	Number
flr-1(ut11)X *unc-84* / *unc-9 flr-1*	Flr	*+ flr-1 +* / *unc-9 flr-1 +*	3
	Unc	*unc-9 + unc-84* / *unc-9 flr-1 +*	4
flr-2(ut5)V *flr-2* / *unc-46 dpy-11*	Unc	*unc-46 + flr-2* / *unc-46 dpy-11 +*	3
	Dpy	*+ dpy-11 +* / *unc-46 dpy-11 +*	2
flr-2 / *unc-23 sma-1*	Unc	*unc-23 flr-2 +* / *unc-23 + sma-1*	5
	Sma	*+ + sma-1* / *unc-23 + sma-1*	4
flr-2 / *sma-l unc-76* ; *flr-1* / *flr-1*	Sma slow grower	*+ sma-1 +* / *+ sma-1 unc-76* ; *flr-1* / *flr-1*	1
	Unc slow grower	*flr-2 + unc-76* / *+ sma-l unc-76* ; *flr-1* / *flr-1*	5
flr-2(ut71)V *flr-2* / *unc-46 dpy-11*	Unc	*unc-46 + flr-2* / *unc-46 dpy-11 +*	5
	Dpy	*+ dpy-11 +* / *unc-46 dpy-11 +*	4
flr-2 / *unc-23 sma-1*	Unc	*unc-23 flr-2 +* / *unc-23 + sma-1*	5
	Sma	*+ + sma-1* / *unc-23 + sma-1*	4
flr-2 / *sma-1 unc-76* ; *flr-1* / *flr-1*	Sma slow grower	*+ sma-1 +* / *+ sma-1 unc-76* ; *flr-1* / *flr-1*	11
	Unc slow grower	*flr-2 + unc-76* / *+ sma-1 unc-76* ; *flr-1* / *flr-1*	8
flr-4(ut7)X *unc-84* / *flr-4 unc-7*	Flr	*+ flr-4 +* / *+ flr-4 unc-7*	2
	Unc	*unc-84 + unc-7* / *+ flr-4 unc-7*	5
flr-5(ut73)V *flr-5* / *let-341 unc-46*	Unc	*+ unc-46 +* / *let-341 unc-46 +*	2
flr-5 / *unc-46 dpy-11*	Unc	*unc-46 + flr-5* / *unc-46 dpy-11 +*	1
	Dpy	*+ dpy-11 +* / *unc-46 dpy-11 +*	6
flr-5 / *dpy-11 unc-23*	Dpy	*dpy-11 flr-5 +* / *dpy-11 + unc-23*	7
	Unc	*+ + unc-23* / *dpy-11 + unc-23*	5

TABLE 3

Continued

Heterozygous parent	Recombinant phenotype	Recombinant genotype	Number
flr-5 / *unc-23 sma-1*	Unc	*+ unc-23 +* / *+ unc-23 sma-1*	5
	Sma	*flr-5 + sma-1* / *+ unc-23 sma-1*	4

Mutations *flr-1*, *flr-3* and *flr-4* were recognized by the slow-growing phenotype, whereas *flr-2* and *flr-5* by the activity of suppressing the slow growing phenotype of *flr-1*. In the latter cases, if the worms did not contain *flr-1*, it was introduced afterward by mating to recognize the phenotype.

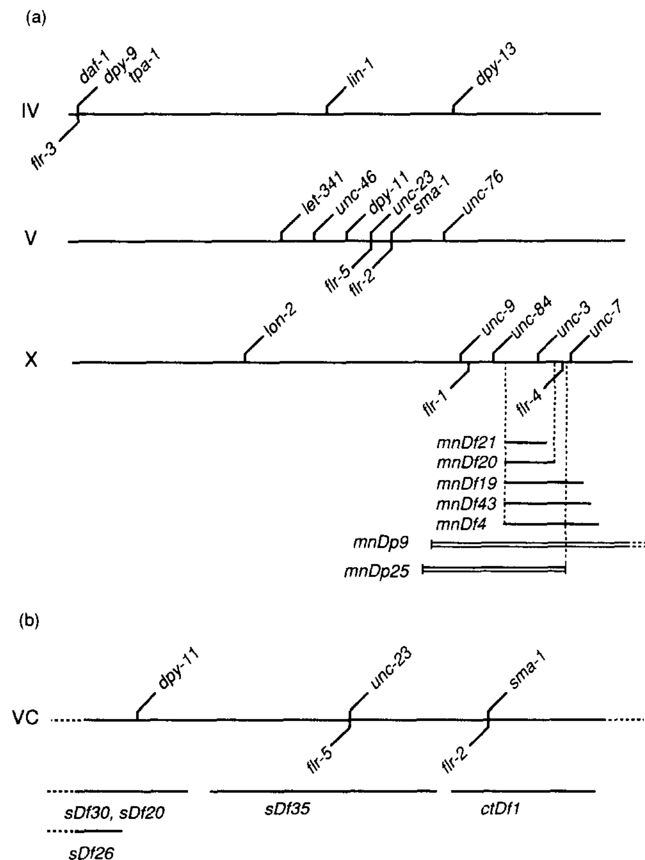

FIGURE 1.—Genetic map of fluoride-resistant mutants. (a) Map of three linkage groups relevant to the *flr* mutations. The bars below the linkage group X show the part of the chromosome deleted in deficiencies (filled bars) and those duplicated in duplications (open bars). (b) Central part of the linkage group V including *flr-5* and *flr-2*. The parts deleted in some deficiencies are shown by solid bars below. The positions of *flr-2* and *flr-5* relative to *sma-1* and *unc-23*, respectively, have not been determined. Although the order of *daf-1*, *dpy-9*, *tpa-1* and *flr-3* was not determined by classical genetic means, the results of DNA cloning show that it is *flr-3*, *daf-1* and *tpa-1* from left to right (M. KAWAKAMI, T. ISHIHARA and I. KATSURA, unpublished data), where the position of *dpy-9* is still unknown.

tation of the pseudo-revertants with *flr-2(ut5); flr-1(ut11)* or *flr-3(ut9); flr-2(ut5)* showed that one of the secondary mutations *(ut73)* complemented *flr-2(ut5)* for suppression of the slow-growing phenotype of the original *flr* mutation, whereas the other mutation *(ut71)* did not complement *flr-2(ut5)*. Mapping of the suppressor mutations showed that the mutation *ut73* is located near *unc-23* on LGV, whereas *ut71* is near *sma-1*, also on LGV (Tables 2, 3 and Figure 1). Mapping of *flr-2(ut5)* was also performed by monitoring its activity of suppressing the slow-growing phenotype of *flr-1*. The results (Tables 2, 3 and Figure 1) are consistent with the above complementation test which showed that it is in the same gene as *ut71*.

Properties of the suppressor mutations: To study the phenotype of the two suppressor mutations *ut71* and *ut73* in the absence of the original mutations *ut9* and *ut11*, respectively, we removed the latter from the pseudo-revertants (see MATERIALS AND METHODS for details). Both of the suppressor mutations by themselves were similar to *flr-2(ut5)* in that they were weakly resistant to NaF (Table 4). Therefore, the allele *ut73* defines a new fluoride-resistant gene, which we call *flr-5*. The weak fluoride-resistant phenotype of *ut71* is consistent with the results of mapping (see above) showing that it is an allele of *flr-2*. Also like *flr-2(ut5)*, those mutants (*flr-2(ut71)* and *flr-5(ut73)*) grow almost as fast as wild-type worms in the absence of fluoride ion, and their brood sizes are only slightly smaller than that of wild-type worms. We therefore include them in class 2 mutants. However, neither *flr-2(ut71)* nor *flr-5(ut73)* has the weak dumpy phenotype of *flr-2(ut5)*. Except for this phenotype those mutants have the same properties as flr-2(ut5) (see *Isolation and characterization of fluoride-resistant mutants* for the properties tested). By making double mutants we confirmed that all the three class 2 mutations suppress the slow-growing phenotype of all of *flr-1*, *flr-3* and *flr-4* (Table 4). We used two alleles of *flr-1*, *flr-2*, *flr-3* and *flr-4* to show that the suppression is not allele specific but specific to the genes. The suppression is weak at 15° in the presence of 400 μg/ml NaF. The *flr-5 flr-2* double mutants (*ut73 ut5* and *ut73 ut71*) were also made and tested for their phenotype. They had essentially the same phenotype as the class 2 single mutants.

Heterozygotes *flr-2/ctDf1* and *flr-5/sDf35*, where *ctDf1* and *sDf35* delete *flr-2* and *flr-5*, respectively, were made by mating *ctDf1/nT1[unc(n754dom) let]* and *sDf35/eT1* hermaphrodites with *flr-2* and *flr-5* males, respectively. They are weakly resistant to fluoride ion and grow as fast as *flr-2* and *flr-5*. Thus, they have essentially the same properties as the *flr-2* and *flr-5* homozygotes except that they segregate dead eggs. Heterozygotes *flr-2/ctDf1 V; flr-1 X* and *flr-5/sDf35 V; flr-1 X* were found among the F_2 progeny of

TABLE 4

Generation time at various concentrations of NaF at various temperatures and brood sizes in the absence of NaF at 20°

	Generation time (days)									
	No NaF			150 μg/ml NaF			400 μg/ml NaF			Brood size
	15°	20°	25°	15°	20°	25°	15°	20°	25°	
(Fluoride-sensitive, normal grower)										
N2	5	3	2	16[a]	6	5	—[b]	—[b]	—[b]	308 ± 22
(Weak fluoride-resistant, normal grower)										
flr-2	5	3	2	6	4	3	10[c]	5[c]	4[c]	300 ± 52
flr-2(ut71)	5	3	2	7	4	3	9[c]	6[c]	4[c]	209 ± 41
flr-5	4	3	2	4	3	2	9[c]	5[c]	4[c]	307 ± 47
flr-5 flr-2	5	3	2	6	4	3	12[c]	6[c]	5[c]	162 ± 54
flr-5 flr-2(ut71)	5	3	3	5	4	3	12[c]	6[c]	5[c]	153 ± 90
(Fluoride-resistant, slow grower)										
flr-1	10	5	5	10	6	6	10	6	5	56 ± 55
flr-1(ut1)	12	8	7	12	7	7	12	7	7	50 ± 38
flr-3	10	6	5	9	5	4	10	7	5	89 ± 32
flr-3(ut8)	12	7	5	12	7	5	11	7	5	61 ± 39
flr-4	12	8	4	15	9	4	12	9	4	31 ± 32
flr-4(ut3)	13	7	5	12	7	5	12	8	5	47 ± 19
flr-3; flr-1	9	5	4	9	5	4	10	6	5	57 ± 49
flr-1 flr-4	9	5	4	10	5	4	10	5	4	60 ± 35
flr-3; flr4	11	7	4	11	8	5	12	7	5	40 ± 43
flr-3; flr-1 flr-4	9	5	4	10	6	5	10	6	4	85 ± 45
(Fluoride-resistant, normal or seminormal grower)										
flr-2; flr-1	5	3	3	5	3	3	7	5	3	204 ± 25
flr-2; flr-1(ut1)	7	4	3	7	4	3	7	4	4	201 ± 10
flr-3; flr-2	6	3	3	6	3	3	6	4	3	184 ± 31
flr-3(ut8); flr-2	6	4	3	6	4	3	7	4	4	125 ± 34
flr-3; flr-2(ut71)	6	3	2	6	3	2	6	3	2	133 ± 42
flr-2; flr-4	6	4	2	6	4	2	6	4	2	150 ± 48
flr-2; flr-4(ut3)	6	4	3	6	4	3	6	4	3	129 ± 35
flr-3; flr-2; flr-1	5	3	2	5	3	2	5	3	2	217 ± 32
flr-2; flr-1 flr-4	6	3	3	6	4	3	7	4	3	203 ± 24
flr-5; flr-1	7	4	3	7	4	4	7	4	3	162 ± 18
flr-5; flr-1(ut1)	6	3	3	7	4	3	8	4	3	205 ± 28
flr-3; flr-5	5	3	3	7	4	3	8	4	3	150 ± 39
flr-3(ut8); flr-5	6	3	3	6	4	3	9	4	3	187 ± 29
flr-5; flr-4	6	4	3	7	4	3	9	4	3	220 ± 9
flr-5; flr-4(ut3)	6	4	3	7	4	4	9	5	4	147 ± 18

The alleles used for the measurements (except those indicated) are *ut11*, *ut5*, *ut9*, *ut7* and *ut73* for *flr-1*, *flr-2*, *flr-3*, *flr-4* and *flr-5*, respectively.

Brood size was measured with five worms, and its average and standard deviation are shown in the table.

[a] Large fluctuation (10–25 days).

[b] Death at L1 larvae.

[c] Less than 5% of the worms can grow to adults.

the cross between *ctDf1/nT1[unc(n754dom) let]* and *sDf35/eT1* hermaphrodites and *flr-2; flr-1* and *flr-5; flr-1* males, respectively (MATERIALS AND METHODS, *Mapping of fluoride-resistant mutants*). They resemble *flr-2; flr-1* and *flr-5; flr-1* and differ from *flr-1*. Namely, they have a generation time of 3 days at 20° and brood size of about 100. These results show that all the class 2 mutations, *flr-2(ut5)*, *flr-2(ut71)* and *flr-5(ut73)*, are null or hypomorphic mutations.

Since class 2 mutations have not been mapped very precisely, and since two different alleles in a gene sometimes produce different phenotypes, it is possible that class 2 mutations may map in known genes. We therefore checked this possibility for *cat-4*, *cat-6*, *osm-6*, *che-11* and *che-12*, all of which map in the central region of LGV. We found that none of the reference alleles of these genes suppressed the slow-growing phenotype of *flr-1(ut11)*. We also showed that the suppression does not occur when these mutations are placed in *trans* to *flr-2* or *flr-5* (*cat-4/flr-2*, *cat-4/flr-5*, etc.) (MATERIALS AND METHODS). Hence, it is very unlikely that class 2 mutations are allelic to these mutations.

DISCUSSION

In this study we isolated 13 fluoride-resistant (*flr*) mutants in five genes, 11 by selection on plates containing NaF and two by reversion of some of the *flr* mutations. These mutants are classified into two groups, class 1 and class 2, according to the phenotype.

Class 1 mutants consist of all the mutants in three new genes, *flr-1*, *flr-3* and *flr-4*. All of them grow almost twice as slowly as wild-type worms and have small brood sizes even in the absence of fluoride ion. They are small and thin and resistant to 400 μg/ml NaF. They were isolated at normal frequency after mutagenesis with ethyl methanesulfonate. The mutations are recessive, and some of them in *flr-1* and *flr-3* are caused by insertion of the transposon Tc1 (M. Kawakami, T. Ishihara, T. Amano, K. Kondo and I. Katsura, unpublished data). Since *flr-4/Df* (where *Df* deletes *flr-4*) has almost the same phenotype as *flr-4/flr-4*, the *flr-4(ut3* and *ut7)* mutations are null or hypomorphic. Although no such data are available for *flr-1* and *flr-3*, their properties mentioned above suggest that they are probably null mutants or hypomorphs. The functions of class 1 genes seem to be important in the normal growth of *C. elegans*. However, slow growth is not the cause of fluoride resistance, since the triple mutant *flr-3; flr-2; flr-1*, for instance, grows as fast as wild-type worms and is still strongly resistant to NaF.

Class 2 mutants consist of *flr-2(ut5)*, *flr-2(ut71)* and *flr-5(ut73)*. These mutations are null or hypomorphs according to the phenotype of *flr-2* (or *flr-5*)/*Df* and *flr-2* (or *flr-5*)/*Df; flr-1*. It was fortuitous that the weakly resistant mutant *flr-2(ut5)* was isolated by selection using 400 μg/ml NaF, since less than 5% of the mutant worms survive this treatment (Table 4). We have not yet succeeded in separating its weak dumpy phenotype from that of weak fluoride resistance. The former may be caused by the same mutation as the latter or by a closely linked extragenic mutation. When we examined the phenotype of double mutants, we found that *flr-2(ut5)* acts as a recessive suppressor of the slow growth rate and small brood size (but not strong fluoride resistance) of class 1 mutations. Therefore, we looked for more class 2 mutations as suppressors of the slow-growing phenotype of *flr-1(ut11)* and *flr-3(ut9)*. Two of the class 2 mutations, *flr-2(ut71)* and *flr-5(ut73)*, were obtained in this way. By separating them from the original mutations *flr-1(ut11)* or *flr-3(ut9)*, we showed that those mutations by themselves cause weak fluoride resistance like *flr-2(ut5)*. All the class 2 mutations suppress the slow-growing phenotype of mutations in all the class 1 genes.

The phenotypes of double and triple mutants suggest a functional relationship between the fluoride-resistant genes. Double and triple mutants of the same class have the same phenotype as the single mutants. Therefore, genes of the same class seem to act in a single process or in different steps of a linear, sequential pathway. It is especially interesting that the class 2 mutations suppress the slow-growing phenotype but not the strong fluoride resistance of the class 1 mutations. We can interpret the relation by either of the two models shown in Figure 2. In model (a) the *flr* gene products form a metabolic, transport or signal transduction pathway that confers fluoride sensitivity on *C. elegans*. A mutation in any of the class 1 genes blocks the pathway and accumulates an intermediate

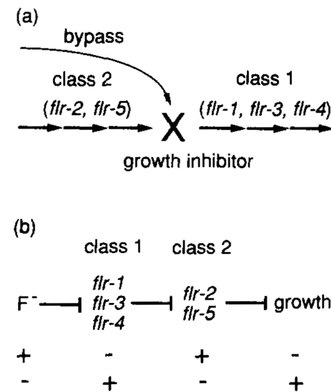

FIGURE 2.—Two models that can explain the epistasis between class 1 and class 2 mutations. (a) A hypothetical pathway that confers fluoride resistance on *C. elegans*. It may concern metabolism, transport or signal transduction and can explain complex epistasis between the fluoride-resistant mutations. The intermediate of the pathway shown as "X," which accumulates by a mutation in *flr-1*, *flr-3* or *flr-4*, has a growth-inhibitory activity. The orders of action of genes in the parentheses have not been determined. (b) A switch circuit that regulates the growth rate. "+" shows presence, active state and normal growth, whereas "−" shows absence, inactive state and slow growth for fluoride ion, genes (or gene products) and growth, respectively. Each component negatively regulates the one on the adjacent right. Action of a high concentration of fluoride ion on the class 1 gene products causes death of worms (not shown in the figure).

that decreases the growth rate of worms without killing them. A mutation in one of the class 2 genes blocks the pathway somewhere upstream of the intermediate and prevents its accumulation. In class 1-class 2 double mutants the intermediate does not accumulate and the pathway is still blocked. Hence, they grow at normal rate and are strongly fluoride resistant. A bypass to the intermediate was assumed to explain the weak fluoride resistance of class 2 mutations as compared with the strong fluoride resistance of class 1 mutations.

In model (b) the *flr* genes form a switching circuit in which each step negatively regulates the following step. This circuit can explain epistasis between class 1 and class 2 genes with respect to the growth rate. Combined action of the products of class 2 genes (*flr-2* and *flr-5*) decreases the growth rate of worms, but in wild-type worms this action is neutralized by the products of class 1 genes (*flr-1*, *flr-3* and *flr-4*). Fluoride ion acts either directly or indirectly on the class 1 gene products and interferes with the neutralizing activity. However, to explain the epistasis concerning degree of fluoride resistance, it is necessary to assume that action of a high concentration of fluoride ion on the class 1 gene products causes death of worms (not shown in the figure).

The pathway shown in Figure 2a resembles that of acetylcholine of *C. elegans* in the logical structure. Triple mutations in all the three acetylcholine esterase genes, *ace-1; ace-2; ace-3* block degradation and accumulate an excess amount of acetylcholine around the post-synaptic membrane, which causes death of worms at early larval stage. Introduction of a strongly defective mutation upstream in the pathway, either in the

synthesis (*cha-1*) or in the release from the pre-synaptic membrane (*unc-17*), suppresses the lethal phenotype most probably by decreasing the amount of acetylcholine around the post-synaptic membrane (C. D. JOHNSON and J. B. RAND, cited in CHALFIE and WHITE 1988).

Although the schemes in Figure 2, a and b, may look different from each other, the same mechanism can be interpreted in both ways. In Figure 2a the action of class 1 genes, which decreases the amount of "X," neutralizes the action of class 2 genes, which increases the amount of "X." This is identical to the relation in which class 1 genes negatively regulates the function of class 2 genes.

The class 1 fluoride-resistant mutations have properties that make them suitable as genetic markers in various experiments. First, they do not interfere with the mating activity of males. Second, the slow-growing phenotype of *flr-1, 3* and *4* is easily discernible, even if they are combined with other mutations such as *dpy* or *unc*. Finally, selection methods in both directions are available: Flr$^+$ to Flr (selection on plates containing 400 μg/ml NaF) and Flr to Flr$^+$ (sequential hypochlorite treatment). Thus, they may have general utility for mapping other genes; as well, fluoride resistance may prove a useful system for mutagenesis studies.

The mechanism of action of fluoride ion has been discussed in the introductory section. This work itself does not give much information in this aspect. However, we recently cloned *flr-3* by the transposon-tagging method and found sequence homology to all the conserved protein kinase catalytic domains (M. KAWAKAMI, T. ISHIHARA and I. KATSURA, unpublished data). We therefore think that the *flr* genes are probably members of a signal transduction system. We hope further studies of the mutants isolated in this study will reveal a new signal transduction system that controls the growth of *C. elegans*.

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