Many Genomic Regions Are Required for Normal Embryonic Programmed Cell Death in *Caenorhabditis elegans*

Asako Sugimoto,*,[1] Ayumi Kusano,* Rebecca R. Hozak,* W. Brent Derry,† Jiangwen Zhu*,†,[2] and Joel H. Rothman*,†

**Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 and †Department of MCD Biology, University of California, Santa Barbara, California 93106*

Manuscript received October 31, 2000
Accepted for publication February 12, 2001

ABSTRACT

To identify genes involved in programmed cell death (PCD) in *Caenorhabditis elegans*, we screened a comprehensive set of chromosomal deficiencies for alterations in the pattern of PCD throughout embryonic development. From a set of 58 deficiencies, which collectively remove ~74% of the genome, four distinct classes were identified. In class I (20 deficiencies), no significant deviation from wild type in the temporal pattern of cell corpses was observed, indicating that much of the genome does not contain zygotic genes that perform conspicuous roles in embryonic PCD. The class II deficiencies (16 deficiencies defining at least 11 distinct genomic regions) led to no or fewer-than-normal cell corpses. Some of these cause premature cell division arrest, probably explaining the diminution in cell corpse number; however, others have little effect on cell proliferation, indicating that the reduced cell corpse number is not a direct result of premature embryonic arrest. In class III (18 deficiencies defining at least 16 unique regions), an excess of cell corpses was observed. The developmental stage at which the extra corpses were observed varied among the class III deficiencies, suggesting the existence of genes that perform temporal-specific functions in PCD. The four deficiencies in class IV (defining at least three unique regions), showed unusually large corpses that were, in some cases, attributable to extremely premature arrest in cell division without a concomitant block in PCD. Deficiencies in this last class suggest that the cell death program does not require normal embryonic cell proliferation to be activated and suggest that while some genes required for cell division might also be required for cell death, others are not. Most of the regions identified by these deficiencies do not contain previously identified zygotic cell death genes. There are, therefore, a substantial number of as yet unidentified genes required for normal PCD in *C. elegans*.

PROGRAMMED cell death (PCD, or apoptosis) is essential for normal development and homeostasis in most multicellular organisms (Jacobson *et al.* 1997). For example PCD is crucial for morphogenesis during embryogenesis and for elimination of excess neurons during construction of the nervous system. PCD also performs critical defensive functions in pathogenesis and carcinogenesis.

The nematode *Caenorhabditis elegans* has proven to be a useful model organism for studying the core machinery involved in PCD. A unique advantage of *C. elegans* is that all cell deaths occur at precisely defined times and locations. Of the 1090 somatic nuclei made during its development, exactly 131 cells die by PCD (Sulston and Horvitz 1977; Sulston *et al.* 1983). Genetic approaches have made it possible to identify large numbers of mutations that affect PCD in this animal. Genetic screens have identified four proteins (CED-3, CED-4, CED-9, and EGL-1) that are central to the execution of the death program (reviewed by Metzstein *et al.* 1998). CED-3, a member of the caspase protease family, induces PCD when activated (Yuan *et al.* 1993; Xue *et al.* 1996). CED-4 (which is similar to mammalian Apaf-1) activates the protease activity of CED-3 (Chinnaiyan *et al.* 1997a; Seshagiri and Miller 1997; Zou *et al.* 1997). In cells in which the death program is blocked, CED-9, the orthologue of mammalian Bcl-2 (Hengartner and Horvitz 1994), binds to CED-4 and prevents it from activating CED-3 (Chinnaiyan *et al.* 1997b; Xue and Horvitz 1997). EGL-1, a BH3 domain containing protein, binds to CED-9 causing it to release CED-4, thereby promoting CED-3-dependent cell death (Conradt and Horvitz 1998; del Peso *et al.* 1998). These core events in PCD appear to be conserved in all metazoans (reviewed in Song and Steller 1999).

Genetic approaches have also identified several genes that are involved in other aspects of PCD. Six genes, *ced-1*, *-2*, *-5*, *-6*, *-7*, and *-10* are involved in engulfment of cell corpses once the death program has been activated

Corresponding author: Joel H. Rothman, Department of MCD Biology and Neuroscience Research Institute, University of California, Santa Barbara, CA 93106. E-mail: rothman@lifesci.ucsb.edu

[1] *Present address:* Department of Biochemistry and Biophysics, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 Japan and PRESTO, Japan Science and Technology Corporation.

[2] *Present address:* Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

(Hedgecock *et al.* 1983; Ellis *et al.* 1991). *ced-8* is required for the timely onset of PCD (Stanfield and Horvitz 2000). *ces-1* and *ces-2*, both of which encode apparent transcription factors, activate PCD in a particular set of cells (Metzstein *et al.* 1996; Metzstein and Horvitz 1999).

Although many factors involved in PCD have been identified, the way in which these functions are integrated with developmental processes, such as cell proliferation and cell-signaling events, are not well understood and it is clear that other components remain to be identified. For example, the comprehensive set of targets of the CED-3 protease is not known. Moreover, it is unclear how EGL-1, the most upstream component known in the core PCD pathway, is regulated. In addition, it is likely that additional *ces*-like genes exist that determine the death fate of subsets of cells. Finally, while the participation of some cell cycle regulators in apoptosis has been reported in other organisms (reviewed in Guo and Hay 1999), their roles in *C. elegans* PCD have not been examined.

Since previous screens for cell death mutants were biased against lethal mutations (Hedgecock *et al.* 1983; Ellis and Horvitz 1986), genes essential for both viability and normal PCD are likely to have been overlooked. To screen the genome for zygotic genes required for normal PCD without bias against lethal genes, we have performed a screen of genetic deficiencies (chromosomal deletions).

Deficiencies can be used to scan segments of the genome for zygotic gene functions. Animals homozygous for most deficiencies arrest during embryogenesis. Their phenotypes usually reflect the null phenotype of one or many zygotic genes. The primary advantage of a deficiency screen is that a large fraction of the genome can be efficiently screened. In addition, deficiencies can identify genes (*e.g.*, very small genes or neighboring genes that perform redundant functions) that are not readily mutated by conventional point mutagenesis (*e.g.*, White *et al.* 1994; Grether *et al.* 1995; Chen *et al.* 1996; Zhu *et al.* 1997). Most deficiencies do not prevent differentiation or cell division and it is possible to screen deficiency homozygotes for defects in a wide range of processes that occur during embryogenesis. Indeed, deficiency screens in Drosophila have been useful for identifying regions of the genome required for morphogenesis and patterning (Merrill *et al.* 1988; Wieschaus and Sweeton 1988; Bilder and Scott 1995), DNA replication (Smith *et al.* 1993), and PCD (White *et al.* 1994) and in *C. elegans* to identify loci required for early cell division patterns (Storfer-Glazer and Wood 1994), embryonic tissue patterning (Chanal and Labouesse 1997; Labouesse 1997; Terns *et al.* 1997; Zhu *et al.* 1997), and muscle differentiation (Ahnn and Fire 1994).

In this study, we report the analysis of 58 chromosomal deficiencies that encompass ~74% of the *C. elegans* genome. We find that these deficiencies can be categorized into four phenotypic classes: (1) normal PCD, (2) no or reduced PCD, (3) excess PCD corpses, and (4) large PCD corpses. We identified 28 new genomic regions involved in PCD. These analyses show that additional genes that participate in execution of PCD and cell corpse engulfment remain to be identified. Finally, our findings suggest that the developmental program for cell death can be uncoupled from cell proliferation, demonstrating that the normal number of cell division rounds is not essential for PCD.

MATERIALS AND METHODS

Worm culture and strains: Techniques for culturing *C. elegans* are described elsewhere (Brenner 1974; Lewis and Fleming 1995). All strains were grown at 20°. The strains used as sources of deficiencies analyzed in this study are listed in Table 1. Most deficiencies used are balanced with a balancer chromosome (*mnC1 II*, *sC1 III*, and *qC1 III*) or a chromosome bearing two visible mutations that closely flank the deficiency; such deficiency strains were used directly for analyses by Nomarski microscopy. Some deficiencies were balanced by reciprocal translocations [*eT1 (III;V)*, *nT1 (IV; V)*, *szT1 (I;X)*], which segregate a high proportion of lethal embryos due to aneuploidy. Strains of this type were crossed with N2 wild-type males and the F_1 progeny that produced ~25% arrested embryos were used as sources for deficiency embryos in the screen. *eDf2*, *rhDf1*, and *mnDf1* are maintained with free duplications and deficiency homozygotes are generated by loss of the duplications during meiotic segregation. To obtain nullo-X embryos we used *him-8(e1489)*, which produces a high fraction of nullo-X ova (Hodgkin *et al.* 1979). When *him-8(e1489)* hermaphrodites are crossed with wild-type XO males, most of the arrested embryos are nullo-X (Ahnn and Fire 1994).

The data in the 1999 Genetic Map of *Caenorhabditis elegans* (published by the Caenorhabditis Genetics Center) were used to estimate the number of mutationally defined genes collectively deleted by the deficiencies. The genes in the genetic map between the leftmost gene and the rightmost gene deleted by each deficiency (confirmed by complementation tests) were regarded as those deleted by the deficiency. Since only a subset of genes was examined by complementation tests, each deficiency is likely to delete more genes than this estimate and this calculation therefore provides a conservative estimate of the fraction of the genome deleted. Collectively, the deficiency collection we screened deleted a minimum of 1213 out of the 1644 genetically defined genes on the genetic map, or ~74% of the genetically defined genome.

Nomarski analysis of deficiency embryos: In general, gravid hermaphrodites heterozygous for each deficiency were dissected in M9 solution and young (<100-cell stage) embryos were collected for cell corpse analysis. Approximately 40 embryos were mounted in M9 solution on agar pads (containing 5% Difco Bacto agar in distilled water) and observed by Nomarski optics. Approximately one-fourth of the embryos are expected to be homozygous for the deficiency and therefore arrest during embryogenesis without hatching. In some strains, >25% of embryos did not hatch owing to haploinsufficiency of genes deleted by the deficiency. Usually such arrested heterozygous embryos were distinguishable from the deficiency homozygotes by comparing their terminal phenotypes; the heterozygotes generally showed a less severe arrest phenotype than the majority class comprising the 25% arrested homozygotes.

TABLE 1

Deficiency strains

Chromosome	Deficiency	Strain	Genotype
I	*hDf10*	KR1557	*hDf10 dpy-5(e61) unc-29(e403)/hT2 dpy-18(h662) I; +/hT2 bli-4(e937) III*
	tDf3	JR66	*fog-1(e2121) unc-11(e47)/tDf3 dpy-5(e61) I*
	qDf3	JK1134	*qDf3/unc-11(e47) dpy-5(e61) I*
	hDf6	KR1737	*hDf6 dpy-5(e61) unc-13(e450) I; hDp31 (I; f)*
	qDf16	JK1726	*qDf16/dpy-5(e61) unc-15(e1402) I*
	ozDf5	BS585	*unc-13(e51) ozDf5 I; nDp4/+ (I; V)*
	nDf24	MT2181	*nDf24/unc-13(e1091) lin-11(n566) I*
	nDf30	MT2139	*nDf30/unc-13(e1091) lin-11(n566) I*
	hDf9	JR58	*hDf9/dpy-14(e188) unc-75(e950) I*
	eDf3	CB2769	*eFd3/eDf24 I*
	eDf9	CB2775	*eDf9/eDf24 I*
II	*nDf3*	MT681	*nDf3/lin-31(n301) bli-2(e768) II*
	ccDf5	PD8605	*ccDf5/dpy-25(e817) II*
	maDf4	VT454	*maDf4/dpy-10(e128) unc-104(e1265) II*
	mnDf30	SP543	*mnDf30 unc-4(e120)/mnC1 II*
	mnDf88	SP754	*unc-4(e120) mnDf88/mnC1 II*
	mnDf63	SP645	*mnDf63/mnC1 II*
	mnDf89	SP755	*mnDf89/mnC1 II*
	jDf2	PJ803	*jDf2/mnC1 II*
III	*unc-45(wc5)*	LV15	*unc-45(wc5) dpy-1(e1)/daf-7(e1372) par-2(it46) III*
	sDf124	BC4330	*unc-45(r450) sDf124(s2670)/sC1(s2023) [dpy-1(s2171)] III*
	nDf11	MT695	*nDf11/unc-79(e1068) dpy-17(e164) III*
	rhDf1	NJ654	*rhDf1 III; sDp3 (III; f)*
	sDf130	BC4637	*sDf130 III; sDp3 (III; f)*
	sDf121	JK2057	*sDf121 unc-32(e189)/unc-93(e1500sd) dpy-17(e169) III*
	nDf40	MT5491	*nDf40 dpy-18(e364am)/eT1 III; eT1/+ (III; V)*
	eDf2	CB1517	*eDf2 III; eDp6 (III; f)*
IV	*mDf5*	DR802	*mDf5/nT1 IV; +/nT1 V*
	nDf41	JR59	*nDf41/dpy-13(e184) unc-24(e138) IV*
	stDf7	JR31	*stDf7/fem-1(e1991) unc-24(e138) unc-22(s12) IV*
	eDf19	CB3824	*eDf19/unc-24(e138) dpy-20(e1282ts) IV*
	mDf7	DR793	*dpy-13(e184) mDf7 IV/nT1[let-?(m435)] (IV; V)*
	nDf27	MT2115	*nDf27/nT1 IV; +/nT1 V*
	sDf21	BC1216	*sDf21 dpy-4/nT1 IV; +/nT1 V*
	sDf23	JR65	*sDf23/unc-26(e205) dpy-4(e1166) IV*
V	*sDf74*	BC3957	*dpy-18(e364)/eT1 III; sDf74 unc-46(e177)/eT1 [let-500(s2165)] V*
	sDf28	BC1289	*dpy-18(e364)/eT1 III; sDf28 unc-46(e177)/eT1 V*
	sDf42	BC3401	*dpy-18(e364)/eT1; sDf42(s1680) unc-46(e177)/let-50(s2165)/eT1 (III; V)*
	sDf45	JR63	*sDf45 unc-46(e177)/let-347(q1035) unc-46(e177) V*
	sDf33	BC1781	*dpy-18(e364)/eT1 III; sDf33 unc-46(e177)/eT1 V*
	sDf26	JR67	*unc-60(e723) dpy-11(e224)/sDf26 V*
	sDf50	JK282	*sDf50 unc-46(e177)/dpy-11(e224) unc-42(e270) V*
	sDf30	BC1785	*dpy-18(e364)/eT1 III; unc-46(e177) sDf30/eT1 V*
	sDf35	BC2511	*dpy-18(e364)/eT1 III; unc-60(e677) dpy-11(e224) sDf35/eT1 V*
	ctDf1	JR61	*ctDf1/unc-42(e270) sma-1(e30) V*
	arDf1	GS357	*+/nT1 (IV); unc-42(e270) arDf1/DnT1 V*
	nDf42	JR106	*nDf42/dpy-11(e224) unc-76(e911) V*
	tDf2	JR56	*itDf2/unc-42(e270) dpy-21(e428) V*
	yDf8	TY974	*unc-42(e270) yDf8/dpy-11(e224) unc-76(e911) V*
	yDf4	TY832	*yDf4/dpy-11(e224) unc-76(e911) V*
	yDf6	TY865	*yDf6/dpy-11(e224) unc-76(e911) V*
	oxDf2	BS509	*ozDf2/dpy-21(e428) par-4(it33)V*
X	nullo-X	CB1489	*him-8(e1489) IV*
	syDf1	PS1032	*syDf1/unc-2(e55) lon-2(e678) X*
	uDf1	TU900	*+/szT1[lon-2(e678)] I; uDf1/szT1 X*
	stDf5	RW2551	*stDp2 (X; II)/+ II; stDf5 X*
	nDf19	MT1401	*+/szT1[lon-2(e678)] I; nDf19/szT1 X*
	mnDf1	SP262	*mnDp1 (X; V)/+ V; mnDf1 X*

The 58 deficiencies analyzed in this study are listed in order of the location of their estimated left breakpoint (proceeding from left to right) on each chromosome.

The number and appearance of cell corpses were analyzed by scoring fields of embryos every 30–60 min. Embryos were scored until the wild-type siblings that started at the same developmental stage reached at least the 2-fold stage. Cell corpses in the terminally arrested deficiency embryos were also scored >14 hr after the first cleavage. Corpses were counted at four stages during embryogenesis: stage I, ("lima bean" stage in wild type, ~340 min after first cleavage); stage II, ("comma" stage in wild type, ~380 min); stage III, ("1.5-fold" stage in wild type, 420 min); and stage IV, ("2-fold" stage in wild type, 460 min) (Sulston *et al.* 1983). Since most deficiency homozygotes show morphological defects, we used the time after first cleavage, instead of morphological characteristics, to determine the age of each embryo. The appearance of the collected embryos varied; the approximate time of the first cleavage of each embryo was estimated retrospectively based on the number of cells at the beginning of the observation.

Quantifying cell corpses: For each deficiency, the number of corpses at each stage in the homozygotes (*Df/Df*) was compared with that of their wild-type-appearing siblings (*Df/+* and +/+) and N2 (wild type) using Student's *t*-test (two-tailed). Nonhomozygotes and N2 were also compared to assess whether the parental strain showed alterations in cell corpse numbers. For *P* values of <0.05, the difference in the means between the two groups was regarded as significant. For *P* values of <0.01, the difference was regarded as highly significant.

Obtaining nuclear counts: The number of nuclei in homozygous deficiency embryos was counted by the squash method (Gossett and Hecht 1980) with some modification. Briefly, *Df/+* adults were placed on NGM agar plates with *Escherichia coli*. After several hours, the adults were removed from the plates. The eggs laid on the plates were allowed to develop for >14 hr. The ~5–10 deficiency homozygous embryos that remained unhatched were recovered from the plates with M9 solution and placed on polylysine-coated slides. A silicone-treated coverslip was laid over the embryos and the slide was inverted onto a tissue and pressed to squash the embryos. The slide was immediately frozen on dry ice. After removing the coverslip by prying off with a razor blade, the slide was fixed in methanol for 5 min and then acetone for 5 min at −20°. The slide was washed in PBS containing 0.5% Tween 20 and mounted in a 25 mg/ml DABCO solution in 90% glycerol:10% PBS with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and sealed with nail polish. The stained nuclei were viewed under a fluorescent microscope and photographed. The number of nuclei per embryo was counted from an image projected from the negative using a slide projector.

Scoring duration of cell corpses: A four-dimensional (4-D) time-lapse recording system (Schnabel 1991; Hird and White 1993; Moskowitz *et al.* 1994) was used to score the duration of cell corpse persistence. The young embryos from *Df/+* adults were collected and mounted on a 5% agar pad as described above. Using the 4-D system, images of 25 focal planes at 1-min intervals were collected for 10 hr. From the recording, the duration of each corpse was scored. We scored all the corpses readily visible in the recording; however, the analysis of some corpses (particularly those located at the edge of the embryos, or in a deep focal plane in the recorded image) were excluded due to optical limitations.

RESULTS

Deficiency screen for genomic regions required for normal PCD: Most previous screens for PCD defects in *C. elegans* have required embryonic viability of the mutant animals. We chose to perform an unbiased screen for zygotic genes involved in PCD by examining embryos homozygous for deficiencies that sample most of the genome. As PCD occurs in cells shortly after their birth, and the corpses are rapidly engulfed, little information can be obtained regarding alterations in PCD based on the appearance of terminally arrested embryos. To screen deficiency homozygotes for defects in PCD, we followed the distribution of cell corpses over the course of embryogenesis. Of the 671 cells born during embryogenesis, 113 undergo PCD (Sulston *et al.* 1983). The first cell death occurs at 220 min after first cleavage (~250-cell stage) and the last embryonic cell deaths at ~630 min (~560-cell stage). Most deaths (109/113) occur between 250 and 450 min of development, the period during which we focused our observations.

We scored cell corpses at four developmental stages: stage I (~340 min after the first cleavage, bean morphology in the wild type), stage II (~380 min, comma), stage III (~420 min, 1.5-fold) and stage IV (~460 min, 2-fold). In addition, we scored terminally arrested embryos for each deficiency homozygote at ~800 min after first cleavage. In wild-type (N2) embryos, the number of corpses seen at each of these stages is relatively reproducible. The average number of cell corpses ± standard deviation was as follows: stage I, 2.2 ± 1.4 ($n = 41$); stage II, 4.4 ± 1.6 ($n = 45$); stage III, 3.6 ± 1.1 ($n = 28$); stage IV, 2.8 ± 1.1 ($n = 41$). Generally no corpses are visible at the time of hatching in wild type (~800 min). The rapid engulfment and degradation of cell corpses (over a period of <20 min) are responsible for the low number of cell corpses visible at any one time.

To examine the profile of cell corpses in deficiency homozygotes, embryos were collected from hermaphrodite adults that were heterozygous for each deficiency. Approximately 25% of these embryos are deficiency homozygotes (*Df/Df*) and arrest during embryogenesis owing to the removal of essential embryonic genes; the remaining 75% (*Df/+* and +/+) are viable. The numbers of corpses in homozygous deficiency embryos were compared with those of their siblings and of wild type and tested for significant deviations.

Fifty-eight deficiencies were examined and divided into four classes based on the number of corpses observed (Table 2). Class I deficiencies (20 total) showed normal cell corpse numbers at all stages. Class II deficiencies (16 total) showed no or significantly fewer cell corpses than wild type. Class III deficiencies (18 total) showed excess numbers of corpses. Class IV deficiencies (4 total) arrested early in embryonic proliferation and showed abnormally large corpses. The deficiencies that led to significant PCD phenotypes are described here.

Deficiencies that delete known cell death genes: Several known genes that function in cell death are deleted by the deficiencies used in this screen. *ced-3*, *ced-4*, and *egl-1* are required for PCD and loss-of-function mutations in these genes result in an absence of cell death throughout development (Ellis and Horvitz 1986;

TABLE 2

Phenotypes of deficiency homozygotes

Deficiency	Terminal arrest stage	Terminal number of nuclei[a]	Overlapping deficiency group[b]	Relevant genes deleted[c]	Comments
N2 (comma)		564 ± 22.4 (n = 6)			
		Class I normal PCD			
hDf10 I	2-fold				
ozDf5 I	2.5-fold				
eDf9 I	1.75-fold				
nDf3 II	1.25-fold				
mnDf89 II	Unenclosed	~300 nuclei			
jDf2 II	Variable				
wc5 III	2-fold				
sDf124 III	2-fold				
nDf11 III	2- to 3-fold				
sDf130 III	1.5- to 2.5-fold				
mDf5 IV	1.75-fold				
stDf7 IV	1.5-fold				
mDf7 IV	Unenclosed				
sDf50 V	2-fold				
sDf30 V	2-fold				Terminal embryos contained 2–4 vacuoles
yDf4 V	Unenclosed				
yDf8 V	1.5-fold				
uDf1 X	1.75-fold				3–4 vacuoles per embryo
nDf19 X	Unenclosed				
nullo-X	Unenclosed				Some arrested embryos had no corpses
		Class II fewer-than-normal or no corpses			
tDf3 I	Bean				Slightly fewer corpses at stage II and III
qDf3 I	Bean	430.7 ± 21.4 (n = 6)			
hDf6 I	2-fold				Fewer corpses at stage III
mnDf63 II	Unenclosed	200~400 nuclei			Fewer corpses at stage II
eDf19 IV	Unenclosed	429.8 ± 15.6 (n = 6)			
nDf27 IV	2- to 3-fold		α	*ced-3*	
sDf21 IV	2- to 2.5-fold		α	*ced-3*	Small vacuole observed at 2-fold stage
sDf23 IV	Unenclosed				
sDf45 V	Unenclosed	172.6 ± 13.6 (n = 7)	β	*emb-29*	
sDf33 V	1.5-fold	~200 nuclei	β	*emb-29*	
sDf28 V	Bean	~200 nuclei	β	*emb-29*	
sDf42 V	2-fold	~200 nuclei	β	*emb-29*	
itDf2 V	Unenclosed		γ	*egl-1*	
nDf42 V	Unenclosed		γ	*egl-1*	
syDf1 X	1.5-fold				Slightly fewer corpses at stages I, II, and III
stDf5 X	2-fold	560.5 ± 15.5 (n = 6)			
		Class III excess corpses			
		Class III-1 corpses accumulate until the terminal stage			
mnDf88 II	Unenclosed	Excess nuclei		*cki-1*, *cki-2*	Many corpses at terminal stage only
sDf121 III	1.25-fold			(*ced-4*)	Many corpses even though *ced-4* is deleted
nDf40 III	Comma			(*ced-9*)	Some large corpses
sDf26 V	Bean to 1.5-fold				
mnDf1 X	1.5-fold				

(*continued*)

Conradt and Horvitz 1998). As expected, the deficiencies that delete *ced-3* (*nDf27* and *sDf21*) completely block PCD (Figure 1). The other region whose removal completely eliminates PCD is defined by the overlapping deficiencies *itDf2* and *nDf42* and excludes the region deleted by the neighboring deficiencies *arDf1*, *yDf8*, *yDf4*, and *yDf6*, which do not block PCD. These observations narrow the relevant region to a small inter-

TABLE 2

(Continued)

Deficiency	Terminal arrest stage	Terminal number of nuclei[a]	Overlapping deficiency group[b]	Relevant genes deleted[c]	Comments
			Class III-2 transiently elevated corpse number		
qDf16 I	1.5-fold				Corpses clustered in the head
nDf24 I	Bean				Many corpses at stage IV
eDf3 I	1.5-fold				Corpses clustered in the head
mnDf30 II	2- to 3-fold				Many corpses seen at stage III and IV
rhDf1 III	1.5-fold				Many corpses at stage III
sDf74 V	2- to 3-fold				Many corpses at stage III and IV
sdf35 V	Unenclosed				Many corpses at stage IV
ozDf2 V	1.5-fold				Many corpses at stage III
			Class III′ excess corpses by maternal effect		
hDf9 I	1.5- to 3-fold		δ		Both Df and [+] had slightly more corpses
nDf30 I	2- to 3-fold		δ		Both Df and [+] had slightly more corpses
yDf6 V	2-fold				Both Df and [+] had slightly more corpses
arDf1 V	2-fold		ε		Both Df and [+] had slightly more corpses
ctDf1 V	1-fold		ε		Both Df and [+] had slightly more corpses
			Class IV large corpses		
ccDf5 II	Unenclosed	130.1 ± 10.7 (n = 8)	ζ		
maDf4 II	Unenclosed	113.7 ± 12.4 (n = 6)	ζ		
eDf2 III	Unenclosed	420.9 ± 25.9 (n = 7)			Some irregular shaped corpses
nDf41 IV	Unenclosed	182.5 ± 9.3 (n = 6)			

The 58 deficiencies were categorized into four major classes based on the PCD phenotypes observed. Class I deficiencies showed no significant difference from wild type in cell corpse number at any stages (see MATERIALS AND METHODS for definition of stages). Class II deficiencies resulted in significantly fewer-than-normal cell corpses for at least one stage. Class III deficiencies resulted in significantly greater-than-normal corpses for at least one stage; these were further subdivided into three classes depending upon their temporal patterns. Class IV deficiencies produced abnormally large corpses. All class IV deficiencies showed significantly fewer corpses than did wild type but were categorized in a separate class because of the aberrant appearance of corpses. The number of cell deaths produced by wild-type embryos at various stages provides a useful comparison: prior to the 200-cell stage wild-type embryos do not undergo cell death. The class II deficiencies that delete *emb-29*, the overlapping class IV deficiencies *ccDf5* and *maDf4*, and the class IV deficiency *nDf41* cause arrest with <200 nuclei. Twelve PCDs occur in wild type between the 200∼400 cell stage (*e.g.*, the stage at which *mnDf63* homozygotes arrest). By the ∼430-cell stage (*e.g.*, around the stage at which *qDf3*, *eDf19*, and *eDf2* homozygotes arrest) ∼20 cell deaths have occurred in wild type. The remaining PCDs in wild type occur between the 430-cell stage and the end of embryogenesis (558 nuclei in hermaphrodite embryos).

[a] Terminal number of nuclei determined by counting DAPI-stained nuclei in squashes of embryos (see MATERIALS AND METHODS) are given as average number ± SD. In the case of approximate numbers, estimates were made by observing terminal embryos under Nomarski optics.

[b] Overlapping deficiencies that show similar PCD phenotypes are indicated in groups denoted with Greek letters. These overlapping deficiencies may delete common genes involved in PCD.

[c] Relevant genes deleted by the indicated deficiency. Deficiencies that remove *ced-4* or *ced-9* (shown in parentheses) do not yield Ced phenotypes owing to maternal rescue.

val on chromosome V. Following our identification of this region, it was reported that loss-of-function mutations in the *egl-1* gene, which resides in this interval, prevent PCD (CONRADT and HORVITZ 1998). Thus, it is likely that the absence of cell death in *itDf2* and *nDf42* is caused by removal of *egl-1*. These two examples of class II deficiencies validate our screen as a means to identify genomic regions involved in PCD.

A limitation of the deficiency screen is that it does not detect maternally rescued genes, as exemplified by *ced-4* and *ced-9*. Although *stDf121* deletes *ced-4*, homozygous embryos, unlike *ced-4(lf)* mutants (ELLIS and HORVITZ 1986; YUAN and HORVITZ 1992) undergo PCD (Figure 2A). We found that this discrepancy is attributable to maternal rescue of the homozygotes, as revealed in the genetic experiments presented in Table 3. Similarly, while *ced-9* is required to suppress cell death, the *ced-9(lf)* homozygotes produced by heterozygous mothers develop normally until late in postembryonic development, owing to maternal supply of the *ced-9(+)* product (HENGARTNER *et al.* 1992). Therefore, although *nDf40* (see below), which deletes *ced-9*, leads to excess numbers of cell corpses, this phenotype is likely not attributable to removal of *ced-9*.

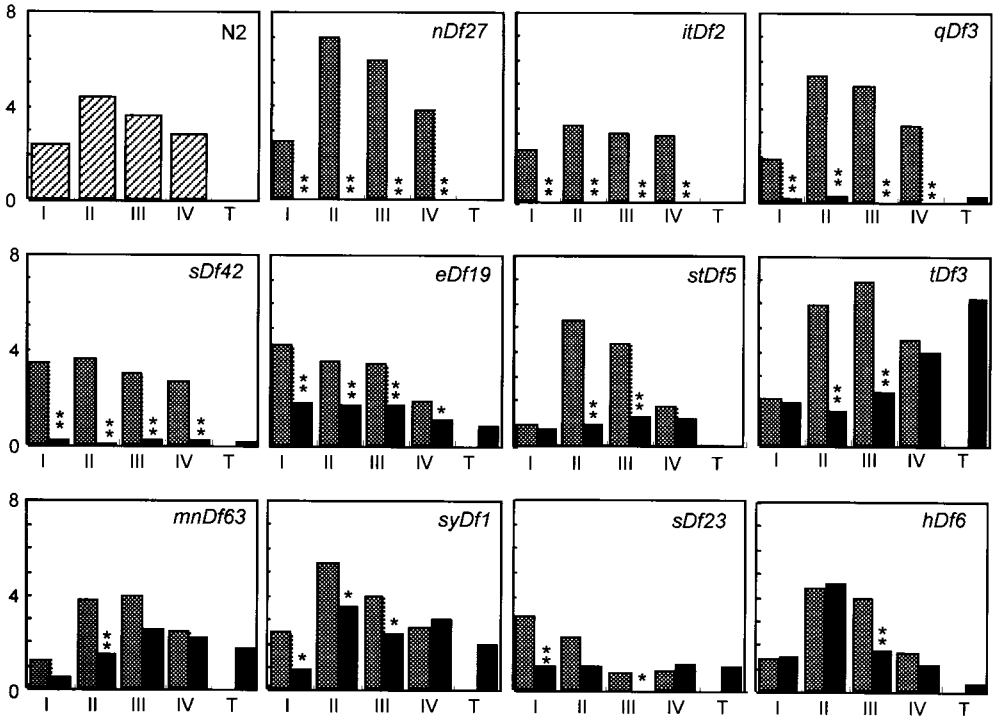


FIGURE 1.—Cell death profiles for class II deficiencies. Average number of cell corpses counted per embryo at each developmental stage is shown for each deficiency strain. Hatched bar, N2 (wild type); stippled bar, +/+ or +/*Df* embryos; solid bar, *Df/Df* embryos. (I) ~340 min after first cleavage (bean stage in wild type); (II) ~380 min (comma stage in wild type); (III) ~420 min (1.5-fold in wild type); (IV) ~460 min (2-fold in wild type); (T: terminally arrested embryo) >800 min (the approximate time at which wild-type embryos hatch). For the terminal stages, cell corpses were scored only in deficiency homozygotes. More than 30 embryos from *Df*/+ mothers were scored for each deficiency, and the average for each category [(+/+ or +/*Df*) and (*Df/Df*)] is shown. The average numbers of corpses between the two categories were compared by *t*-test. For data marked with one asterisk, $0.01 \leq P < 0.05$; for those with two asterisks, $P < 0.01$. For overlapping deficiencies that delete common relevant regions, only one representative deficiency is shown.

All described engulfment mutants, with the exception of *ced-1*, are maternally rescued (ELLIS *et al.* 1991). Since there was no available deficiency that deletes *ced-1*, we were unable to evaluate the efficacy of our screen in identifying engulfment genes. However, we found that several of the deficiencies we analyzed are profoundly defective in cell corpse engulfment (see DISCUSSION).

Some class II deficiencies suggest a link between PCD and cell cycle progression: As described above, deletion of the two genomic regions that contain *ced-3* and *egl-1* result in the complete absence of PCD. All other class II deficiencies cause a partial reduction of cell corpse number (Figure 1). For some of the deficiencies, a diminution in cell corpse number appeared to result from mitotic arrest. Four overlapping deficiencies (*sDf45*, *sDf33*, *sDf28*, and *sDf42*) cause a profound decrease in cell corpse number at all stages. All four caused embryos to arrest with <200 nuclei, compared to the ~560 nuclei present in wild-type embryos at hatching (Table 2); PCD does not occur before the 200-cell stage in wild-type embryos. (The number of PCDs that have been generated by various stages in wild-type development are noted in Table 2). By contrast, three nearby deficiencies (*sDf74*, *sDf26*, and *sDf50*) did not result in decreased cell corpse number or reduced number of nuclei. Thus, the PCD and cell proliferation phenotypes are closely linked and are not separable by the deficiencies that we examined. One of the genes in the relevant region is *emb-29*, mutations in which result in arrest of embryos with 150–200 differentiated cells (HECHT *et al.* 1987), suggesting that the arrest in proliferation is due to loss of *emb-29*. To test whether the absence of PCD in these deficiencies could be explained by the mitotic block caused by loss of *emb-29*, we examined the pattern of cell corpses in *emb-29* mutants. Embryos carrying any of three mutant alleles of *emb-29* (*s819*, *g52ts*, and *b262ts*), invariably arrested with <200 cells and showed depressed cell corpse numbers comparable to that seen with the deficiencies that delete *emb-29*. Although most cells in wild-type 200-cell stage embryos do not show obvious differentiation, arrested *emb-29* embryos and deficiencies that delete *emb-29* contain a number of differentiated cell types (*e.g.*, intestine, neurons, and muscles); thus, differentiation is not blocked in these embryos despite the early arrest of cell proliferation. Therefore, the diminished cell corpse number in these deficiencies is likely to be explained by the mitotic block. EMB-29 may be directly required both for mitotic progression and activation of the cell death program; alternatively, it may not be possible to initiate PCD from the particular stage in the cell cycle at which *emb-29* mutants arrest.

Embryos homozygous for *qDf3*, *eDf19*, *stDf5*, and *sDf23* showed fewer corpses at all stages. *stDf5* maps near the *ced-8* gene, mutations in which cause cell corpses to appear later than they did in wild type (STANFIELD and HORVITZ 2000). However, we found that *ced-8(n1891)* complements the cell death phenotype of *stDf5*, indicat-

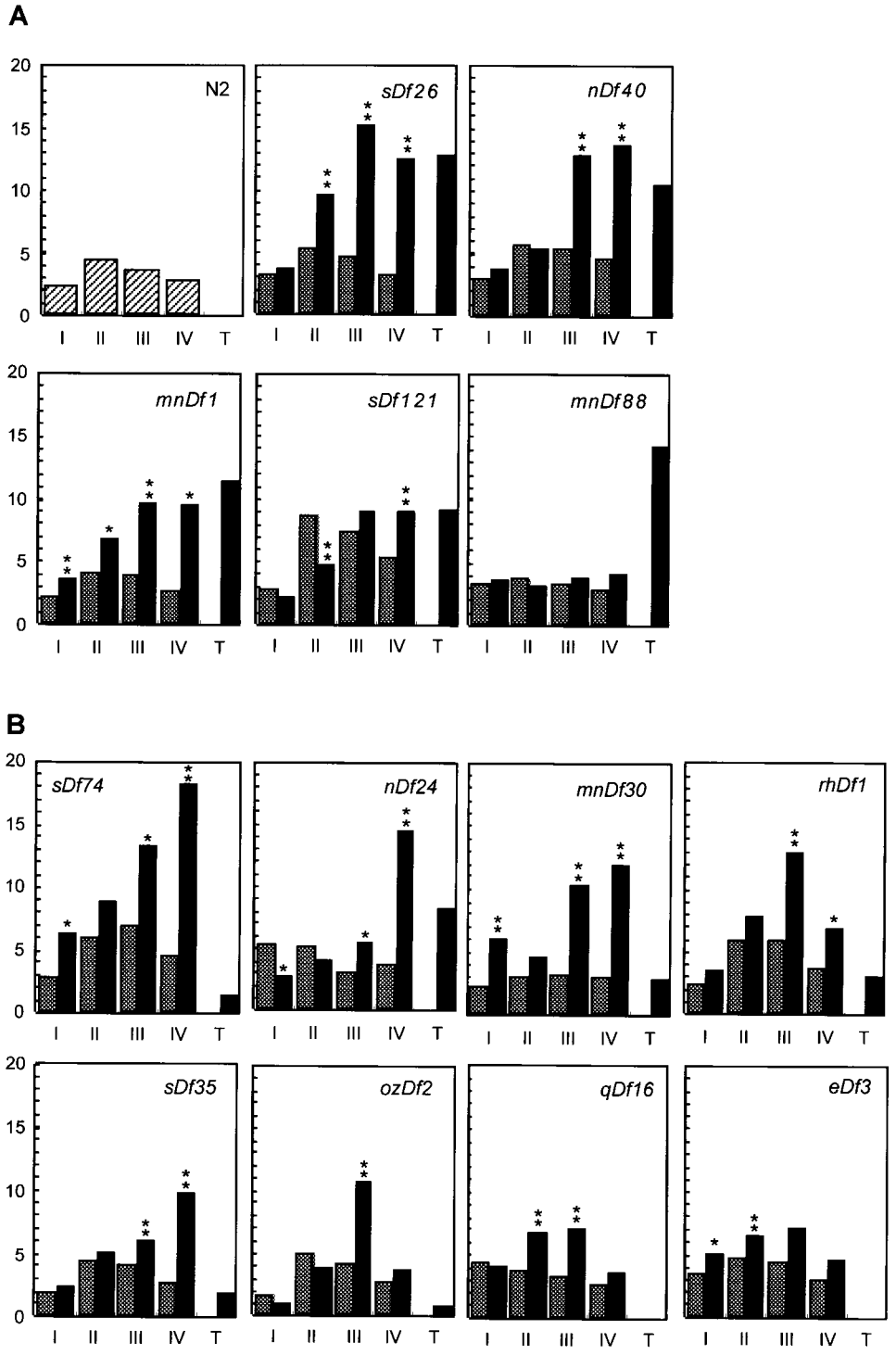


FIGURE 2.—Cell death profiles for class III deficiencies. (A) Class III-1: cell corpses accumulated toward the end of embryogenesis. (B) Class III-2: excess corpses were observed at specific developmental stages. Data presented as described in Figure 1.

ing that the reduced number of cell corpses is not due to deletion of *ced-8*. *qDf3* and *eDf19* homozygotes arrest with slightly fewer (420–430) nuclei than are present in fully developed embryos. This partial block in cell proliferation may contribute to the reduction in cell corpse number in these deficiencies. However, based on their morphology, these embryos undergo differentiation and the reduction of corpses cannot, therefore, be explained by a general failure to differentiate.

Four deficiencies (*mnDf63*, *syDf1*, *hDf6*, and *tDf3*) lead to stage-specific decreases in cell corpse number. Most notably, *tDf3* embryos showed fewer corpses during stages II and III, while corpses accumulated in the arrested embryos. This apparent delay in the production of corpses may be caused by the delayed execution of cell death in these embryos, as seen in *ced-8* mutant embryos.

Possible involvement of some, but not all, Ras path-

TABLE 3

Maternal rescue of the *ced-4* and *sDf121* PCD phenotype

Genotype of mother	Genotype of F_1 embryos	Average no. corpses per embryo
unc-79(e1068) ced-4(n1162)	*unc-79(e1068) ced-4(n1162)*	0 (20)
unc-79(e1068) ced-4(n1162)/+	*unc-79(e1068) ced-4(n1162)/+* or *unc-79(e1068) ced-4(n1162)/+* or *+/+*	6.3[a] (23)
sDf121 unc-32(e189)/unc-93(e1500) dpy-17(e164)	*sDf121 unc-32(e189)*	3.6[b] (7)
	sDf121 unc-32(e189)/unc-93(e1500) dpy-17(e164) or *unc-93(e1500) dpy-17(e164)*	5.0 (12)
sDf121 unc-32(e189)/unc-79(e1068) ced-4(n1162)	*sDf121 unc-32(e189)*	0 (4)
	sDf121 unc-32/unc-79(e1068) ced-4(n1162) or *unc-79(e1068) ced-4(n1162)*	0 (20)

Embryos derived from mothers of the indicated genotype were scored for PCD corpses at stage II. Number of embryos scored is shown in parentheses.

[a] Every embryo contained at least three corpses.

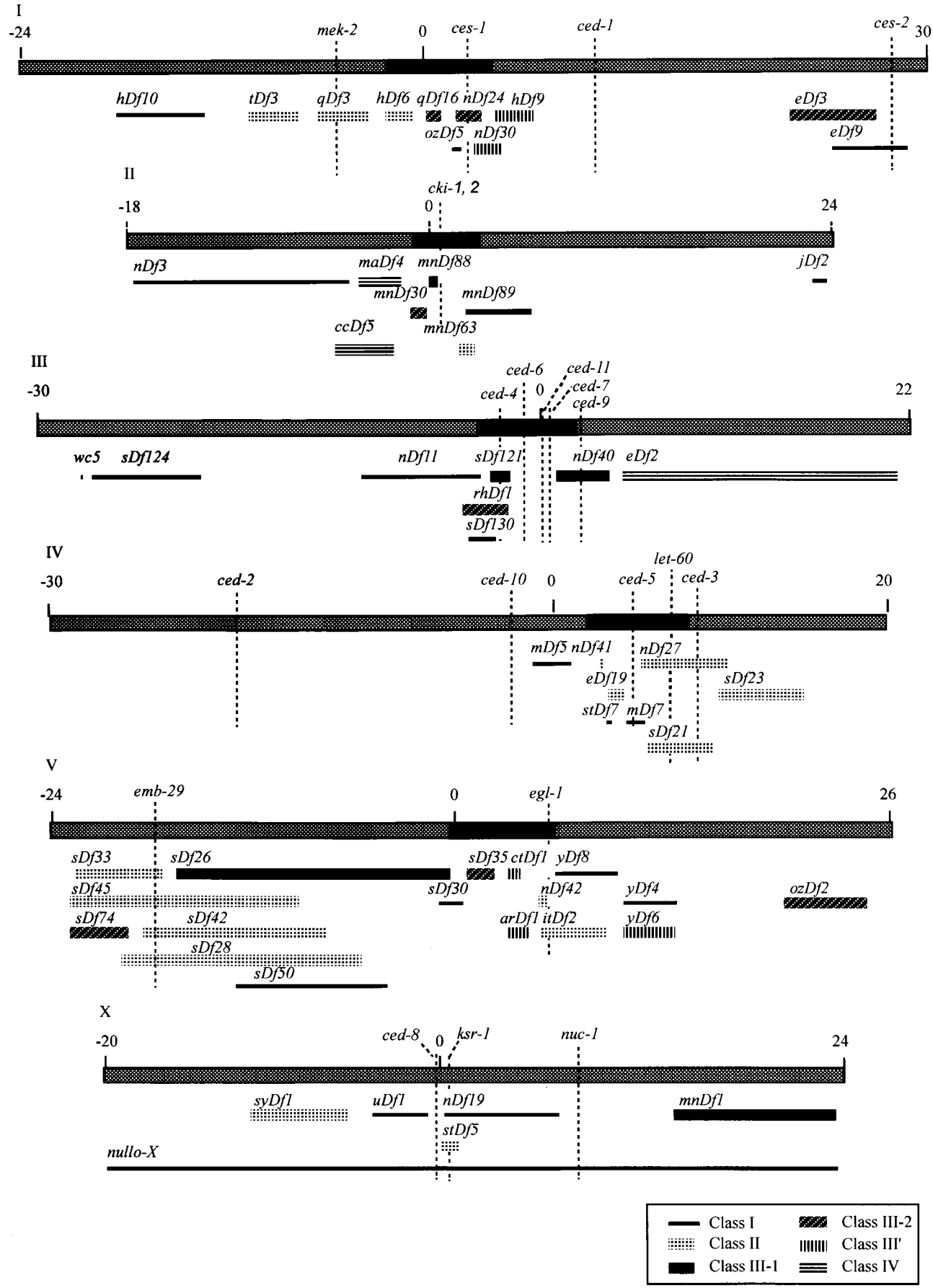
[b] Every embryo contained at least two corpses.

way components in embryonic PCD: Among the class II deficiencies that produce diminished numbers of corpses, two delete genes of the *ras* signaling pathway: *stDf5* deletes *ksr-1* (Kornfeld *et al.* 1995b; Sundaram and Han 1995), and *tDf3* deletes *mek-2* (Church *et al.* 1995; Kornfeld *et al.* 1995a; Wu *et al.* 1995; Figure 3). The Ras signaling pathway has been implicated in the regulation of apoptotic cell death in cultured mammalian cells (reviewed by Pritchard and McMahon 1997), and an activated form of Ras causes hyperplasia and increased cell death in a process that is dependent on the mitogen-activated protein kinase (MAPK) cascade in Drosophila (Karim and Rubin 1998). In *C. elegans*, germline cell death requires activation of the Ras/MAPK pathway (Gumienny *et al.* 1999). Unlike somatic cells, germ cells are produced by indeterminant rounds of cell division and PCD in the germline is subject to different control mechanisms than in somatic cells. It is not known whether the *ras* pathway is involved in PCD of cells arising from a defined cell lineage.

To investigate the possible role of Ras pathway components in embryonic PCD, we scored the profile of cell corpses during embryogenesis in *ksr-1* mutants. Mutations in *ksr-1* suppress the multivulva phenotype caused by activated *let-60 ras* but have no apparent phenotype in a wild-type background (Kornfeld *et al.* 1995b; Sundaram and Han 1995). We found that ~20% of the *ksr-1 (ku68)* embryos arrest between the 2-fold and early pretzel stages and these arrested embryos contained fewer cell corpses than did wild-type or the *ksr-1* embryos that did not arrest (Figure 4). By contrast, we found that *let-60 ras* mutants did not show dramatically altered developmental cell corpse profiles. Thus, while some components of the *ras* pathway may affect embryonic PCD, we could not obtain evidence for involvement of the entire pathway; this is likely to reflect a fundamental difference between the regulation of PCD in the germline and in somatic cells.

Stage-specific elevation of cell corpse number in class III deficiencies: Thirteen deficiencies result in abnormally elevated cell corpse numbers at one or more stages. We further subdivided these deficiencies into two subclasses based on their temporal effects. In class III-1 deficiencies (*sDf26*, *nDf40*, *mnDf1*, *sDf121*, and *mnDf88*), the number of corpses was elevated particularly in later and terminal embryos (Figure 2). Of these, *sDf26* and *nDf40* showed the strongest effect: the number of corpses at stage III and IV was approximately three times higher than that in wild type. By contrast, *mnDf88* showed normal corpse numbers through stage IV, but a large number were seen in terminal embryos.

As mentioned earlier, *nDf40* deletes *ced-9*, a cell death inhibitor. The maternal supply of CED-9 is sufficient to suppress PCD during embryogenesis, and homozygous *ced-9(−)* progeny of *ced-9(−)/+* mothers live and become sterile adults (Hengartner *et al.* 1992). Therefore, the excess cell corpses observed in *nDf40* embryos are unlikely to be caused by increased PCD resulting from the lack of *ced-9* function. To examine further whether the elevated cell corpse numbers result from increased numbers of dying cells or accumulation of corpses due to a defect in their engulfment, we scored for the persistence of individual cell corpses in 4-D time-lapse video images of recorded embryos (Schnabel 1991; Hird and White 1993; Moskowitz *et al.* 1994). In wild-type embryos, most corpses remain visible for <30 min (average 20.2 min). By contrast, in *nDf40* embryos, many corpses persisted for >30 min (Figure 5). The engulfment defect in *nDf40* homozygotes was more severe after stage II: while only one-third (11 out of 33) of the corpses persisted longer than 50 min prior to stage II, all 20 corpses remained for such an extended


I
-24
mek-2
0
ces-1
ced-1
ces-2
30
hDf10
tDf3
qDf3
hDf6
qDf16
nDf24
hDf9
ozDf5
nDf30
eDf3
eDf9
II
-18
cki-1, 2
0
24
nDf3
maDf4
mnDf88
jDf2
mnDf30
mnDf89
ccDf5
mnDf63
III
-30
ced-6
ced-11
ced-4
0
ced-7
ced-9
22
wc5
sDf124
nDf11
sDf121
nDf40
eDf2
rhDf1
sDf130
IV
-30
ced-2
ced-10
0
let-60
ced-5
ced-3
20
mDf5
nDf41
nDf27
eDf19
sDf23
stDf7
mDf7
sDf21
V
-24
emb-29
0
egl-1
26
sDf33
sDf26
sDf35
ctDf1
yDf8
sDf45
sDf30
nDf42
yDf4
ozDf2
sDf74
sDf42
arDf1
itDf2
yDf6
sDf28
sDf50
X
-20
ced-8
0
ksr-1
nuc-1
24
syDf1
uDf1
nDf19
mnDf1
stDf5
nullo-X
Class I
Class II
Class III-1
Class III-2
Class III'
Class IV

period after stage II. This observation explains why embryos homozygous for *nDf40* contained a normal number of corpses up until stage II, with a striking increase in corpse number thereafter. The defect in cell corpse engulfment can at least partially explain the elevated corpse numbers seen during embryogenesis, further supporting the view that removal of *ced-9* is not responsible for this phenotype.

In class III-2 deficiencies (*sDf74*, *nDf24*, *mnDf30*, *rhDf1*, *sDf35*, *ozDf2*, *qDf16*, and *eDf3*), cell corpse numbers are transiently elevated, but decrease in terminal embryos (Figure 2). *sDf74* shows the most drastic effect: *sDf74* homozygotes contain more corpses than do wild type even at stage I, and the numbers progressively increase through stage IV, reaching a peak average of 18.2 corpses per embryo; the number then drops to only 1.4 corpses in terminal embryos. By contrast, *nDf24* homozygotes show almost normal numbers until stage III; the number rapidly increased to an average of 14.5 corpses at stage IV, diminishing to an average of 8.3 in terminal embryos. In *qDf16* and *eDf3* embryos, there was a slight elevation in cell corpse number during early stages, but no corpses were observed in terminal embryos.

Possible maternal effects of class III′ deficiencies: Although the deficiency screen was designed to find regions required zygotically, we found three regions (*yDf6*, the overlapping region defined by *nDf30* and *hDf9*, and the overlapping region defined by *ctDf1* and *arDf1*) that, when deleted, show phenotypes suggesting a maternal requirement for normal PCD. In all three cases, embryos produced by heterozygous mothers show a higher number of corpses at each stage compared to wild type, even though only 25% were expected to be homozygous for the deficiency (Figure 6). In fact, arrested (*Df/Df*) and viable (*Df/+* or *+/+*) embryos showed similar numbers of cell corpses. The elevated cell corpse numbers seen in all embryos derived from such heterozygotes may be attributable to haploinsufficiency of the gene(s) responsible for the cell death phenotype: heterozygous mothers may contribute insufficient levels of gene product to the zygote to provide for normal function. An alternative explanation, *i.e.*, that the strains used in the screen were homozygous for an unknown mutation responsible for the phenotype, is unlikely, at least in the case of the regions defined by overlapping deficiencies: it is improbable that independently isolated deficiencies carry additional mutations that act similarly. Instead, it seems likely that these regions include genes that are maternally required in the cell death process.

Class IV deficiencies suggest that cell division and cell death can be uncoupled: Embryos homozygous for *eDf2*, *nDf41*, *ccDf5*, and *maDf4*, defining at least three genomic regions, showed prominent corpses that were abnormally large (Figure 7). In all cases, the number of corpses was greatly reduced compared to wild type (Figure 8). In addition, the arrested embryos contained fewer nuclei than the 558 of fully developed wild-type embryos: *eDf2* embryos arrested with ~420 cells, *nDf41* with ~180, and *ccDf5* and *maDf4* with <140. In the case of at least the two overlapping deficiencies, *ccDf5* and *maDf4*, the large cell corpse size is probably attributable to the programmed death of abnormally early (therefore larger) cells. Such a conclusion is consistent with the observation that *ccDf5* and *maDf4*, which arrested with the lowest number of nuclei, exhibited exclusively large corpses. Corpses in *eDf2* and *nDf41* embryos, though generally larger than those in wild type, were occasionally normal in size. These latter two deficiencies result in multinucleate hypodermal cells, suggesting defects in cytokinesis (Terns *et al.* 1997; M. Furuya and A. Sugimoto, unpublished observation). Moreover, *eDf2* deletes *cyk-4*, and *nDf41* deletes *zen-4*; loss-of-function mutations in either of these genes lead to multinucleate cells and both are required for completion of cytokinesis (Powers *et al.* 1998; Raich *et al.* 1998; Jantsch-Plunger *et al.* 2000; M. Furuya and A. Sugimoto, unpublished data). Thus, the large corpses in *eDf2* and *nDf41* are likely to be caused by the death of these multinucleate cells. However, we did not observe multinucleate cells in embryos homozygous for *ccDf5* and *maDf4*. The first PCD in wild-type *C. elegans* embryos occurs when there are ~250 cells (Sulston *et al.* 1983) and most arise after the 400-cell stage. Our finding that embryos containing as few as ~140 nuclei can nonetheless give rise to several cell corpses implies that activation of the cell death program is not absolutely dependent on the normal number of rounds of mitosis during embryogenesis.

DISCUSSION

New regions required for embryonic PCD: As an approach for identifying new genes required zygotically (and in some cases maternally), a major advantage of this deficiency screen is that extensive genomic regions,

Figure 3.—Map positions of deficiencies screened. Chromosomes are indicated by horizontal bars, the central gene clusters by black bars. The positions of known cell death genes and other genes relevant to this study are indicated above each chromosome. The approximate location of each deficiency is indicated below its corresponding chromosome. The pattern of the horizontal line representing each deficiency is coded according to the cell death phenotype it exhibited (see annotations in the figure). *yDf4* and *yDf6* are coextensive based on available map data, yet they exhibit different phenotypes; however, since the precise endpoints of these deficiencies have not been determined, it is certainly reasonable to suppose that the latter removes genes that the former does not.

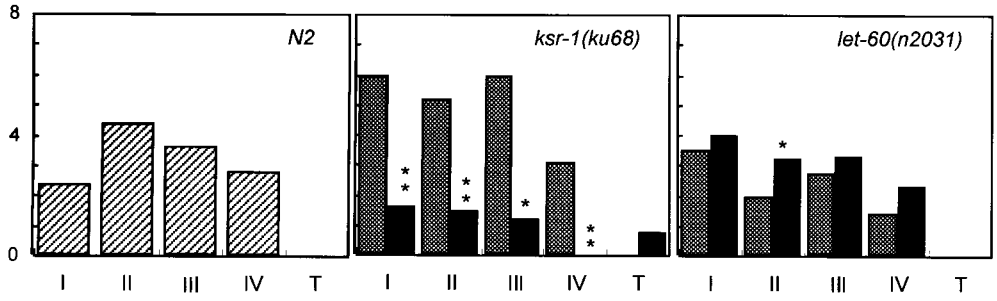


FIGURE 4.—Cell death profiles for Ras pathway mutants. For *ksr-1(ku68)*, arrested embryos (solid bars) are compared with embryos that developed normally (stippled bars). For *let-60(n2031)*, homozygotes are compared with +/+ or +/*let-60 (n2031)*. Asterisks represent *P* values, as described in Figure 1.

and therefore a large number of genes, can be screened simultaneously. From a screen of 58 deficiencies, covering an estimated 74% of the genome, we identified many genomic regions involved in embryonic PCD. At least 30 regions required for the normal number or appearance of embryonic PCDs were found, nearly all of which (28) correspond to genomic segments in which no known cell death genes reside. These results suggest that, although extensive screens for point mutations leading to abnormal PCD have been performed (*e.g.*, ELLIS and HORVITZ 1986; ELLIS *et al.* 1991; HENGARTNER *et al.* 1992; METZSTEIN *et al.* 1996; CONRADT and HORVITZ 1998; METZSTEIN and HORVITZ 1999), many cell death genes have yet to be identified and characterized in *C. elegans*.

The failure of past genetic screens to identify the relevant genes in the regions revealed by this study might be attributable to the lower sensitivity of the assay for aberrant PCD and/or the bias toward viable mutations in the screens for point mutations. By characterizing cell corpse numbers at multiple times during embryogenesis, our assay was sensitive to transient alterations in PCD, enabling us to identify subtle abnormalities (such as the stage-specific reduction, or increase, in corpse number), which might be missed by previous screening methods. In addition, all PCD-related genes previously reported in *C. elegans* were originally identified from viable mutants (ELLIS and HORVITZ 1986; ELLIS *et al.* 1991; CONRADT and HORVITZ 1998). Even the *ced-9* gene, for which loss-of-function alleles produce a maternal lethal phenotype, was originally identified from a viable gain-of function allele (HENGARTNER *et al.* 1992). The deficiency screen is not biased toward genes that mutate to viability and it is therefore likely that some of the relevant genes in the regions we identified may be essential for viability.

New genomic regions required for PCD: Many of the regions identified in the screen may contain previously unidentified genes that are directly involved in controlling PCD.

Deletion of nine distinct genomic regions (12 deficiencies) not containing a known PCD gene resulted in reduction of cell corpse number (class II). The class IV

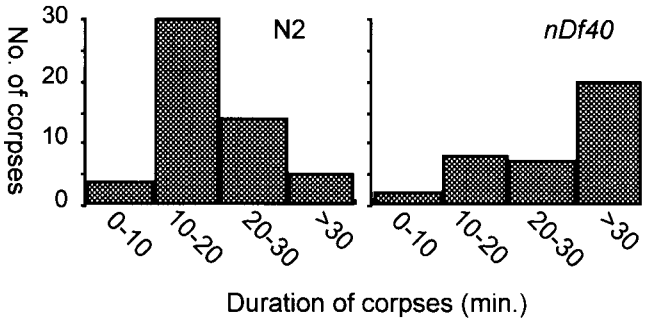


FIGURE 5.—Analysis of cell corpse duration in *nDf40*. Embryos homozygous for the indicated deficiency were recorded throughout embryogenesis by 4-D time lapse. All visible corpses from the recorded images were scored for their duration and histograms of their persistence time are shown.

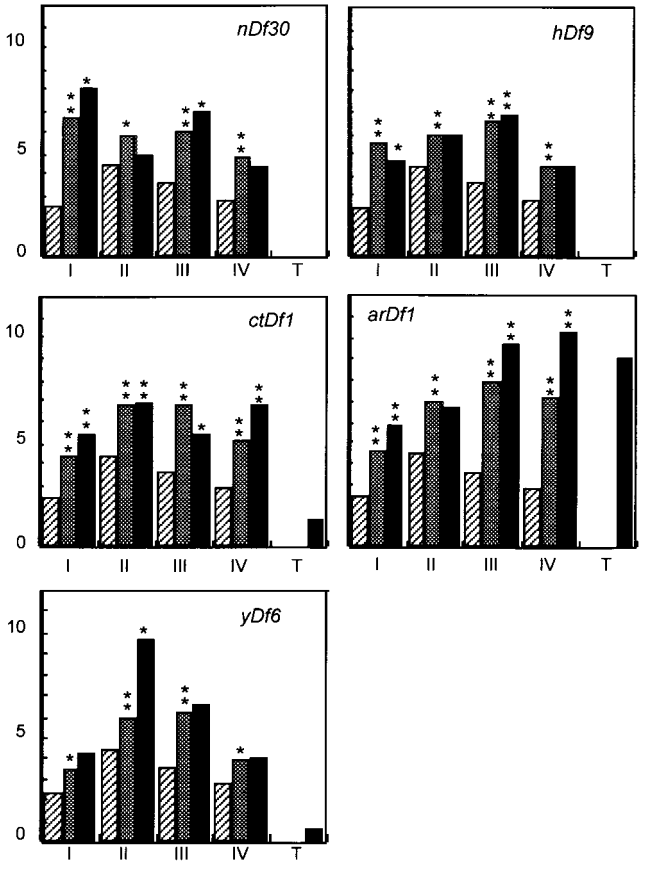


FIGURE 6.—Cell death profiles for class III′ deficiencies. Data presented as described in Figure 1.

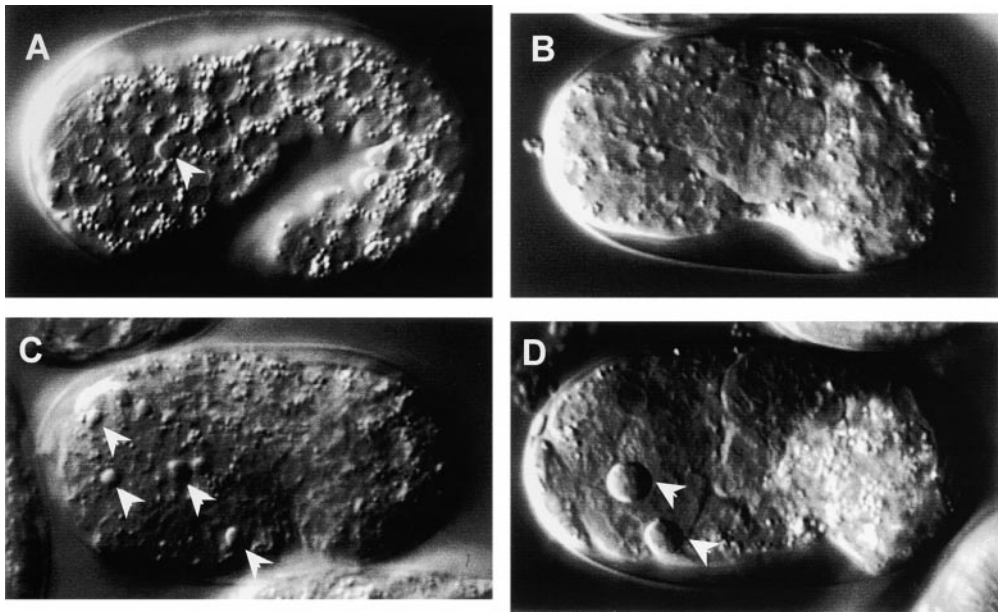


FIGURE 7.—Examples of cell death phenotypes seen in deficiency homozygotes. Nomarski image of a representative embryo from each class is shown. Cell corpses are indicated by arrowheads. (A) Wild-type embryo at the 1.5-fold stage. (B) Terminal *itDf2* homozygote (class II). No cell corpses are visible. (C) Terminal *stDf74* homozygote (class III). Many corpses are apparent. (D) Terminal *nDf41* homozygote (class IV). Abnormally large corpses are visible.

deficiencies (three regions; 4 deficiencies) also resulted in fewer corpses. However, none of these deficiencies completely eliminated PCD; thus, in the genomic regions we investigated there appear to be no genes that are zygotically essential for all PCD. It is possible, therefore, that all genes that are zygotically essential for PCD in *C. elegans* have been identified.

The deficiencies that delete *emb-29* (*sDf45*, *sDf33*, *sDf28*, and *sDf42*) and the class IV deficiencies (*eDf2*, *ccDf5/maDf4*, and *nDf41*) resulted in arrest with reduced numbers of nuclei; this premature arrest is likely to contribute to, but may not be entirely responsible for, the general decrease in corpse number. For those deficiencies that do not decrease nuclear numbers, the observed decrease in PCD might be the result of defects in mechanisms that determine the fate of cells destined for death. To date, only two genes, *ces-1* and *ces-2*, are known to be involved in such cell death determination processes in *C. elegans* (METZSTEIN *et al.* 1996; METZSTEIN and HORVITZ 1999). Both genes regulate a small subset of PCDs. The genomic regions defined by the deficiencies that result in normal cell numbers and reduced PCD may provide a starting point to identify other *ces*-like genes that determine the death fate of particular cells.

The removal of 13 genomic regions results in the excess accumulation of corpses. Five deficiencies (*mnDf88*, *sDf121*, *nDf40*, *sDf26*, and *mnDf1*) cause excess accumulation of corpses that persisted to the end of embryogenesis, while eight (*qDf16*, *nDf24*, *eDf3*, *mnDf30*, *rhDf1*, *sDf74*, *sDf35*, and *ozDf2*) led to a transient excess during embryogenesis. The excess corpse number may be the result of increased numbers of dying cells [as in *ced-9(lf)* mutants] or of defects in engulfment of corpses (as in the many known engulfment-defective mutants; HEDGECOCK *et al.* 1983; ELLIS *et al.* 1991). Our temporal analysis revealed that *nDf40* is defective in engulfment (Figure 5). Similar analysis of two other class III deficiencies, *sDf74* and *rhDf1*, revealed that these also lead to a severe defect in engulfment (our unpublished observations), suggesting that most, and possibly all, of the class III deficiencies might be engulfment mutants. Additional analyses of cell corpse persistence with the other class III deficiencies will reveal whether the increased cell corpse number is caused by excessive PCD or defective engulfment.

Although the deficiencies described here exhibited PCD defects, we cannot exclude the possibility that the aberrant numbers of PCD corpses are caused by dramatic changes in the embryonic cell lineage. In such a case, the relevant genes might play no role in the cell death program *per se*. Analyses of cell lineages will be

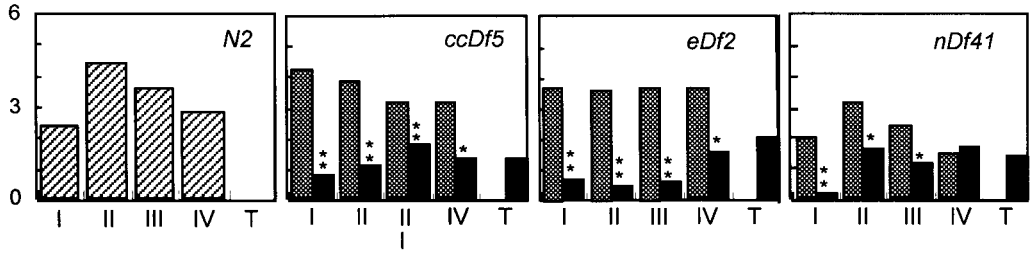


FIGURE 8.—Cell death profiles for class IV deficiencies. Data presented as described in Figure 1.

required to determine whether the altered PCD numbers are the result of direct defects in PCD or of dramatic transformations in embryonic cell fates.

Embryonic PCD and Ras signaling: In wild-type *C. elegans*, female germ cells undergo PCD near the region where cells exit the pachytene stage. It has been shown that the members of the Ras signaling pathway [*let-60* (ras), *lin-45* (raf), *mek-2* (MAPKK), and *mpk-1* (MAPK)] are required for exit from pachytene arrest, as well as for PCD in the female germline (Church *et al.* 1995). Thus, it was suggested that the Ras pathway might directly or indirectly regulate germline PCD. We have shown that a *ksr-1* mutant, and deficiencies that delete *ksr-1* or *mek-2*, depress PCD during embryogenesis. KSR-1 is a conserved Raf-related kinase and has been implicated as a positive regulator in the Ras signaling pathway, functioning in parallel to, or downstream of, Ras (Kornfeld *et al.* 1995b; Sundaram and Han 1995). Our results raise the possibility that this component of the Ras signaling pathway might participate not only in regulating PCD of female germ cells, but also in embryonic PCD. However, we were unable to find evidence that *ras* itself affects embryonic PCD, perhaps indicating that some, but not all, components in this pathway function in the embryo to regulate cell death.

Cell cycle and PCD: A number of studies have suggested a link between the regulatory mechanisms controlling cell proliferation and PCD (for review, Guo and Hay 1999; King and Cidlowski 1998; Lundberg and Weinberg 1999). For example, some cell cycle regulators also control apoptosis. The p53 tumor suppressor promotes either cell cycle arrest or apoptosis in response to DNA damage (Levine 1997). Survivin, an inhibitor of apoptosis (IAP) protein, is a cell cycle-regulated caspase inhibitor, suggesting that caspases may be active normally during particular phases of the cell cycle (Li *et al.* 1998). However, IAP homologues of *C. elegans* have not been implicated in PCD (Fraser *et al.* 1999) and the link between cell proliferation and PCD in *C. elegans* is not well understood.

Previous analysis of *lin-5* mutants demonstrated that nuclear deaths occur even when cell division does not occur (Albertson *et al.* 1978). In *lin-5* mutants, nuclear and cell divisions are blocked during postembryonic development, although DNA replication occurs. Despite the block in cytokinesis, the correct timing of developmental processes is retained, and cells exhibit varying characteristics of the differentiated descendants they would normally generate, including PCD. In the ventral nerve cord of *lin-5* larva, multinucleate cells were found to undergo programmed death. We have found that embryos homozygous for *eDf2* and *nDf41* similarly generate multinucleate cells that undergo PCD, probably accounting for the large corpse phenotype.

Our deficiency screen contributed two additional findings regarding the relationship between regulation of the cell cycle and PCD in *C. elegans*. First, we found that the program controlling the proper number of cell division rounds and that controlling PCD are separable. Two class IV deficiencies (*ccDf5* and *maDf4*) result in arrest with far fewer than the normal number of nuclei, do not appear to be defective in cytokinesis, and yet do not block PCD. Presumably, the regulatory machinery that normally activates PCD in particular lineages during embryogenesis is able to function even when too few cell divisions have occurred. This might indicate that PCD-promoting regulators are activated in response to a clock that is dependent upon absolute time rather than on rounds of cell division. By contrast, we also found that some specific cell cycle defects can apparently prevent cell death. The *emb-29* mutants and embryos homozygous for deficiencies that delete *emb-29* arrest with <200 nuclei but, unlike class IV deficiencies, few or no corpses were observed in these mutants.

One possible explanation for these contrasting phenotypes might be a difference in the stage of the cell cycle at which arrest occurs. Cells in *emb-29* mutants and in homozygous deficiencies that delete *emb-29* arrest at M phase (Hecht *et al.* 1987; our unpublished data). By contrast, cells of the class IV deficiencies appeared not to arrest at M phase, as judged by DAPI staining of chromosomes (data not shown). These observations suggest that it might not be possible to initiate PCD at M phase. While it is apparent that PCD might be regulated in a cell cycle-specific manner, the embryos lacking *emb-29* function differentiate various cell types, including neurons, muscles (twitching), and intestine, implying that M-phase arrest does not prevent all aspects of cellular differentiation. It will be interesting to identify the genes that simultaneously block both PCD and cell proliferation, in hopes of learning which cell cycle regulatory functions are also required for initiation of the apoptotic program.

We are grateful to the Caenorhabditis Genetics Center (Theresa Stiernagle) and to Ralph Hecht, David Baillie, and Andy Fire for strains. Some of the strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources of the National Institutes of Health (NIH). We thank Richard Halberg for initial assistance with analyses of deficiencies. A.S. was supported by a Naito Foundation Fellowship and a Leukemia Society of America Special Fellowship. R.H. was supported by a Cell and Molecular Biology predoctoral training grant from the NIH. W.B.D. was supported by a Cancer Foundation of Santa Barbara postdoctoral fellowship. This work was supported by grants from the NIH (GM-48137 and AG-13736), a Searle Scholars Award from the Chicago Community Trust, and a Shaw Scientists Award from the Milwaukee Foundation to J.H.R.

LITERATURE CITED

Ahnn, J., and A. Fire, 1994 A screen for genetic loci required for body-wall muscle development during embryogenesis in *Caenorhabditis elegans*. Genetics **137:** 483–498.

Albertson, D. G., J. E. Sulston and J. G. White, 1978 Cell cycling and DNA replication in a mutant blocked in cell division in the nematode Caenorhabditis elegans. Dev. Biol. **63:** 165–178.

Bilder, D., and M. P. Scott, 1995 Genomic regions required for

morphogenesis of the Drosophila embryonic midgut. Genetics **141:** 1087–1100.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Chanal, P., and M. Labouesse, 1997 A screen for genetic loci required for hypodermal cell and glial-like cell development during *Caenorhabditis elegans* embryogenesis. Genetics **146:** 207–226.

Chen, P., W. Nordstrom, B. Gish and J. M. Abrams, 1996 grim, a novel cell death gene in Drosophila. Genes Dev. **10:** 1773–1782.

Chinnaiyan, A. M., D. Chaudhary, K. O'Rourke, E. V. Koonin and V. M. Dixit, 1997a Role of CED-4 in the activation of CED-3. Nature **388:** 728–729.

Chinnaiyan, A. M., K. O'Rourke, B. R. Lane and V. M. Dixit, 1997b Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science **275:** 1122–1126.

Church, D. L., K. L. Guan and E. J. Lambie, 1995 Three genes of the MAP kinase cascade, *mek-2, mpk-1/sur-1* and *let-60 ras*, are required for meiotic cell cycle progression in Caenorhabditis elegans. Development **121:** 2525–2535.

Conradt, B., and H. R. Horvitz, 1998 The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell **93:** 519–529.

del Peso, L., V. M. Gonzalez and G. Nunez, 1998 Caenorhabditis elegans EGL-1 disrupts the interaction of CED-9 with CED-4 and promotes CED-3 activation. J. Biol. Chem. **273:** 33495–33500.

Ellis, H. M., and H. R. Horvitz, 1986 Genetic control of programmed cell death in the nematode C. elegans. Cell **44:** 817–829.

Ellis, R. E., D. M. Jacobson and H. R. Horvitz, 1991 Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. Genetics **129:** 79–94.

Fraser, A. G., C. James, G. I. Evan and M. O. Hengartner, 1999 *Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. Curr. Biol. **9:** 292–301.

Gossett, L. A., and R. M. Hecht, 1980 A squash technique demonstrating embryonic nuclear cleavage of the nematode Caenorhabditis elegans. J. Histochem. Cytochem. **28:** 507–510.

Grether, M. E., J. M. Abrams, J. Agapite, K. White and H. Steller, 1995 The head involution defective gene of Drosophila melanogaster functions in programmed cell death. Genes Dev. **9:** 1694–1708.

Gumienny, T. L., E. Lambie, E. Hartweig, H. R. Horvitz and M. O. Hengartner, 1999 Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Development **126:** 1011–1022.

Guo, M., and B. A. Hay, 1999 Cell proliferation and apoptosis. Curr. Opin. Cell. Biol. **11:** 745–752.

Hecht, R. M., M. Berg-Zabelshansky, P. N. Rao and F. M. Davis, 1987 Conditional absence of mitosis-specific antigens in a temperature-sensitive embryonic-arrest mutant of *Caenorhabditis elegans*. J. Cell Sci. **87:** 305–314.

Hedgecock, E. M., J. E. Sulston and J. N. Thomson, 1983 Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. Science **220:** 1277–1279.

Hengartner, M. O., and H. R. Horvitz, 1994 C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell **76:** 665–676.

Hengartner, M. O., R. E. Ellis and H. R. Horvitz, 1992 Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature **356:** 494–499.

Hird, S. N., and J. G. White, 1993 Cortical and cytoplasmic flow polarity in early embryonic cells of Caenorhabditis elegans. J. Cell Biol. **121:** 1343–1355.

Hodgkin, J., H. R. Horvitz and S. Brenner, 1979 Nondisjunction mutants of the nematode *C. elegans*. Genetics **91:** 67–94.

Jacobson, M. D., M. Weil and M. C. Raff, 1997 Programmed cell death in animal development. Cell **88:** 347–354.

Jantsch-Plunger, V., P. Gonczy, A. Romano, H. Schnabel, D. Hamill *et al.*, 2000 CYK-4: A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. J. Cell Biol. **149:** 1391–1404.

Karim, F. D., and G. M. Rubin, 1998 Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. Development **125:** 1–9.

King, K. L., and J. A. Cidlowski, 1998 Cell cycle regulation and apoptosis. Annu. Rev. Physiol. **60:** 601–617.

Kornfeld, K., K. L. Guan and H. R. Horvitz, 1995a The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. Genes Dev. **9:** 756–768.

Kornfeld, K., D. B. Hom and H. R. Horvitz 1995b The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. Cell **83:** 903–913.

Labouesse, M., 1997 Deficiency screen based on the monoclonal antibody MH27 to identify genetic loci required for morphogenesis of the Caenorhabditis elegans embryo. Dev. Dyn. **210:** 19–32.

Levine, A. J., 1997 p53, the cellular gatekeeper for growth and division. Cell **88:** 323–331.

Lewis, J. A., and J. T. Fleming, 1995 Basic culture methods, pp. 3–29 in *Caenorhabditis elegans: Modern Biological Analysis of an Organism*, edited by H. F. Epstein and D. C. Shakes. Academic Press, San Diego.

Li, F., G. Ambrosini, E. Y. Chu, J. Plescia, S. Tognin *et al.*, 1998 Control of apoptosis and mitotic spindle checkpoint by survivin. Nature **396:** 580–584.

Lundberg, A. S., and R. A. Weinberg, 1999 Control of the cell cycle and apoptosis. Eur. J. Cancer **35:** 1886–1894.

Merrill, P. T., D. Sweeton and E. Wieschaus, 1988 Requirements for autosomal gene activity during precellular stages of Drosophila melanogaster. Development **104:** 495–509.

Metzstein, M. M., and H. R. Horvitz, 1999 The C. elegans cell death specification gene ces-1 encodes a snail family zinc finger protein. Mol. Cell **4:** 309–319.

Metzstein, M. M., M. O. Hengartner, N. Tsung, R. E. Ellis and H. R. Horvitz, 1996 Transcriptional regulator of programmed cell death encoded by Caenorhabditis elegans gene ces-2. Nature **382:** 545–547.

Metzstein, M. M., G. M. Stanfield and H. R. Horvitz, 1998 Genetics of programmed cell death in C. elegans: past, present and future. Trends Genet. **14:** 410–416.

Moskowitz, I. P., S. B. Gendreau and J. H. Rothman, 1994 Combinatorial specification of blastomere identity by glp-1-dependent cellular interactions in the nematode Caenorhabditis elegans. Development **120:** 3325–3338.

Powers, J., O. Bossinger, D. Rose, S. Strome and W. Saxton, 1998 A nematode kinesin required for cleavage furrow advancement. Curr. Biol. **8**(20): 1133–1136.

Pritchard, C., and M. McMahon, 1997 Raf revealed in life-or-death decisions. Nat. Genet. **16:** 214–215.

Raich, W. B., A. N. Moran, J. H. Rothman and J. Hardin, 1998 Cytokinesis and midzone microtubule organization in Caenorhabditis elegans require the kinesin-like protein ZEN-4. Mol. Biol. Cell **9**(8): 2037–2049.

Schnabel, R., 1991 Cellular interactions involved in the determination of the early C. elegans embryo. Mech. Dev. **34:** 85–99.

Seshagiri, S., and L. K. Miller, 1997 Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. Curr. Biol. **7:** 455–460.

Smith, A. V., J. A. King and T. L. Orr-Weaver, 1993 Identification of genomic regions required for DNA replication during Drosophila embryogenesis. Genetics **135:** 817–829.

Song, Z., and H. Steller, 1999 Death by design: mechanism and control of apoptosis. Trends Cell Biol. **9:** M49–M52.

Stanfield, G. M., and H. R. Horvitz, 2000 The ced-8 gene controls the timing of programmed cell deaths in C. elegans. Mol. Cell **5:** 423–433.

Storfer-Glazer, F. A., and W. B. Wood, 1994 Effects of chromosomal deficiencies on early cleavage patterning and terminal phenotype in *Caenorhabditis elegans* embryos. Genetics **137:** 499–508.

Sulston, J. E., and H. R. Horvitz, 1977 Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. **56:** 110–156.

Sulston, J. E., E. Schierenberg, J. G. White and J. N. Thomson, 1983 The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. **100:** 64–119.

Sundaram, M., and M. Han, 1995 The *C. elegans ksr-1* gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:** 889–901.

Terns, R. M., P. Kroll-Conner, J. Zhu, S. Chung and J. H. Rothman, 1997 A deficiency screen for zygotic loci required for establishment and patterning of the epidermis in *Caenorhabditis elegans*. Genetics **146:** 185–206.

White, K., M. E. Grether, J. M. Abrams, L. Young, K. Farrell *et al.*, 1994 Genetic control of programmed cell death in Drosophila. Science **264:** 677–683.

Wieschaus, E., and D. Sweeton, 1988 Requirements for X-linked zygotic gene activity during cellularization of early Drosophila embryos. Development **104:** 483–493.

Wu, Y., M. Han and K. L. Guan, 1995 MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes Dev. **9:** 742–755.

Xue, D., and H. R. Horvitz, 1997 Caenorhabditis elegans CED-9 protein is a bifunctional cell-death inhibitor. Nature **390:** 305–308.

Xue, D., S. Shaham and H. R. Horvitz, 1996 The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev. **10:** 1073–1083.

Yuan, J., and H. R. Horvitz, 1992 The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. Development **116:** 309–320.

Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis and H. R. Horvitz, 1993 The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell **75:** 641–652.

Zhu, J., R. J. Hill, P. J. Heid, M. Fukuyama, A. Sugimoto *et al.*, 1997 end-1 encodes an apparent GATA factor that specifies the endoderm precursor in Caenorhabditis elegans embryos. Genes Dev. **11:** 2883–2896.

Zou, H., W. J. Henzel, X. Liu, A. Lutschg and X. Wang, 1997 Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell **90:** 405–413.

Communicating editor: P. Anderson