Genes Required for the Engulfment of Cell Corpses During Programmed Cell Death in *Caenorhabditis elegans*

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ABSTRACT

After programmed cell death, a cell corpse is engulfed and quickly degraded by a neighboring cell. For degradation to occur, engulfing cells must recognize, phagocytose and digest the corpses of dying cells. Previously, three genes were known to be involved in eliminating cell corpses in the nematode *Caenorhabditis elegans*: *ced-1*, *ced-2* and *nuc-1*. We have identified five new genes that play a role in this process: *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10*. Electron microscopic studies reveal that mutations in each of these genes prevent engulfment, indicating that these genes are needed either for the recognition of corpses by other cells or for the initiation of phagocytosis. Based upon our study of double mutants, these genes can be divided into two sets. Animals with mutations in only one of these sets of genes have relatively few unengulfed cell corpses. By contrast, animals with mutations in both sets of genes have many unengulfed corpses. These observations suggest that these two sets of genes are involved in distinct and partially redundant processes that act in the engulfment of cell corpses.

CELL death plays an important role in the normal development of most animals (SAUNDERS 1966; COWAN *et al.* 1984; TRUMAN 1984; ELLIS, YUAN and HORVITZ 1992). Usually the corpses of dead cells are quickly eliminated from the body, because they are engulfed and degraded by other cells (*e.g.* STOCKER, EDWARDS and TRUMAN 1978; WYLLIE, KERR and CURRIE 1980; LOCKSHIN 1981). Because dying cells, but not living cells, are engulfed, there must be signals that mark cell corpses for engulfment. To understand how cell corpses are recognized by other cells, and how the process of phagocytosis is initiated, we have begun a genetic analysis of engulfment in the nematode *Caenorhabditis elegans*.

Programmed cell death plays a major role in the development of a *C. elegans* hermaphrodite–of the 1090 cells formed, 131 undergo cell death (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). These programmed cell deaths require the products of the genes *ced-3* and *ced-4*, which act within cells that die to cause their deaths (ELLIS and HORVITZ 1986; YUAN and HORVITZ 1990). The dying cells are quickly engulfed and degraded by neighboring cells, and their corpses remain visible for only a few minutes (SULSTON and HORVITZ 1977; ROBERTSON and THOMSON 1982; SULSTON *et al.* 1983). The process of engulfment can begin even before a cell that will die has been completely separated from its sister by cell division. As engulfment proceeds, the dying cell splits into membrane-bound fragments, and the nuclear membrane degenerates. The last recognizable features of the dead cell are whorls of membrane contained in vacuoles within the engulfing cell. After dead cells have been engulfed, a nuclease controlled by the gene *nuc-1* digests the DNA of the dead cell (SULSTON 1976; HEDGECOCK, SULSTON and THOMSON 1983), and the remaining cellular debris is degraded.

Mutations in the genes *ced-1* and *ced-2* prevent dead cells from being engulfed by neighboring cells, and in these mutants cell corpses remain visible for many hours (HEDGECOCK, SULSTON and THOMSON 1983). That cells die in the absence of engulfment indicates that the engulfment process is not the cause of most programmed cell deaths. The single previously known *ced-2* allele has a maternal effect. Since earlier mutant hunts could not efficiently detect maternal-effect mutations that prevent the degradation of cell corpses, we developed a screen to identify more such mutations. In this manuscript, we describe the isolation and characterization of 24 new mutations that affect the engulfment of cell corpses generated by programmed cell death in *C. elegans*.

MATERIALS AND METHODS

Strain maintenance and nomenclature: Techniques for culturing *C. elegans* are described by BRENNER (1974). Unless otherwise indicated, strains were grown at 20°. *C. elegans* genetic nomenclature is described by HORVITZ *et al.* (1979).

Strains used: The wild-type parent of most of the strains we used is the *C. elegans* Bristol strain N2 (BRENNER 1974). However, some strains contain the *ced-1(n1506)* mutation, which was isolated in a strain derived from TR679 (COLLINS, SAARI and ANDERSON 1987; FINNEY 1987). We also used strains containing the following mutations: LG*I*: *sem-4(n1378)* (M. STERN, personal communication), *ces-1(n703)* (ELLIS and HORVITZ 1991), *unc-75(e950)*, *ced-1(e1735)* (HEDGECOCK, SULSTON and THOMSON 1983), *unc-59(e261)*; LG*II*: *clr-1(e1745)* (E. HEDGECOCK, personal communication); LG*III*: *dpy-17(e164)*, *lon-1(e185)*, *lon-1(e1820)*, *daf-4(e1364)*

(RIDDLE, SWANSON and ALBERT 1981), *sma-3(e491)*, *par-3(it62)* (KEMPHUES *et al.* 1988), *sma-4(e729)*, *sma-2(e502)*, *unc-32(e189)*, *glp-1(q231)* (AUSTIN and KIMBLE 1987), *unc-16(e109)*, *unc-69(e587)*, *unc-50(e306)*, *dpy-18(e364)*; LG*IV*: *dpy-9(e12)*, *ced-2(e1752)* (HEDGECOCK, SULSTON and THOMSON 1983), *lin-1(e1275)* (HORVITZ and SULSTON 1980), *dpy-13(e184)*, *unc-5(e53)*, *unc-24(e138)*, *mec-3(e1338)* (CHALFIE and SULSTON 1981), *let-61(s65)* (ROGALSKI, MOERMAN and BAILLIE 1982), *let-63(s170)* (ROGALSKI, MOERMAN and BAILLIE 1982), *let-69(s684)* (ROGALSKI and BAILLIE 1985), *let-72(s685)* (ROGALSKI and BAILLIE 1985), *let-96(s1112)* (CLARK *et al.* 1988), *let-307(s1171)* (CLARK *et al.* 1988), *let-308(s1705)* (CLARK *et al.* 1988), *let-651(s1185)* (CLARK *et al.* 1988), *him-8(e1489)* (HODGKIN, HORVITZ and BRENNER 1979), *dpy-20(e1282)* (HORVITZ and SULSTON 1980), *unc-22(s7)* (MOERMAN and BAILLIE 1979), *unc-31(e169)*, *unc-30(e191)*, *ced-3(n717)* (ELLIS and HORVITZ 1986); LG*V*: *unc-42(e270)*, *egl-41(n1077)* (DESAI and HORVITZ 1989), *him-5(e1467*, *e1490)* (HODGKIN, HORVITZ and BRENNER 1979); LG *X*: *unc-10(e102)*, *xol-1(y9)* (MILLER *et al.* 1988), *dpy-6(e14)*, *unc-27(e155)*, *unc-9(e101)*. Mutations without a reference are described by BRENNER (1974).

In addition, we used strains containing the following chromosomal aberrations: *nDf17 III* (FINNEY 1987), *sDf2 IV* (MOERMAN and BAILLIE 1981), *sDf64 IV* (D. BAILLIE, personal communication), *nT1(IV;V)* (FERGUSON and HORVITZ 1985), *uDf1 X* (SAVAGE *et al.* 1989), and *yDf5 X* (MILLER *et al.* 1988). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center.

Isolation of new *ced* alleles: We mutagenized *sem-4* hermaphrodites with ethyl methanesulfonate (EMS) as described by BRENNER (1974), and then transferred individual L4 larvae or young adults to 35 mm plates, which were incubated at either 20° or 25°. The *sem-4* mutation prevents worms from laying eggs due to the absence of sex muscles, so that the mother fills with eggs and larvae, forming a bag-of-worms (M. STERN, personal communication). To screen for new *ced* mutants, we transferred 100 to 200 F_2 bags-of-worms from a single P_0 to a microscope slide, prepared for observation by Nomarski optics as described by SULSTON and HORVITZ (1977). The worms were mounted in a drop of M9 salt solution (SULSTON and BRENNER 1974) containing 30 mM NaN_3 (AVERY and HORVITZ 1987) to prevent them from moving. Using Nomarski microscopy (SULSTON and HORVITZ 1977), we screened for bags-of-worms containing eggs or larvae with undegraded cell corpses. Cell corpses are round and highly refractile, and can be quickly identified in embryos or young larvae (HEDGECOCK, SULSTON and THOMSON 1983). Most of the cells that die during embryogenesis do so several hours before morphogenesis is complete and are quickly degraded (SULSTON *et al.* 1983), so we scored only fully developed embryos or young larvae, which normally contain no cell corpses. Mutants were recovered from the slide after the cover slip had been gently pushed to one side.

We screened 13,488 F_2 bags-of-worms by this procedure. These worms were selected randomly from the progeny of about 6,420 F_1 animals. For maternal-effect mutations, only F_2 homozygotes will contain visibly mutant F_3 progeny, so this screen represents 13,488/2 = 6,744 haploid genomes scored out of the total of 12,840 haploid genomes present among the F_1. To correct for the fact that some chromosomes from the F_1 animals would have been homozygous in more than one of the F_2 bags-of-worms we scored, we estimated the probability of our scoring one particular haploid genome out of the 12,840 present among the F_1, given that we screened 6,744 haploid genomes: $1 - (12{,}839/12{,}840)^{6744} = 0.41$. Thus, we actually screened about 0.41(12, 840) = 5,264 haploid genomes for recessive mutations that show a maternal effect. Zygotic mutations can be recognized in both F_2 homozygous and heterozygous bags-of-worms, so this screen represents about (13,488)(3)/(2) = 20,232 haploid genomes scored for zygotic mutations. Correcting for the number of haploid genomes scored more than once indicates that we screened about 10,150 haploid genomes for recessive, zygotic mutations.

In addition, we have characterized three other *ced* mutations: the mutation *n1506* was found by G. GARRIGA in a *mut-2* strain in which several *C. elegans* transposons are active (COLLINS, SAARI and ANDERSON 1987; FINNEY 1987), *n1812* is a spontaneous mutation discovered by C. DESAI in an *egl-41(e1077) him-5(e1467)* strain, and the mutation *n1813* was found by M. STERN in a strain of *sup(n1789)* worms isolated from a γ-ray mutagenesis of *clr-1(e1745)* animals.

Genetic mapping: *ced-1* was mapped to linkage group *I* near the gene *unc-75* by HEDGECOCK, SULSTON and THOMSON (1983). We showed that *ced-1* lies between *unc-75* and *unc-59* by three-factor mapping: 8/10 Unc-75 non-Unc-59 and 3/29 Unc-59 non-Unc-75 recombinant chromosomes from *unc-75 ced-1(n1506) unc-59/+ + +* heterozygotes carried *ced-1(n1506)* (see Figure 3).

The map position of *ced-2* was determined by HEDGECOCK, SULSTON and THOMSON (1983).

The mutation *ced-5(n1812)* was mapped to linkage group *IV* by linkage to *unc-5* (data not shown). The following cross demonstrates that *ced-5* is located between *unc-5* and *dpy-20*: from a *ced-5(n1812)/unc-5 dpy-20* hermaphrodite 2/3 Unc non-Dpy and 2/9 Dpy non-Unc recombinant progeny segregated *ced-5*. A four-factor cross demonstrates that *ced-5* must lie very near the gene *mec-3*: from a *ced-5(n1812)/unc-24 mec-3 dpy-20* hermaphrodite, 21/21 Unc non-Mec non-Dpy, 0/7 Dpy Mec non-Unc, 0/1 Unc Mec non-Dpy, and 7/7 Dpy non-Mec non-Unc recombinants segregated *ced-5*. Recent work in our laboratory has shown that *ced-5* lies to the right of *mec-3* and to the left of *him-8* (S. GLASS and R. ELLIS, unpublished results). Deficiency mapping places *ced-5* in the region uncovered by *sDf2*, but not uncovered by *sDf64*: 28/28 *ced-5(n1812)/sDf2* animals had unengulfed cell corpses, whereas 12/12 *ced-5(n1812)/sDf64* animals were wild type. The presence of the deficiencies in these strains was confirmed by using 1% nicotine to test for deletion of the nearby gene *unc-22* (MOERMAN and BAILLIE 1979).

The mutation *ced-6(n1813)* was mapped to linkage group *III* by linkage to *unc-32* (data not shown). The following cross confirms this linkage, and demonstrates that *ced-6* lies to the left of *unc-32*: from a *ced-6(n1813)/dpy-17 unc-32* hermaphrodite 1/8 Dpy non-Unc and 9/9 Unc non-Dpy recombinant progeny segregated *ced-6*. A four-factor cross shows that *ced-6* lies between *lon-1* and *sma-3*, near the gene *daf-4*: from a *lon-1(e185) ced-6(n1813) sma-3/daf-4* hermaphrodite 0/16 Sma Daf non-Lon and 3/3 Sma non-Daf non-Lon recombinant chromosomes carried the *ced-6* mutation. The genes *par-3* and *sma-4* are probably located to the right of *ced-6*: from a *ced-6(n1813)/lon-1(e185) par-3* hermaphrodite 1/2 Lon non-Par recombinant animals segregated *ced-6*, and from a *ced-6(n1813)/lon-1 sma-4* hermaphrodite 2/3 Sma non-Lon recombinant animals segregated *ced-6*. Finally, in *lon-1 ced-6(n1813)/nDf17* animals cell corpses are engulfed normally, so *ced-6* is probably not removed by the deletion *nDf17*.

The mutation *ced-7(n1892)* was mapped to linkage group *III* by linkage to *unc-32* (data not shown). The following three-factor cross confirms this linkage, and shows that *ced-7* is located near the gene *unc-32*: from a *ced-7(n1892)/unc-32 dpy-18* hermaphrodite 0/11 Unc non-Dpy and 10/11 Dpy non-Unc recombinant progeny segregated *ced-7*. The following four-factor cross shows that *ced-7* is located to the

right of *glp-1* and to the left of *unc-69*: from a *sma-2 ced-7(n1892) unc-69/glp-1* hermaphrodite 5/6 Sma non-Glp non-Unc and 1/2 Unc Glp non-Sma recombinant chromosomes carried the *ced-7* mutation. Finally, a five-factor cross demonstrated that *ced-7* lies to the left of *unc-16*: from a *ced-7(n1892)/dpy-17 lon-1(e1820) unc-16 unc-50* hermaphrodite 6/8 Dpy non-Unc and 0/10 Unc-16 Unc-50 non-Dpy non-Lon recombinant progeny segregated *ced-7*. The mutation *ced-7(n1893)* is also linked to *unc-32*: from a *ced-7(n1893)/unc-32 dpy-18* hermaphrodite 0/3 Unc non-Dpy and 7/7 Dpy non-Unc recombinant progeny segregated *ced-7*.

The mutation *ced-8(n1891)* was mapped to the X chromosome because it showed sex-linkage (data not shown). The following cross confirms this linkage: from *ced-8(n1891)/dpy-6 unc-9* hermaphrodites 0/7 Dpy non-Unc and 9/9 Unc non-Dpy recombinant progeny segregated *ced-8*. Although we do not know on what side of *dpy-6* the gene *ced-8* is located, it must be very close: from a *ced-8(n1891)/unc-10 xol-1 dpy-6* hermaphrodite 21/21 Unc non-Dpy recombinant progeny segregated *ced-8*, and from a *ced-8(n1891)/dpy-6 unc-27* hermaphrodite 8/8 Unc non-Dpy and 0/9 Dpy non-Unc recombinant progeny segregated *ced-8*. The mutation *ced-8(n1999)* is also linked to *dpy-6*: from a *ced-8(n1999)/dpy-6 unc-9* hermaphrodite 14/14 Unc non-Dpy and 0/3 Dpy non-Unc recombinant progeny segregated *ced-8*. Finally, *unc-42/+; ced-8(n1891)/uDf1* animals segregated only 6/21 Ced eggs, but *unc-42/+; ced-8(n1891)/yDf5* animals segregated 19/19 Ced eggs, showing that *ced-8* is uncovered by *yDf5* but not by *uDf1*.

The mutation *ced-10(n1993)* was mapped to linkage group *IV* by linkage to *unc-5* (data not shown). The following cross confirms this linkage, and shows that *ced-10* lies to the left of *unc-24*: from a *ced-10(n1993)/unc-24 mec-3 dpy-20* hermaphrodite 0/12 Unc non-Dpy and 5/5 Dpy non-Unc recombinant progeny segregated *ced-10*, and two Unc Dpy chromosomes with an unselected recombination event also carried the *ced-10* mutation. Furthermore, *ced-10* lies between *lin-1* and *dpy-13*: from a *ced-10(n1993) him-8/lin-1 dpy-13* heterozygote, 6/12 Lin non-Dpy recombinant progeny segregated *ced-10*.

Complementation tests: In general, we assigned each new *ced* mutation to a complementation group by crossing males mutant for a known *ced* gene with *sem-4* hermaphrodites homozygous for a new *ced* mutation. Because *sem-4* animals cannot lay eggs (M. STERN, personal communication), cross-progeny hermaphrodites laid all the F_2 eggs present on the test plates. We viewed several of these F_2 eggs using Nomarski microscopy to see if the older embryos had unengulfed cell corpses. If the new *ced* mutation fails to complement the *ced* gene being tested, then all the eggs will have unengulfed corpses. Otherwise, many of the F_2 eggs will appear wild-type. Furthermore, if the *ced* mutations complement and both show a maternal effect, then all of the F_2 eggs will appear wild type. In these crosses, all of the mutations tested showed segregation typical of recessive mutations.

In a few complementation tests, *sem-4* was not used as a marker. The mutation *n1893* was assigned to the *ced-7* gene by showing that all of the eggs laid by *n1893/ced-7(n1892)* heterozygotes had unengulfed corpses, and the mutation *n1999* was assigned to the *ced-8* gene by showing that all of the eggs laid by *n1999/ced-8(n1891)* heterozygotes had unengulfed corpses. Besides the *ced* mutations isolated in the *sem-4* mutagenesis, we also characterized three *ced* mutations isolated by colleagues. Based on map position, the mutations *ced-5(n1812)* and *ced-6(n1813)* defined new *ced* genes. The third such mutation, *n1506*, failed to complement *ced-1(e1735)*: all cross-progeny of *ces-1(n703*sd*); ced-1(e1735); him-5* males mated with *ced-1(n1506)* hermaphrodites had unengulfed corpses.

We also tested mutations in each new gene for complementation with mutations in nearby genes (data not shown). The mutation *ced-5(n1812)* complements mutations in the following genes: *mec-3, let-61, let-63, let-69, let-72, let-96, let-307, let-308* and *let-651*. The mutation *ced-6(n1813)* complements mutations in *lon-1, par-3*, and *daf-4*. The mutation *ced-7(n1892)* complements mutations in *unc-16* and *glp-1*. Finally, the mutation *ced-8(n1891)* complements mutations in *xol-1, dpy-6* and *unc-27*.

Strain constructions: To build double mutants using the various *ced* genes, we relied on two different approaches. In some cases we made the appropriate double heterozygotes, and then screened their offspring for animals with an increased number of undegraded corpses. We used this technique only when we knew or suspected that two *ced* genes would combine to have a much stronger effect on the engulfment of cell corpses than either single mutant alone. If we could not screen for an enhanced phenotype, we used marker mutations, either in *cis* or in *trans* to one of the *ced* genes, to aid in building the double mutants. In all cases where the phenotype of a double mutant was not different from that of the single mutants, we used complementation tests to confirm the genotype of the putative double mutant strains. The fact that the *ced-1* and *ced-8* genes do not show maternal effects aided in these strain constructions by allowing us to identify animals homozygous for either *ced-1* or *ced-8* one generation before a Ced phenotype is expressed by mutants for any of the other genes. As an example, to construct the *ced-1; ced-7* double mutant we used the *unc-32* gene as a *cis* marker for the presence of *ced-7*. From *ced-1/+; unc-32 ced-7/+ +* heterozygotes we isolated Ced progeny, which must be homozygous for *ced-1* because the *ced-7* gene shows a maternal effect. In the next generation we isolated an Unc animal, which was putatively *ced-1; unc-32 ced-7*, and verified its genotype by complementation tests. We used similar techniques to construct triple mutants.

Cell corpse assays: To study the number and distribution of cell corpses in the pharynges of *ced* mutants, we anesthetized the worms by mounting them in a drop of 30 mM NaN_3, and then examined the animals using Nomarski microscopy, as described above. Except where stated otherwise, we scored only young L1 animals with four cells in the gonad, the number present from hatching until the middle of the first larval stage (KIMBLE and HIRSH 1979). For each animal scored, we noted the exact position in the pharynx of each corpse. From these data we derived the average number of corpses in the pharynx for each strain, and also the frequency at which NSM sister corpses were present. For mutations with an average number of corpses in the pharynx between 0.4 and 1.2, we also determined how many corpses were present in four separate regions of the pharynx: the procorpus, the metacorpus, the isthmus, and the terminal bulb (ALBERTSON and THOMSON 1976), as well as how often the NSM sister corpses were preserved (see Figure 6). The 95% confidence limits for the average number of corpses were determined by the *t*-test, performed with the *StatView II* program (Abacus Concepts, Inc., Berkeley, California).

In our cell corpses assays shown in Tables 5 and 6, the following strains contained additional mutations besides the two *ced* mutations indicated:

ced-1(e1735); unc-32 ced-7(n1892)
ced-1(e1735); ced-8(n1891) unc-27
dpy-9 ced-2(e1752) ced-5(n1812) unc-30
ced-2(e1752); ced-8(n1891) unc-27

dpy-9 ced-2(e1752) ced-10(n1993) him-8
lon-1(e185) ced-6(n1813); ced-5(n1812)
ced-5(n1812); ced-8(n1891) unc-27
lon-1(e185) ced-6(n1813) unc-32 ced-7(n1892)
ced-6(n1813); ced-8(n1891) unc-27
lon-1(e185) ced-6(n1813); ced-8(n2093)
lon-1(e185) ced-6(n1813); ced-10(n1993)
unc-32 ced-7(n1892); ced-8(n1891)
unc-32 ced-7(n1892); ced-8(n2093)
unc-32 ced-7(n1892); ced-10(n1993)
ced-10(n1993); ced-8(n1891) unc-27

In the assays described in Table 6 we used these strains:

ced-1(e1735); unc-32 ced-7(n1892); ced-5(n1812)
ced-1(e1735); unc-32 ced-7(n1892); ced-8(n1891)
unc-32 ced-7(n1892); ced-5(n1812); ced-8(n1891)
lon-1(e185) ced-6(n1813); dpy-9 ced-2(e1752) ced-5(n1812) unc-30
lon-1(e185) ced-6(n1813) unc-32 ced-7(n1892); ced-8(n2093).

Electron microscopy: We used Nomarski optics to select worms of an appropriate age that contained several undegraded cell corpses. These worms were recovered from the slide, immobilized by chilling on ice, and decapitated with a razor blade to improve subsequent fixations. The worms were fixed in 2% glutaraldehyde and 2% paraformaldehyde, 0.1 M Na cacodylate (pH 7.4) at 20° for one hour (KARNOVSKY 1965), then rinsed twice in 0.1 M Na cacodylate (pH 7.4), and placed in 1% osmium tetroxide, 0.1 M Na cacodylate (pH 7.4) at 4° for 1 hr. The worms were stained *en bloc* for 2 hr at 4° in 2% uranyl acetate, and subsequently dehydrated through an ethanol series and embedded in Epon 812. We collected pale gold sections, stained them with uranyl acetate and lead citrate (ROBERTSON and THOMSON 1982), and examined them with a JEOL JEM-1200EXII transmission electron microscope.

Laser microsurgery: Laser microsurgery (SULSTON and WHITE 1980) was performed using an optical system similar to that used by STERNBERG and HORVITZ (1981). Specifically, we used a VSL-DYE (mirror) dye laser excited by a VSL-337 nitrogen laser (LaserScience, Inc., Cambridge), as described by AVERY and HORVITZ (1987).

Most living cells can absorb several pulses of a laser microbeam before they are killed, and irradiated cells very rarely burst even when dying. For example, when we irradiated eight HSN neurons (WHITE *et al.* 1986) with five pulses each from our laser microbeam, none of the cells exploded and two of the eight cells survived. Under identical conditions, the random cell corpses we tested in the head and pharynx usually exploded when we fired a single pulse of a laser beam at the cell surface.

RESULTS

***ced-2* shows a maternal-rescue effect:** H. ELLIS (personal communication) observed that the single previously known *ced-2* allele, *e1752*, shows a maternal-rescue effect: homozygous *ced-2* progeny of a homozygous *ced-2* mother have unengulfed cell corpses, but homozygous *ced-2* progeny of a *ced-2/+* mother appear wild type, as do all *ced-2/+* animals themselves (Figure 1). Mutations in the gene *ced-1* do not show any maternal effects. These results suggested

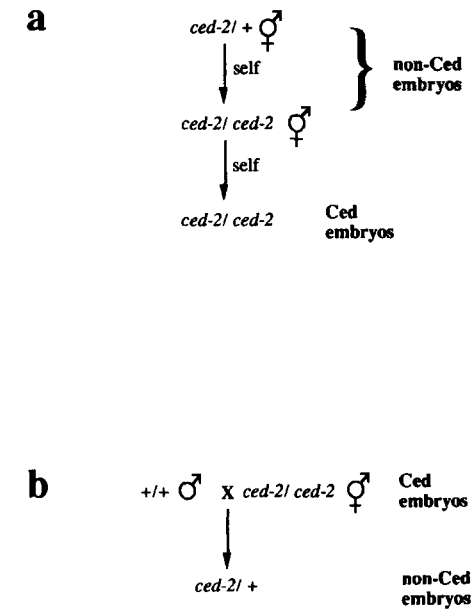

FIGURE 1.—*ced-2* mutations show a maternal-rescue effect. (a) *ced-2* homozygotes from a *ced-2/+* mother appear wild type. (b) *ced-2/+* progeny from a *ced-2* mother also appear wild type. In the zygote a *ced-2(+)* allele contributed by the father is sufficient to rescue the mutant phenotype, so *ced-2* mutations do not show a strict maternal effect.

that other genes like *ced-2* might not have been identified in previous screens for *ced* mutants, because these screens were of F_2 progeny from mutagenized animals, and maternal-effect mutations can be recognized only in the following generation.

Isolation of new *ced* mutations: Because living animals with undegraded cell corpses can be recognized using Nomarski optics but appear normal in general morphology and behavior (HEDGECOCK, SULSTON and THOMSON 1983), we used Nomarski microscopy to screen for new *ced* mutants. To allow the identification of both maternal-effect and zygotic mutations, we examined the F_3 offspring of individual F_2 animals after EMS mutagenesis. We mutagenized *sem-4* animals, which are unable to lay eggs because they lack sex muscles (M. STERN, personal communication); these animals eventually fill with eggs and newly hatched larvae and die, forming bags-of-worms (Figure 2). If an F_2 animal is homozygous for a maternal-effect mutation, then all of the F_3 progeny within that F_2 bag-of-worms will show the mutant phenotype.

This protocol has several advantages. (1) Mutations that are weak or that show incomplete penetrance can be identified reliably, because several siblings within a bag-of-worms will show the mutant phenotype. (2) In older embryos and very young larvae undegraded cell corpses are particularly prominent. Individuals of both of these stages can be easily examined, because the F_3 population within a single F_2 bag-of-worms comprises a range of ages. (3) The body of the mother protects the young animals during screening and recovery from the microscope slide, reducing the chance

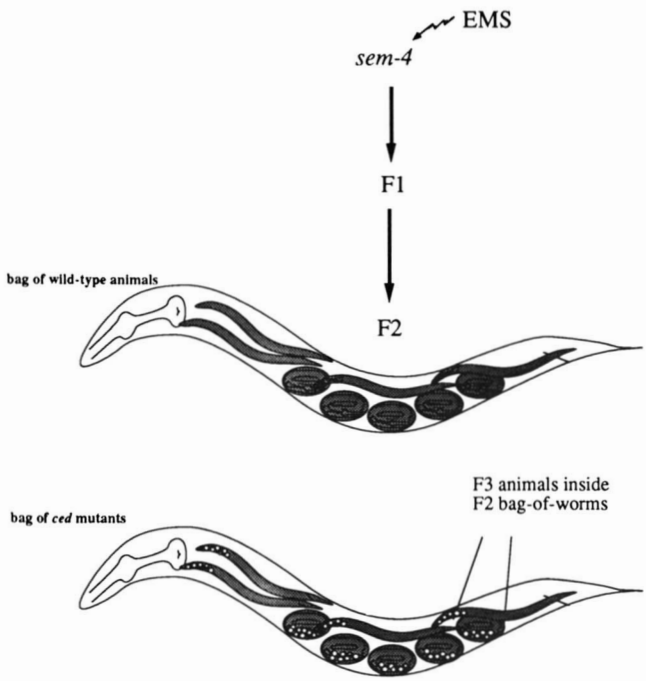

FIGURE 2.—The screen for new mutations affecting the degradation of cell corpses. After mutagenizing *sem-4* animals with EMS, we used Nomarski microscopy to examine individual F_3 animals, held within the body of their mother, for undegraded cell corpses. All of the F_2 animals are generated either by +/+ F_1 mothers, or by rare *ced*/+ F_1 animals carrying a mutation in one of the *ced* genes. If a new mutation shows a maternal effect, the *ced*/*ced* F_2 animals will themselves appear wild type, but their progeny contained within the bag-of-worms will all have undegraded cell corpses. If a new *ced* mutation is zygotic, *ced*/+ F_2 bags-of-worms will contain about 25% F_3 animals with undegraded cell corpses, and *ced*/*ced* F_2 bags-of-worms will contain 100% F_3 animals with undegraded cell corpses.

of killing new mutants. (4) Mutant strains can be easily mated and analyzed genetically, because the *sem-4* mutation blocks egg-laying by preventing sex muscle development; by contrast, many other mutations that cause animals to become bags-of-worms block development of the vulva, which is required for mating (FERGUSON and HORVITZ 1985).

We screened the F_3 progeny within 13,488 F_2 bags-of-worms after EMS mutagenesis of *sem-4* animals. This screen represents about 5,000 haploid genomes examined for mutations that show a recessive maternal-effect phenotype. Such mutations must be homozygous in F_2 animals to be detected. This screen also represents about 10,000 haploid genomes examined for recessive zygotic mutations, which can be detected in progeny from either homozygous or heterozygous F_2 animals (see MATERIALS AND METHODS). From this screen we isolated 21 mutations that prevent the degradation of cell corpses. A few additional strains might have carried mutations with very weak effects on the degradation of cell corpses, but the phenotypes of these strains were difficult to score and the strains were therefore discarded. We also studied three *ced* mutations isolated by other members of our laboratory (see MATERIALS AND METHODS).

TABLE 1

New *ced* mutations

Gene	Alleles
ced-1	*n1506, n1995, n2000, n2089, n2091, n2092*
ced-2	*n1994*
ced-5	*n1812, n2002*
ced-6	*n1813, n2095*
ced-7	*n1892, n1893, n1996, n1997, n1998, n2001, n2094, n2096*
ced-8	*n1891, n1999, n2090, n2093*
ced-10	*n1993*

The 24 *ced* mutations we have characterized are all recessive. Complementation tests (see MATERIALS AND METHODS) show that these mutations define seven genes–the previously known genes *ced-1* and *ced-2* (HEDGECOCK, SULSTON and THOMSON 1983), and the new genes *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10*.

We have characterized these 24 mutations. All are recessive, and the homozygotes are viable. These mutations define seven complementation groups, which include the previously known genes *ced-1* and *ced-2*, and five new genes, *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10* (Table 1, MATERIALS AND METHODS). The map locations of these genes are shown in Figure 3.

Phenotypes of the new mutants: Each of the 24 new mutations we studied prevents many cell corpses from being degraded (*e.g.*, Figure 4). In each of the mutants the appearance of the corpses, as visualized using Nomarski optics, is the same as in the *ced-1* and *ced-2* mutants isolated by HEDGECOCK, SULSTON and THOMSON (1983). These corpses resemble those seen in wild-type animals during the stage of programmed cell death immediately before degradation (SULSTON and HORVITZ 1977). Occasionally, older corpses in these mutants contain vacuoles, lose the shiny appearance of fresh corpses, or even seem to be slowly disintegrating.

For each *ced* mutation, we examined the progeny of *ced*/+ heterozygotes, and determined the proportion that contain undegraded cell corpses (Table 2). About one fourth of the progeny of each *ced-1* or *ced-8* heterozygote appear mutant, indicating that these mutations are recessive and that the wild-type gene is required in the zygote. By contrast, all alleles of the genes *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* show a maternal-rescue effect–almost all of the offspring from these heterozygotes appear wild type.

The new *ced* mutations prevent engulfment: The new *ced* mutations might prevent either the engulfment of cell corpses or the degradation of cell corpses after they are engulfed. To distinguish between these alternatives, we used electron microscopy to examine cell corpses in *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10* mutants. Because previous ultrastructural analysis of cell death in wild-type *C. elegans* focused on cells that

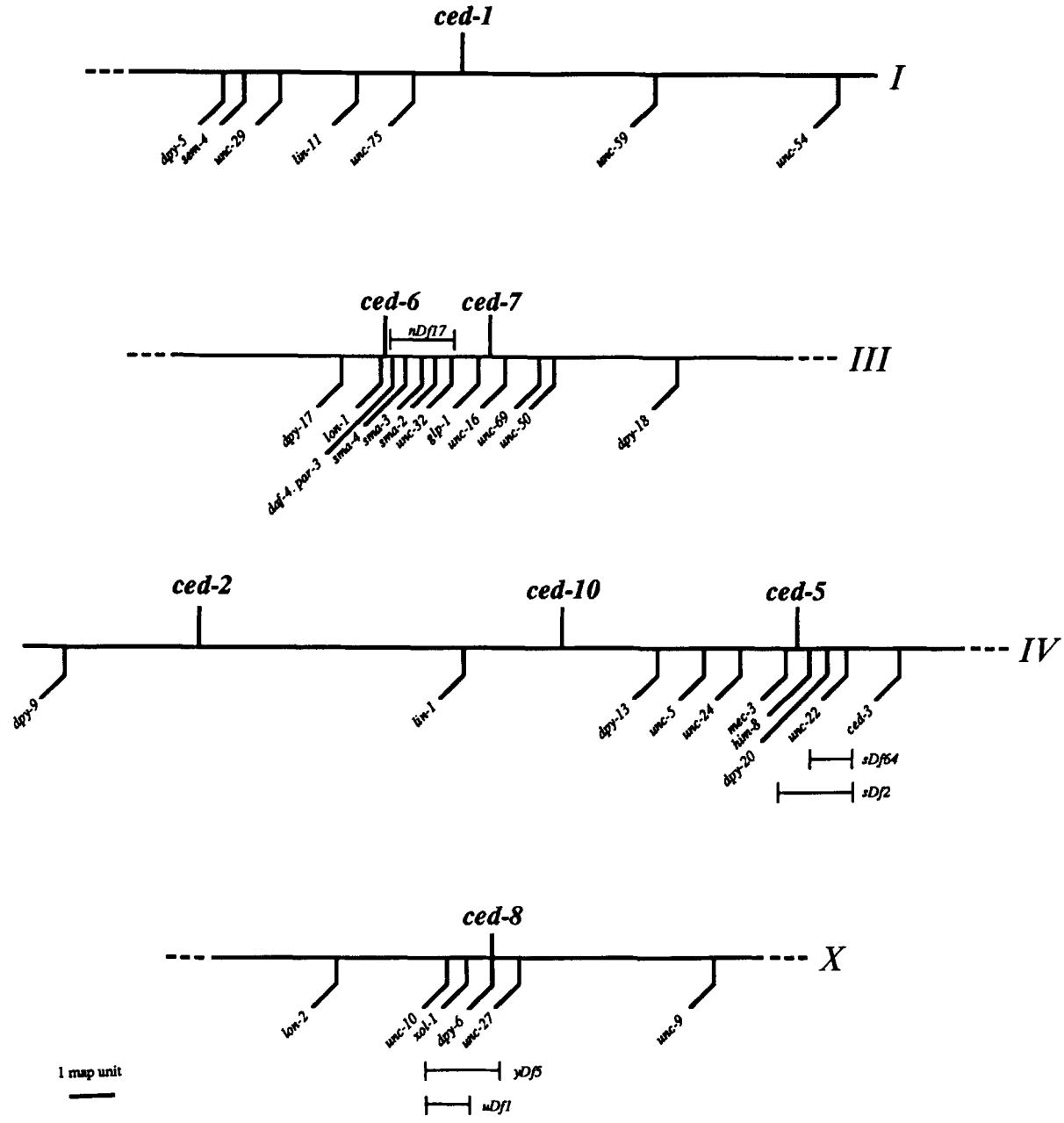

FIGURE 3.—A genetic map showing portions of linkage groups *I*, *III*, *IV* and *X*. The positions of the *ced* genes involved in the engulfment of cell corpses are based upon data presented in MATERIALS AND METHODS.

die in the ventral nervous system during larval development (ROBERTSON and THOMSON 1982), we fixed late L1 and early L2 larvae and examined dying cells descended from the ventral cord precursor cells P9 through P12. Some of the corpses we examined were in early stages of cell death, whereas others had remained undegraded for several hours. None of the dead cells we observed had been engulfed in the *ced-5(n1812)*, *ced-6(n1813)*, *ced-7(n1892)* and *ced-10(n1993)* mutants, whereas all of the corpses found in wild-type animals were being engulfed normally (n = 8, 4, 4, 3 and 6 corpses, respectively; see Figure 5 for representative photographs).

Undegraded corpses are visible in *ced-8* embryos viewed with Nomarski optics, but not in L2 larvae, so it is possible that *ced-8* does not affect postembryonic cell deaths (see below). We therefore examined both embryos and larvae of *ced-8(n1891)* mutants by electron microscopy. Of five undegraded embryonic corpses visible in the heads of newly hatched *ced-8* animals, none had been engulfed. By contrast, the two corpses we examined in the ventral cords of *ced-8* larvae were being engulfed normally. These observations indicate that mutations in *ced-8* affect the engulfment of cell corpses during embryogenesis. We therefore conclude that all of our newly discovered *ced* genes, like *ced-1* and *ced-2* (HEDGECOCK, SULSTON and THOMSON 1983), affect the process of engulfment rather than a later step of degradation.

Expressivity of the *ced* mutations: All alleles of

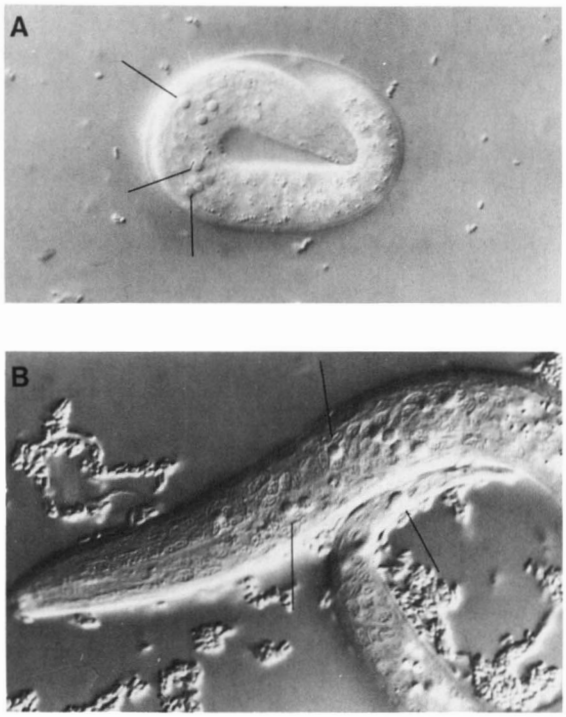

FIGURE 4.—Two Nomarski photomicrographs of a representative mutant defective in the degradation of cell corpses. (A) a *ced-5(n1812)* embryo, and (B) a *ced-5(n1812)* L1 larvae. Many undegraded cell corpses are visible as bright, round refractile objects. Some of these corpses are indicated with lines.

these seven *ced* genes appear fully penetrant; in each animal some undegraded corpses are visible in the head during late embryogenesis. By contrast, 8 of the 10 wild-type embryos we examined contained no cell corpses in this region, and the remaining two embryos each had only a single corpse. However, the expressivity of each *ced* mutation appeared low and varied from animal to animal. Although 71 cells die during development of the head (SULSTON *et al.* 1983), preliminary observations revealed an average of around 30 corpses in the heads of late embryos of the strongest mutant strains, and an average of 2 to 3 such corpses in embryos of the weakest mutant strains (*e.g.*, see Table 9). To quantify the effects of the *ced* mutations on the engulfment of cell corpses, and also to look for cell-specific effects of mutations in the *ced* genes, we focused on cell deaths in one organ of the animal–the pharynx (Figure 6).

The pharynx has several advantages for these studies: (1) Only 22 cells die during pharyngeal development, which is a small and easily countable number. (2) Almost all cell deaths in the pharynx occur within a two hour period during mid-embryogenesis (SULSTON *et al.* 1983). By counting the number of corpses present in L1 larvae shortly after hatching (about nine hours after the pharyngeal cells die), we can study the effects of the different *ced* mutations on a variety of cell corpses after essentially the same interval of time has passed for each corpse. (3) The pharynx is surrounded by a basement membrane, and has a well

TABLE 2

Many *ced* mutations show maternal-rescue effects

Zygotic mutations			Maternal-rescue mutations		
Gene	Allele	Percent Ced eggs from *ced*/+ parents (*n*)	Gene	Allele	Percent Ced eggs from *ced*/+ parents (*n*)
ced-1	*n2089*	25 (60)	*ced-2*	*e1752*	0 (56)
	n1506	26 (46)		*n1994*	4 (27)
	e1735	26 (42)			
	n691	24 (25)	*ced-5*	*n1812*	1 (127)
	n2092	15 (102)		*n2002*	0 (27)
	n2091	29 (56)			
	n2000	33 (36)	*ced-6*	*n2095*	0 (60)
	n1951	29 (28)		*n1813*	2 (235)
	n1995	15 (34)			
			ced-7	*n1892*	2 (155)
ced-8	*n2093*	17 (97)		*n1997*	0 (23)
	n2090	26 (91)		*n1998*	0 (24)
	n1891	20 (30)		*n2096*	0 (60)
	n1999	20 (45)		*n2001*	0 (24)
				n2094	1 (68)
				n1996	0 (23)
				n1893	0 (17)
			ced-10	*n1993*	2 (41)

To determine which mutations show a maternal effect, we used Nomarski microscopy to count the number of eggs (produced by self-fertilization from *ced*/+ hermaphrodites) that had unengulfed cell corpses. All eggs produced by self-fertilization from *ced/ced* hermaphrodites appear mutant (data not shown). We include data for the mutations *ced-1(e1735)* and *ced-2(e1752)*, previously characterized by HEDGECOCK, SULSTON and THOMSON (1983); *ced-1(n691)*, a spontaneous mutation (H. ELLIS, personal communication); and *ced-1(n1951)* a mutation we isolated in a separate EMS mutagenesis experiment.

defined structure (Figure 6; ALBERTSON and THOMSON 1976), making it easy to observe the distribution of cell corpses. (4) Although it is difficult to determine the exact identity of most embryonic corpses, because of their characteristic positions we can reliably identify the corpses formed by the deaths of two specific cells in the pharynx, the sisters of the two NSM neurons (ELLIS and HORVITZ 1991).

To measure the expressivity of each *ced* mutation we developed two assays using the pharynx. First, we determined the average number of cell corpses found in the pharynges of young larvae. Second, we studied the effects of these mutations on a specific pair of dying cells, by determining the frequencies at which the NSM sister corpses remain undegraded. Table 3 shows the results of these two assays.

The most striking feature of Table 3 is that all of the *ced* mutations are very low in expressivity. These mutants contain 0–4 corpses per pharynx at hatching, far fewer than the total of 22 corpses formed as the pharynx develops. Furthermore, the NSM sister corpses are engulfed most or all of the time in these *ced* mutants. HEDGECOCK, SULSTON and THOMSON (1983) previously noted that many cells were still engulfed in *ced-1* and *ced-2* mutants. These results suggest that (1) none of these mutations completely

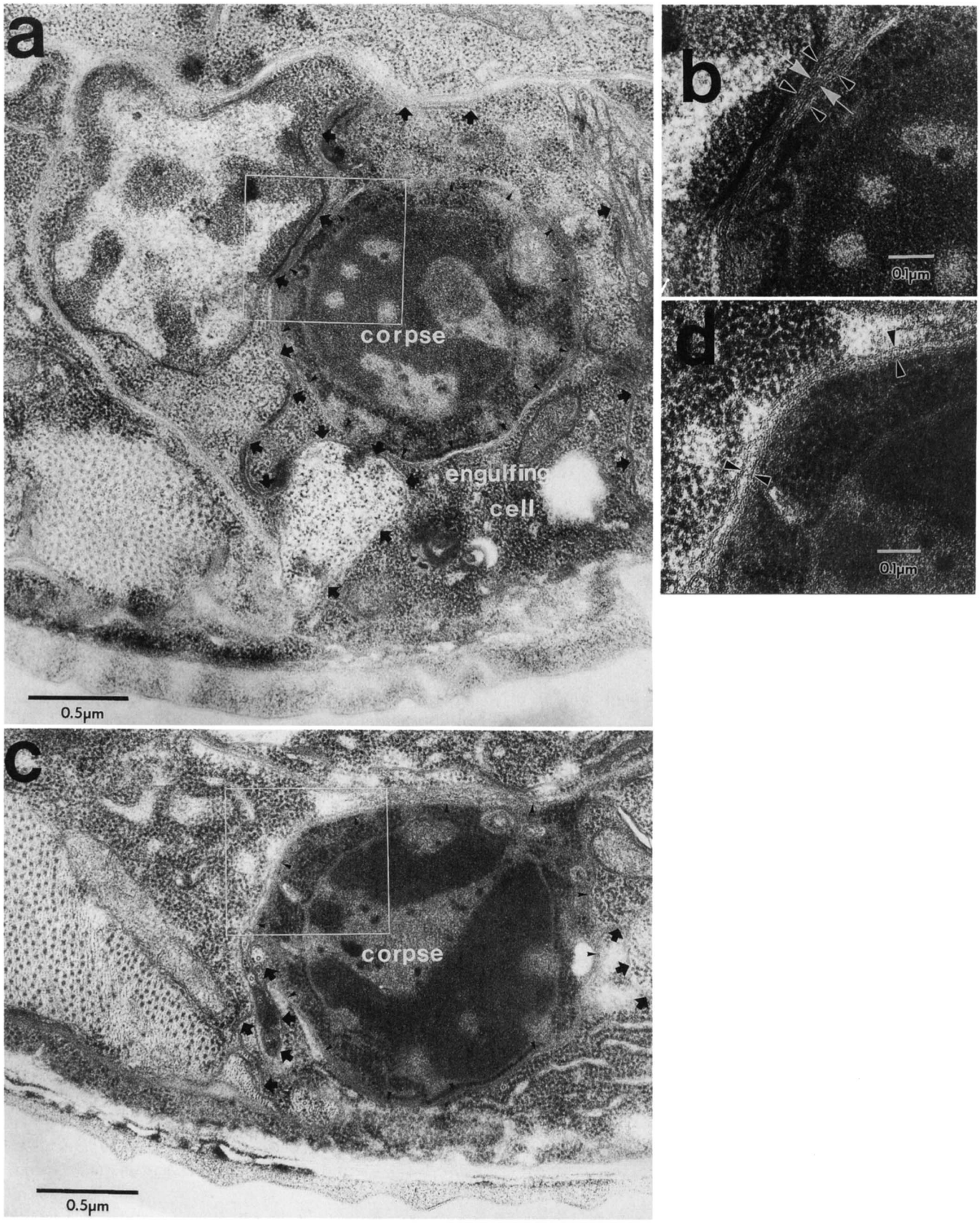

FIGURE 5.—Electron micrographs of cell corpses in wild-type and *ced-10* animals. (a) An engulfed corpse in a wild-type larva at about the time of the L1 molt. The corpse is P11.aap (SULSTON and HORVITZ 1977), which is engulfed by the hypodermis (ROBERTSON and THOMSON 1982). The ventral cuticle is visible at the bottom of the micrograph. The membrane of the corpse is indicated with small arrowheads, and the membrane of the engulfing pseudopod from the hypodermal cell is marked with black arrows where it faces neighboring cells. The

TABLE 3

Expressivities of the *ced* mutants

Gene	Allele	Total corpses in the pharynx	Undegraded NSM sister corpses (%)
ced-1	*n2089*	1.0 ± 0.2	0
	n1506	0.9 ± 0.2	0
	e1735	0.8 ± 0.2	0
	n691	0.8 ± 0.2	0
	n2092	0.7 ± 0.2	0
	n2091	0.6 ± 0.2	0
	n2000	0.6 ± 0.2	0
	n1951	0.4 ± 0.1	0
	n1995	0.04 ± 0.04	0
ced-2	*e1752*	1.4 ± 0.2	2
	n1994	1.4 ± 0.3	3
ced-5	*n1812*	3.9 ± 0.3	28
	n2002	3.8 ± 0.4	14
ced-6	*n2095*	0.5 ± 0.1	0
	n1813	0.4 ± 0.1	0
ced-7	*n1892*	2.7 ± 0.3	1
	n1997	1.6 ± 0.3	0
	n1998	1.6 ± 0.3	2
	n2096	1.4 ± 0.3	0
	n2001	1.2 ± 0.2	0
	n2094	1.1 ± 0.2	1
	n1996	0.7 ± 0.2	1
	n1893	0.07 ± 0.08	0
ced-8	*n2093*	1.0 ± 0.2	5
	n2090	0.7 ± 0.2	5
	n1891	0.7 ± 0.1	2
	n1999	0.5 ± 0.1	1
ced-10	*n1993*	1.9 ± 0.2	3
Wild type (N2)		0	0

The numbers of corpses present in the pharynges of L1 larvae were assayed as described in MATERIALS AND METHODS. We present the total number of corpses observed (22 cells die during formation of the pharynx), and also the percentage of NSM sister corpses remaining undegraded (two of the cells that die during pharyngeal development are the sisters of the NSM neurons). The data shown for *ced-2(n1994)*, *ced-5(n2002)* and the *ced-7* alleles *n1997*, *n1998*, *n2096* and *n1893* represent the average of 50–60 animals; all other data represent the average of 100 animals. The 95% confidence limits were calculated with the *t*-test, using the *StatView II* program (Abacus Concepts, Inc., Berkeley, California).

eliminates the function of its particular *ced* gene, or (2) there is redundancy in the system for engulfing dead cells, so that more than one gene must be inactivated to prevent all engulfments from occurring, or (3) some corpses might disappear without being engulfed. We consider these possibilities below.

Are any of the *ced* mutations null alleles? The effects of all of the *ced* mutations we describe are recessive, which suggests that each mutation might cause a reduction or loss of gene function. Table 3 reveals that there are significant differences in expressivity among these mutations, and these differences provide hints about which mutations might cause a complete loss of gene function. For example, the *ced-1* alleles form a series, with weak alleles such as *n1951* and *n1995*, and a number of strong alleles. These strong alleles include *ced-1(n1506)*, a potential transposon insertion (our unpublished observations), and *ced-1(n691)*, a spontaneous mutation. Furthermore, we isolated five *ced-1* mutations from a screen of about 10,000 haploid genomes, a frequency equivalent to the value of 1/2,000 expected for loss-of-function mutations in typical *C. elegans* genes (BRENNER 1974; MENEELY and HERMAN 1979; GREENWALD and HORVITZ 1980). These observations suggest that the strong *ced-1* alleles might result in a complete loss of *ced-1* function. Because many cell corpses are engulfed in strong *ced-1* mutants, the null phenotype of *ced-1* probably is of low expressivity.

We isolated eight *ced-7* mutations from a screen of about 5,000 haploid genomes, so *ced-7* alleles occur very frequently. However, this series of alleles does not end with a group of strong mutations. Rather, *ced-7(n1892)* stands alone as the strongest allele. Thus the *ced-7* gene is very sensitive to mutation, but we do not know which mutations, if any, eliminate *ced-7* function. We recovered four *ced-8* alleles from a screen of about 10,000 haploid genomes, which is also close to the frequency expected for loss-of-function mutations. Furthermore, *ced-8* homozygotes and *ced-8(n1891)/yDf5* animals resemble one another–both live and contain undegraded cell corpses–so it is possible that some *ced-8* alleles completely eliminate gene function. Similarly, *ced-5(n1812)/sDf2* animals live and resemble *ced-5* mutants, so this *ced-5* mutation also might be a null allele. Our data do not indicate if any existing mutations in *ced-2*, *ced-6* and *ced-10* result in partial or complete losses of gene function.

The *ced* genes fall into two functional sets: To determine if some of the *ced* genes that act in the engulfment of cell corpses are functionally redundant, which would account for the low expressivities seen in the single mutants, we built various double mutant combinations. We then measured the expressivity of the Ced phenotype of each of the double mutants, using the assays described above. Our results are summarized in Table 4. We also constructed additional double mutants to test for allele-specific effects. These data, shown in Table 5, indicate that the inter-

engulfing pseudopod is visible in some regions, whereas in other regions it is squeezed between the corpse and a neighboring cell and no cytoplasm can be seen. One such compressed region is enlarged in (b), which shows four parallel membranes, one from the neighboring cell and one from the corpse (indicated with black arrowheads), and two from the engulfing cell (indicated with white arrows). (c) An unengulfed corpse in a *ced-10* larva at about the time of the L1 molt. The corpse is P11.aap, and the ventral cuticle is visible at the bottom of the micrograph. The membrane of the corpse is indicated with small arrowheads, and the membrane of the hypodermal cell is marked with black arrows where it faces neighboring cells. Note that the hypodermal cell has not produced an engulfing pseudopod. (d) An enlargement of (c), showing the membrane of the dead cell next to that of a neighbor, and the absence of membranes from an engulfing pseudopod.

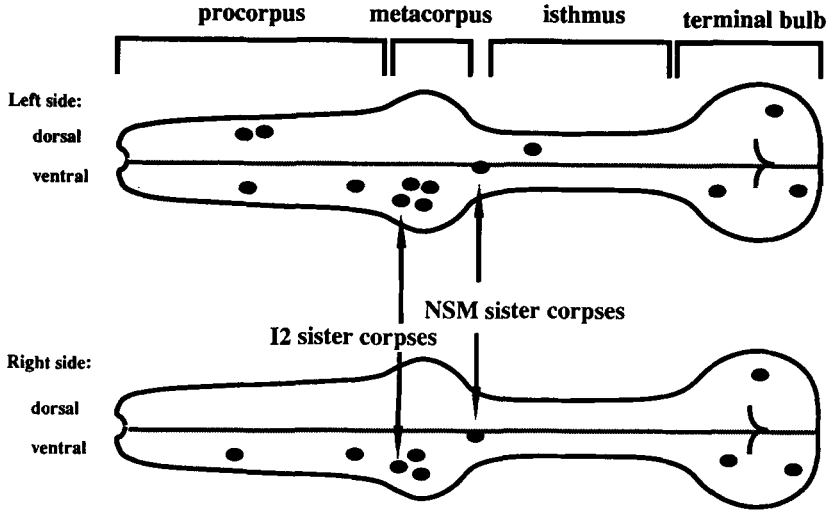

FIGURE 6.—The approximate positions of the cell corpses formed during pharyngeal development. The locations of these corpses vary somewhat, and are drawn based upon (1) the anatomy of the pharynx (ALBERTSON and THOMSON 1976), (2) the cell lineage of the pharynx (SULSTON *et al.* 1983) and (3) our observations of *ced* mutants (data not shown). Because of this variation, the I2 sister corpses cannot be distinguished by location from those of adjacent dying cells. The identification of the NSM sister corpses is described by ELLIS and HORVITZ (1991).

TABLE 4

Expressivities of *ced* double mutants

	ced-1	*ced-6*	*ced-7*	*ced-8*	*ced-2*	*ced-5*	*ced-10*
ced-1(e1735)	0.8 0%	1.4 0%	4.1 3%	3.6 5%	4.5 50%	6.8 52%	4.2 23%
ced-6(n1813)		0.4 0%	2.8 0%	2.3 2%	3.0 22%	5.9 55%	3.6 13%
ced-7(n1892)			2.7 1%	4.1 18%	9.3 47%	10.1 73%	7.7 23%
ced-8(n1891)				0.7 2%	6.2 53%	9.2 58%	7.5 32%
ced-2(e1752)					1.4 2%	3.7 20%	4.3 30%
ced-5(n1812)						3.9 28%	4.4 18%
ced-10(n1993)							1.9 3%

The upper value in each box is the average number of corpses in the pharynges of young L1 larvae, and the lower value represents the percentage of NSM sister corpses remaining undegraded in these animals; the values for each single mutant represent the average of at least 100 animals, and are taken from Table 3. The assays are described in MATERIALS AND METHODS.

actions between different *ced* genes do not depend on the particular alleles we used to construct the strains. The results of both assays show that the expressivities of certain double mutants are much greater than those of any single *ced* mutant. For example, *ced-7; ced-5* worms have an average of 10 corpses in the pharynx, and we have observed individuals of this genotype with up to 17 pharyngeal corpses. By contrast, *ced-7* and *ced-5* single mutants have an average of three to four pharyngeal corpses, with a maximum of seven observed in some individuals. Furthermore, the NSM sister corpses remain unengulfed 73% of the time in the *ced-7; ced-5* double mutant, but only 28% of the time in *ced-5* animals and 1% of the time in *ced-7* animals.

Analysis of the double mutants suggests that two distinct sets of genes might operate in the engulfment of dead cells. Double mutants among *ced-2*, *ced-5* and *ced-10* show the same expressivity as *ced-5* single mutants, whereas double mutants between *ced-2*, *ced-5* or *ced-10*, and *ced-1*, *ced-6*, *ced-7* or *ced-8* generally display a much greater expressivity. This generalization applies to the results of both our assays, but is particularly striking for the data concerning the NSM sister corpses. These results suggest that the genes *ced-2*, *ced-5* and *ced-10* act in one process important for engulfing cell corpses, and that the genes *ced-1*, *ced-6*,

TABLE 5

Expressivities of double mutant combinations are not allele-specific

Genotype	Total corpses in the pharynx	Undegraded NSM sister corpses (%)
ced-1(e1735); ced-5(n1812)	6.8	52
ced-1(n1506); ced-5(n1812)	6.1	60
ced-1(e1735); ced-2(e1752)	4.5	50
ced-1(e1735); ced-2(n1994)	4.4	62
ced-7(n1892); ced-2(e1752)	9.3	47
ced-7(n1892); ced-2(n1994)	9.2	56
ced-2(e1752) ced-5(n1812)	3.7	20
ced-2(n1994) ced-5(n2002)	4.1	15
ced-7(n1892); ced-5(n1812)	10.1	73
ced-7(n1997); ced-5(n1812)	9.7	57
ced-7(n1892); ced-5(n2002)	10.2	65
ced-6(n1813); ced-5(n1812)	5.9	55
ced-6(n2095); ced-5(n1812)	6.1	70
ced-6(n1813); ced-8(n1891)	2.3	2
ced-6(n1813); ced-8(n2093)	2.9	2
ced-7(n1892); ced-8(n1891)	4.1	18
ced-7(n1892); ced-8(n1999)	3.9	23
ced-7(n1892); ced-8(n2093)	2.8	17

Each set represents double mutants containing different alleles of the same two *ced* genes. The assays used are described in MATERIALS AND METHODS. Each value represents the average of 30 animals. The strains used in the studies presented in Table 4 are listed first in each set shown in this table.

ced-7 and *ced-8* act in a second process. Some of the double mutant combinations within the *ced-1*, *ced-6*, *ced-7*, *ced-8* set are stronger than any of the single mutants within this set, which could be either because some of these mutations are not null alleles, or because these four genes do not act in a single process.

Because genotypes with the strongest effects on engulfment do not prevent all 22 corpses formed during development of the pharynx from disappearing, we examined the triple mutants shown in Table 6 to see if any showed an even more severe phenotype. None of these triple mutants is more severe than the strongest double mutants. Instead, each triple mutant appears similar to the strongest double mutant combination that it contains. The fact that triple mutants are not more severe than double mutants supports the idea that there are only two processes of engulfment defined by the seven *ced* genes.

Some cell corpses probably disappear without engulfment: Two observations suggest that cell corpses might occasionally disappear without being engulfed. First, preliminary experiments with a laser microbeam indicate that corpses are more fragile than living cells, suggesting that some unengulfed corpses might burst (see MATERIALS AND METHODS). All that is left of a corpse after destruction with a laser beam is a small amount of debris, similar to material we have sometimes seen where we expect cell corpses in the various *ced* mutants (our unpublished observations). Such debris was not counted in our assays for the number of cell corpses. Second, HEDGECOCK, SULSTON and THOMSON (1983) noticed that some cell corpses separate from the developing embryo and drift into the egg fluid. Based on these two observations, we conclude that some corpses probably disappear in the absence of engulfment, which could explain why we never find the corpses of all dead cells in the severe double and triple mutants we have assayed.

The *ced* genes act on the same corpses, but with different relative effects: Analysis of double mutant combinations suggests that the two engulfment processes do not act on entirely distinct groups of cells. Mutations in each of the *ced* genes show synergistic effects on the engulfment of the NSM sister corpses in some double mutant combinations (Table 4). Therefore all of these genes must play a role in the engulfment of the NSM sister corpses. We also studied the roles of the different *ced* genes in the engulfment of two other pharyngeal corpses, those formed by the sisters of the two I2 neurons. The mutation *ces-1(n703)* prevents both the I2 sisters and the NSM sisters from dying but does not affect other cell deaths (ELLIS and HORVITZ 1991), so we compared *ces-1; ced* double mutants with the corresponding *ced* single mutants (Table 7). When the I2 sisters survive, there are fewer corpses in the metacorpus of all of the *ced* mutants (although the decrease for *ced-8* is small and might not be significant), which indicates that most or all of the *ced* genes affect the engulfment of the I2 sister corpses. As a control, we also measured the number of corpses found in the procorpus, the isthmus, the terminal bulb, and the anterior end of the isthmus, where the NSM sisters die (Figure 6). In general, the control values were unaffected by the presence of a *ces-1* mutation.

Although the *ced* genes do not act on distinct groups of corpses, mutations in the *ced* genes appear to have different relative effects on different corpses. For example, seven cells, including the sisters of the two I2 neurons, die in the metacorpus of the pharynx. In *ces-1; ced-2(e1752)* animals, in which the I2 sisters survive, there is an average of only 0.04 corpses in the metacorpus, whereas *ced-2(e1752)* animals have an average of 1.05 corpses in this region. Thus, in the metacorpus, *ced-2(e1752)* affects mainly the engulfment of the I2 sisters (Table 7). By contrast, *ced-7(n1892)* appears to affect other corpses in the metacorpus in addition to the I2 sisters, because in these mutants a *ces-1* mutation decreases by only half the number of corpses in this location.

To see if the different relative effects on different corpses are gene-specific, we compared the distributions of corpses in the pharynx for several alleles of four *ced* genes. To control for the differences in expressivity among the *ced* mutations, we only analyzed mutations with expressivities similar to those of

TABLE 6

Expressivities of triple mutants

Genotype	Total corpses in the pharynx	Undegraded NSM sister corpses (%)
ced-1(e1735); ced-7(n1892); ced-5(n1812)	11.1	67
ced-7(n1892); ced-5(n1812)	10.1	73
ced-1(e1735); ced-5(n1812)	6.8	52
ced-1(e1735); ced-7(n1892)	4.1	3
ced-1(e1735); ced-7(n1892); ced-8(n1891)	3.9	12
ced-7(n1892); ced-8(n1891)	4.1	18
ced-1(e1735); ced-8(n1891)	3.6	5
ced-1(e1735); ced-7(n1892)	4.1	3
ced-7(n1892); ced-5(n1812); ced-8(n1891)	8.2	78
ced-7(n1892); ced-5(n1812)	10.1	73
ced-5(n1812); ced-8(n1891)	9.2	58
ced-7(n1892); ced-8(n1891)	4.1	18
ced-6(n1813); ced-2(e1752) ced-5(n1812)	5.5	60
ced-6(n1813); ced-5(n1812)	5.9	55
ced-6(n1813); ced-2(e1752)	3.0	22
ced-2(e1752) ced-5(n1812)	3.7	20
ced-6(n1813); ced-7(n1892); ced-8(n2093)	4.5	10
ced-7(n1892); ced-8(n2093)	2.8	17
ced-6(n1813); ced-8(n2093)	2.9	2
ced-6(n1813); ced-7(n1892)	2.8	0

The expressivities of each triple mutant and of its constituent double mutants are listed. Each value represents the average of 30 animals. The assays used are described in MATERIALS AND METHODS.

TABLE 7

Distributions of pharyngeal corpses in *ces-1(n703); ced* double mutants

Genotype	Average number of corpses observed: Procorpus	Metacorpus[a]	NSM sisters	Isthmus	Terminal bulb	Total pharynx
ced-1(e1735)	0.03	0.57	0.00	0.02	0.16	0.8
ces-1 ced-1(e1735)	0.00	0.16	0.00	0.00	0.07	0.2
ced-6(n1813)	0.03	0.29	0.00	0.00	0.03	0.4
ces-1; ces-6(n1813)	0.00	0.07	0.00	0.01	0.06	0.1
ced-7(n1892)	0.32	1.02	0.01	0.01	1.36	2.7
ces-1; ced-7(n1892)	0.20	0.52	0.01	0.01	1.15	1.9
ced-8(n1891)	0.16	0.28	0.03	0.01	0.19	0.7
ces-1; ced-8(n1891)	0.13	0.22	0.01	0.04	0.33	0.7
ced-2(e1752)	0.01	1.05	0.04	0.02	0.32	1.4
ces-1; ced-2(e1752)	0.02	0.04	0.00	0.00	0.25	0.3
ced-5(n1812)	0.42	1.71	0.55	0.13	1.04	3.9
ces-1; ced-5(n1812)	0.27	0.62	0.01	0.09	2.03	3.0
ced-10(n1993)	0.08	1.22	0.05	0.03	0.53	1.9
ces-1; ced-10(n1993)	0.10	0.49	0.01	0.00	0.36	1.0

[a] The metacorpus contains the I2 sisters.

Cell corpse assays are described in MATERIALS AND METHODS, the anatomy of the pharynx is described by ALBERTSON and THOMSON (1976), and a diagram showing the distribution of dying cells in the pharynx is shown in Figure 6. Note that the I2 sister corpses are located in the metacorpus. The total numbers of dying cells in each region are: procorpus (6); metacorpus, which contains the two I2 sisters (7); NSM sisters (2); isthmus (1); and terminal bulb (6). The final column shows the average number of corpses found in the whole pharynx, as presented in Table 3. We scored 100 animals of each genotype for each mutation, except for *ced-8(n1891)*, for which we scored 150 animals.

the four *ced-8* alleles (Table 8). Our results show, for example, that of the mutations listed, those in *ced-8* have the greatest relative effects on the NSM sister corpses, and those in *ced-7* have the greatest effects on corpses in the terminal bulb. In general, the effects on particular corpses of mutations in these seven genes are gene-specific and not allele-specific. There are also overall variations in expressivity among the *ced* genes. For example, all alleles of *ced-1* are weaker than either allele of *ced-2*, and both alleles of *ced-2* are much weaker than either allele of *ced-5* (Table 3).

***ced-7* and *ced-8* show stage-specificity during de-**

TABLE 8

The spatial distributions of corpses in *ced* mutants are gene-specific

Genotype	Average number of corpses observed: Procorpus	Metacorpus[a]	NSM sisters	Isthmus	Terminal bulb	Total pharynx
ced-1(n1506)	0.04	0.77	0.00	0.00	0.12	0.9
ced-1(e1735)	0.03	0.57	0.00	0.02	0.16	0.8
ced-1(n691)	0.00	0.60	0.00	0.00	0.17	0.8
ced-1(n2092)	0.03	0.60	0.00	0.01	0.09	0.7
ced-1(n2091)	0.02	0.51	0.00	0.00	0.11	0.6
ced-1(n2000)	0.01	0.51	0.01	0.00	0.08	0.6
ced-1(n1951)	0.01	0.24	0.00	0.00	0.17	0.4
ced-6(n2095)	0.03	0.42	0.00	0.00	0.08	0.5
ced-6(n1813)	0.03	0.29	0.00	0.00	0.03	0.4
ced-7(n2001)	0.04	0.62	0.00	0.01	0.55	1.2
ced-7(n2094)	0.11	0.40	0.02	0.00	0.58	1.1
ced-7(n1996)	0.02	0.21	0.01	0.02	0.44	0.7
ced-8(n2093)	0.10	0.56	0.10	0.02	0.26	1.0
ced-8(n2090)	0.12	0.39	0.10	0.00	0.13	0.7
ced-8(n1891)	0.16	0.28	0.03	0.01	0.19	0.7
ced-8(n1999)	0.04	0.33	0.02	0.03	0.11	0.5

[a] The metacorpus contains the I2 sisters.

The assays are described in Table 7. We scored 100 animals of each genotype for each mutation, except for *ced-8(n1891)*, for which we scored 150 animals.

velopment: We determined the role the *ced* genes play in cell death at different stages of development. Mutations in *ced-1*, *ced-2*, *ced-5*, *ced-6* and *ced-10* affect cell corpses formed in the head and pharynx during embryogenesis, and also corpses formed in the ventral nerve cord during larval development (Table 9). By contrast, mutations in the genes *ced-7* and *ced-8* affect corpses mainly during embryogenesis, since there are many undegraded corpses in *ced-7* and *ced-8* embryos, but not in the ventral nerve cord during larval development. Furthermore, the embryonic corpses in *ced-7* and *ced-8* animals disappear during larval development, so that these mutants eventually contain far fewer corpses than even *ced-1(n1995)* mutants, although in other respects *ced-1(n1995)* is the weakest *ced* mutation we have characterized (Table 3). It seems possible either that *ced-7* and *ced-8* play a role specific to embryogenesis, or else that mutations in these genes slow down but do not prevent engulfment, and so are most easily observed during the relatively short embryonic period. In our ultrastructural studies (see above), both of the larval *ced-8* corpses we examined were being engulfed, but the four *ced-7* corpses were unengulfed, which indicates that mutations in *ced-7* can affect larval corpses, although these corpses eventually disappear.

TABLE 9

Number of corpses in embryos and L3/L4 larvae

Genotype	Embryos: Head	L3/L4 larvae: Embryonic deaths: Head	Pharynx	Tail[a]	Larval deaths: posterior ventral cord
ced-1(n1995)	11.3	5.5	0.5	1.2	1.2
ced-1(e1735)	22.2	>10	0.5	2.2	5.8
ced-2(e1752)	9.0	~9	1.5	1.5	3.7
ced-5(n1812)	27.4	>10	3.2	4.0	3.8
ced-6(n1813)	22.6	>10	0	1.8	3.0
ced-10(n1993)	18.3	~7	1.5	1.8	4.3
ced-7(n1997)	28.1	0.2	0.2	0	0.0
ced-7(n1892)	26.8	0.3	0.5	0	0.2
ced-8(n1891)	6.3	0.2	0.2	0	0.0

[a] The tail contains both embryonic and larval deaths.

We used Nomarski microscopy to determine the average number of corpses in the heads of three embryos of each genotype, and also to determine the average number of corpses found in different body regions of six larvae of each genotype during the L3/L4 molt. The wild-type pattern of cell deaths was determined by SULSTON and HORVITZ (1977) and by SULSTON *et al.* (1983). The 71 cells that die during formation of the head (excluding the pharynx) and the 22 cells that die during formation of the pharynx all die embryonically. The tail contains 13 cells that die embryonically, and two that die during larval development. In the posterior ventral cord, one descendant of P9, one descendant of P10, two descendants of P11 and three descendants of P12 die during larval development. >10, greater than 10 (in these animals the number of corpses also appeared roughly less than or equal to the embryonic value); ~, approximately equal to.

DISCUSSION

After cells die by programmed cell death, their corpses are quickly engulfed by other cells and then degraded (WYLLIE, KERR and CURRIE 1980; HEDGECOCK, SULSTON and THOMSON 1983). In a few cases, engulfment can even cause cell death (SULSTON, ALBERTSON and THOMSON 1980). To understand the

mechanism of engulfment, we have isolated and characterized 24 mutations that affect this process in *C. elegans*. These mutations define seven genes: *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10*. Two of these genes act zygotically–animals homozygous for *ced-1* or *ced-8* mutations have unengulfed corpses regardless of their maternal genotypes. Mutations in *ced-1* and *ced-8* are recessive and occur at frequencies expected for loss-of-function mutations. Of the nine zygotic mutations isolated in our screen for mutants with undegraded cell corpses, five are *ced-1* alleles and four are *ced-8* alleles, so *ced-1* and *ced-8* might well be the only zygotic genes for which a loss-of-function specifically prevents cell corpses from being degraded.

The other five genes all show a recessive maternal-rescue effect–homozygous mutants look wild type if their mother was a heterozygote. Of the 15 *ced* mutations that show maternal effects, eight are alleles of *ced-7*, two are alleles of *ced-2*, two are alleles of *ced-5*, two are alleles of *ced-6*, and a single mutation defines the gene *ced-10*. It seems likely that we have identified most genes of this type.

In each of these *ced* mutants many cell corpses appear to be engulfed normally, since there are relatively few undegraded corpses present. By contrast, in several *ced* double mutants there is a large increase in the number of undegraded corpses. We consider three possible explanations for this result. First, the genes *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10* might all play essential roles in a single process responsible for engulfment, but because none of the mutations we are studying is a null allele, this process is impaired only partially in the individual *ced* mutants. Our results suggest that at least some *ced-1* alleles result in a complete loss of gene function, but we cannot be certain that these or any of the other mutations we have isolated are null alleles. Second, different *ced* genes might function in the engulfment of different corpses. We believe that this explanation is incorrect, because all of these genes affect many of the same corpses, for example those of the 12 sisters and of the NSM sisters in the pharynx. Third, these seven genes might act in multiple, partially redundant processes, any one of which is sufficient to cause the engulfment of many cell corpses.

Our observations are most consistent with this third hypothesis. If there are two distinct processes that can each cause engulfment, and two genes are essential for the same process, then eliminating the functions of both genes should have the same consequence as eliminating the function of either one. By contrast, if two genes are essential for different processes, then eliminating both should cause an enhanced phenotype. Our studies of double mutants indicate that the *ced* engulfment genes behave in this way. For example, mutations in *ced-5* cause the strongest phenotype of all of these *ced* genes. The *ced-2 ced-5* and *ced-10 ced-5* double mutants look like *ced-5* mutants, whereas all other double mutants with *ced-5* have a much more severe phenotype than do *ced-5* animals. These results suggest that *ced-2*, *ced-10* and *ced-5* all act in the same process, and that *ced-1*, *ced-6*, *ced-7* and *ced-8* act in a different process. In general, combinations of double mutants between the genes *ced-2*, *ced-5* or *ced-10* and the genes *ced-1*, *ced-6*, *ced-7* or *ced-8* have greater expressivities than double mutants within these sets, which supports this hypothesis. Furthermore, both *ced-5* mutants, both *ced-2 ced-5* double mutants, and the *ced-10 ced-5* and *ced-2 ced-10* double mutants all have the same expressivity, which suggests that this phenotype may reflect the complete absence of the process controlled by *ced-2*, *ced-5* and *ced-10*. Additional evidence that *ced-2*, *ced-5* and *ced-10* act together in a process that does not involve *ced-1*, *ced-6*, *ced-7* and *ced-8* has recently been obtained by S. C. KIM (personal communication). Mutations in the gene *lin-24* cause some larval cells that normally divide to die instead; these deaths differ morphologically from programmed cell deaths (FERGUSON and HORVITZ 1985). S. C. KIM has observed that mutations in *ced-2*, *ced-5* or *ced-10* can prevent these abnormal deaths, but that mutations in the other *ced* genes do not affect these deaths. Because *ced-2*, *ced-5* and *ced-10* are necessary for the process that causes *lin-24*-induced cell deaths, it is possible that these three genes act not only in the engulfment of cells that have died by programmed cell death, but in some more general process of cell engulfment. By contrast, *ced-1*, *ced-6*, *ced-7* and *ced-8* might act only during programmed cell death.

Similar considerations do not resolve whether the genes *ced-1*, *ced-6*, *ced-7* and *ced-8* act in one process or multiple processes, because most double mutants involving these genes are more severe in phenotype than are the individual *ced* mutants. Thus, we can conclude only that there are at least two processes that are important in engulfing dead cells, one that involves *ced-2*, *ced-5* and *ced-10*, and one or more that involve the other *ced* genes. Nonetheless, in all cases tested, triple mutants involving the different *ced* genes are no more severe than double mutants, which is consistent with the hypothesis that only two different processes are defined by these seven *ced* genes. If *ced-1*, *ced-6*, *ced-7* and *ced-8* do act in a common process, then either we do not have null mutations in the genes essential for this process, or some of these genes are partially redundant with each other.

Thus, we propose that there are two distinct and partially redundant processes that can cause a cell corpse to be engulfed. One engulfment process involves the genes *ced-2*, *ced-5* and *ced-10*, and the other involves the genes *ced-1*, *ced-6*, *ced-7* and *ced-8*. Even when one of these processes does not function, the other can cause the engulfment of cell corpses. If both

processes are completely blocked, engulfment does not occur, although some corpses might be lost by lysis or detachment.

What might these two different processes be? Phagocytosis involves the recognition of a corpse by an engulfing cell, followed by the extension of pseudopodia and the envelopment of the corpse. Because the two processes defined by the *ced* genes are partially redundant, and hence presumably act in parallel, they cannot define two sequential steps in a pathway in which the second step depends on the completion of the first. Thus it is unlikely that one process controls the recognition of corpses, and the other controls the extension of pseudopodia. Instead, these two processes probably represent either two different systems for recognizing cell corpses or two different systems for extending pseudopodia. In *C. elegans*, the amoeboid sperm crawl by extending a large pseudopod (WOLF, HIRSH and MCINTOSH 1978); because none of the *ced* single or multiple mutants is infertile, this system of pseudopod extension must be functional in these mutants. Similarly, many cells, such as the HSN neurons and sex myoblasts, migrate during wild-type development (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983), and electron microscopic studies indicate that at least some cell migrations as well as the growth of various neuronal axons involve extensions that resemble pseudopods (R. DURBIN, personal communication). Because the *ced* mutants appear normal in anatomy and behavior, cell migration and axonal outgrowth are probably not affected. Thus if these *ced* genes act in the extension of pseudopodia, they play a role specific to phagocytosis.

Alternatively, these *ced* genes might act in the recognition of a corpse by an engulfing cell. SAVILL *et al.* (1990) have shown that vitronectin receptors on the surface of macrophages mediate engulfment of dying lymphocytes. Similarly, in nematodes, engulfment might require that specific molecules on the surface of a dying cell be recognized by specific receptors on the surface of the engulfing cell. If so, then mutations either in a gene for marking a corpse or in a gene encoding the receptor of an engulfing cell will prevent recognition of corpses. In addition, nonspecific changes on the surface of a dying cell might elicit engulfment; for example, DUVALL, WYLLIE and MORRIS (1985) propose that macrophages might recognize a variety of proteins that become abnormally presented on the cell-surface during the process of cell death. This second hypothesis predicts that mutations that inactivate such a receptor system will prevent engulfment, but that mutations that prevent dying cells from being marked for engulfment by this system might not exist, since any of a wide variety of proteins might elicit engulfment. If there are indeed two parallel processes in *C. elegans* that mediate engulfment, it is possible that one involves the recognition of specific cell-surface markers expressed during cell death, and that the other allows recognition of nonspecific changes that occur on the surface of a dying cell.

To understand the mechanisms that cause the engulfment of cell corpses in *C. elegans*, we must know which *ced* genes act within dying cells and which act within engulfing cells. The only hint we currently have is that *ced-1* and *ced-8* are required in the zygote, whereas the other genes display a maternal-rescue effect. For a gene that controls a specific cell-surface marker on dying cells to show a maternal effect requires that its product be provided by the mother and sequestered to, or specially activated in, only dying cells. By contrast, since many and possibly all cells in *C. elegans* have the ability to engulf corpses (SULSTON, ALBERTSON and THOMSON 1980; ROBERTSON and THOMSON 1982; SULSTON *et al.* 1983; HEDGECOCK, SULSTON and THOMSON 1983), it would not be surprising if a gene that acts in engulfing cells were expressed in the egg and its product segregated to all cells. This reasoning suggests, for example, that the *ced-2*, *ced-5* and *ced-10* genes, all of which show a maternal-rescue effect, might act in engulfing cells as part of a receptor system; perhaps this system recognizes nonspecific changes on the surface of the dying cell. On the other hand, zygotic genes like *ced-1* and *ced-8* might control a specific marker expressed by dying cells and recognized by a receptor on the engulfing cell controlled by the genes *ced-6* and *ced-7*.

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