Mosaic Analysis Using a *ncl-1* (+) Extrachromosomal Array Reveals That *lin-31* Acts in the Pn.p Cells During *Caenorhabditis elegans* Vulval Development

Leilani M. Miller,*,[1] David A. Waring[†] and Stuart K. Kim*

**Department of Developmental Biology, Stanford University Medical Center, Stanford, California 94305 and [†]Fred Hutchinson Cancer Research Center, Seattle, Washington 98104*

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ABSTRACT

We describe a genetic mosaic analysis procedure in which *Caenorhabditis elegans* mosaics are generated by spontaneous loss of an extrachromosomal array. This technique allows almost any *C. elegans* gene that can be used in germline transformation experiments to be used in mosaic analysis experiments. We identified a cosmid clone that rescues the mutant phenotype of *ncl-1,* so that this cell-autonomous marker could be used to analyze mosaic animals. To determine the sites of action for *unc-29* and *lin-31,* an extrachromosomal array was constructed containing the *ncl-1*(+) cosmid linked to *lin-31*(+) and *unc-29*(+) cosmids. This array is mitotically unstable and can be lost to produce a clone of mutant cells. The specific cell division at which the extrachromosomal array had been lost was deduced by scoring the Ncl phenotypes of individual cells in genetic mosaics. The Unc-29 and Lin-31 phenotypes were then scored in these animals to determine in which cells these genes are required. This analysis showed that *unc-29,* which encodes a subunit of the acetylcholine receptor, acts in the body muscle cells. Furthermore, *lin-31,* which specifies cell fates during vulval induction and encodes a putative transcription factor similar to HNF-3/fork head, acts in the Pn.p cells.

MANY developmental and behavioral processes involve intercellular interactions in which one cell signals another cell, such as the induction of target cell differentiation by regulatory cells or the stimulation of muscle cell contraction by nerve cells. In using genetic analysis to dissect these intercellular interactions, it is important to determine whether mutations that disrupt the interactions cause defects in the inducing or target cell. This can be accomplished by genetic mosaic analysis. In *Caenorhabditis elegans,* one previous approach has been to study genetic mosaic animals that were generated when an unattached fragment of a chromosome, called a free duplication, was spontaneously lost in a fraction of the cells during development (HERMAN 1984, 1989). In a strain carrying a free duplication, the free duplication itself contains wild-type copies of a cell lineage marker and a gene of interest, whereas the chromosomal copies of these genes are mutant. Mitotic loss of the free duplication in a single founder cell results in a clone of cells that lacks the activities of both the marker gene and the gene of interest. The identity of the founder cell that gave rise to the entire clone of mutant cells can be inferred by determining the marker gene phenotype of specific lineages and correlating the pattern of mutant cells with the complete cell lineage of *C. elegans* (see Figure 1). Cells generated by the founder cell must be mutant for both the marker gene and the gene of interest. Finally, scoring the phenotype of the gene of interest in the mosaic animal allows one to deduce which cells require its activity.

We have used loss of an extrachromosomal array, rather than loss of a free duplication, to generate genetic mosaic animals. In *C. elegans,* extrachromosomal arrays are generated when DNA is injected into hermaphrodite gonads. These arrays are mixed concatamers of each of the microinjected DNA clones that behave genetically like free duplications (STINCHCOMB *et al.* 1985). We reasoned that extrachromosomal arrays could be used to generate genetic mosaic animals in a manner similar to free duplications. One advantage of using extrachromosomal arrays rather than free duplications for mosaic analysis is that desirable mosaic markers can be used even if they are not near the gene of interest on the genetic map, since DNA clones of marker gene(s) and the gene of interest can be artificially linked on an extrachromosomal array. *ncl-1* is an especially useful cell lineage marker because *ncl-1* mutations alter the nuclear morphology of a large number of cell types in a cell-autonomous fashion. Most cells mutant for *ncl-1* display enlarged nucleoli and are referred to as Ncl (HEDGECOCK and HERMAN 1995). We identified a cosmid clone that rescues the mutant phenotype of *ncl-1* animals to use it as a cell lineage marker on extrachromosomal arrays.

We have used this approach to study one example of intercellular signaling by determining where the *lin-31* gene acts during induction of the *C. elegans* vulva. In

Corresponding author: Stuart K. Kim, Department of Developmental Biology, Stanford University Medical Center, Stanford, CA 94305. E-mail: kim@cmgm.stanford.edu

[1] *Present address:* Department of Biology, Santa Clara University, Santa Clara, CA 95053.

vulval development, each of the six vulval precursor cells (P3.p–P8.p; hereafter referred to as Pn.p) adopts a cell fate (1°, 2°, or 3°) in response to extracellular signals (reviewed in HORVITZ and STERNBERG 1991; EISENMANN and KIM 1994). LIN-3 is thought to be an inductive signal from the anchor cell that causes the nearest Pn.p cell (P6.p) to adopt the 1° cell fate (HILL and STERNBERG 1992). Subsequently, a lateral signal from P6.p causes the two adjacent Pn.p cells (P5.p and P7.p) to adopt 2° cell fates (STERNBERG 1988; KOGA and OHSHIMA 1995; SIMSKE and KIM 1995). Pn.p cells may also express the 2° cell fate in response to low levels of signal from the anchor cell (STERNBERG and HORVITZ 1986; THOMAS *et al.* 1990; KATZ *et al.* 1995). P3.p, P4.p and P8.p do not respond to inductive signals and adopt an uninduced (3°) cell fate. In addition, a signal from the hyp7 hypodermal syncytium, which surrounds most of the worm, may inhibit Pn.p cells from adopting induced cell fates (HERMAN and HEDGECOCK 1990). The cells generated by the 1° and 2° lineages form the vulva, while the cells generated by the 3° lineages contribute to hyp7 (SULSTON and HORVITZ 1977).

The *lin-31* gene is required for the Pn.p cells to adopt appropriate vulval cell fates (FERGUSON and HORVITZ 1985; MILLER *et al.* 1993). In *lin-31* mutants, the three cells that normally express 1° or 2° cell fates (P5.p, P6.p and P7.p) may sometimes express the uninduced 3° cell fate, and the three cells that normally express the uninduced 3° cell fate (P3.p, P4.p and P8.p) may instead express 1° or 2° cell fates. *lin-31* encodes a protein with significant similarity to the DNA binding domain of the HNF-3/fork head transcription factor family (MILLER *et al.* 1993). We used genetic mosaic analysis to show that *lin-31* acts in the Pn.p cells. This site-of-action, combined with the presence of a DNA binding domain, indicates that *lin-31* may be involved in the transcriptional regulation of vulval gene expression in response to extracellular signals.

MATERIALS AND METHODS

Genes, alleles and general procedures: The animals described as wild type were *C. elegans*, variety Bristol, strain N2. Animals were grown at 20° and standard techniques were used for maintenance and handling of *C. elegans* strains (BRENNER 1974). The following genes and mutations were used: LGI, *unc-29(e1072)* (WOOD 1988); LGII, *lin-31(n1053)* (FERGUSON and HORVITZ 1985); LGIII, *ncl-1(e1942, e1865)* (WOOD 1988); LGX, *let-2(g37ts)* (J. KRAMER, personal communication). Standard techniques were used for all molecular biology experiments (SAMBROOK *et al.* 1989). The plasmid pLM268 contains a 14-kb *Spe* I fragment spanning the entire *lin-31* gene (including 4 kb of sequence upstream of the mRNA start site) inserted at the *Spe* I site of the Bluescript II SK+ vector (Stratagene). Individual cells were identified and Pn.p cell lineages were analyzed as described in SULSTON and HORVITZ (1977), using the criteria for the assignment of 1°, 2°, and 3° cell fates described in STERNBERG and HORVITZ (1986).

Germline transformation: Animals of genotype *unc-29 (e1072); lin-31(n1053); ncl-1(e1942); let-2(g37ts)* were injected with cosmid C45D10 (rescues the Unc-29 mutant phenotype; LACKNER *et al.* 1994), cosmid C33C3 (rescues the Ncl-1 mutant phenotype; this paper), and pLM268 (rescues the Lin-31 mutant phenotype; MILLER *et al.* 1993) at DNA concentrations of ~50 μg/ml each, using the method of FIRE (1986), as modified by MELLO *et al.* (1991). Cosmid C33C3 rescued the mutant phenotypes of both *ncl-1(e1942)* and *ncl-1(e1865)*. The *let-2(g37ts)* mutation does not cause a mutant phenotype at the permissive temperature of 20° and was originally included in this strain so that DNA containing *let-2(+)* could be used as a tranformation marker. For these experiments, *unc-29* was used as a coinjection marker. Other markers (*e.g., unc-30* and *unc-36*) have also been used successfully with this technique (HERMAN *et al.* 1995; SIMSKE and KIM 1995). We obtained one transgenic strain (SD167) with an extrachromosomal array (*gaEx25*) that rescued the Unc-29, Lin-31, and Ncl-1 mutant phenotypes. The *lin-31(+)* gene in the *gaEx25* extrachromosomal array efficiently rescues the *lin-31* mutant phenotype, since every Pn.p cell of each of five non-Unc, nonmosaic animals (30 Pn.p cells total) adopted a wild-type cell fate.

We determined the approximate frequency with which the array is transmitted through the germline (transmission frequency) in SD167. We scored the Unc-29 phenotype in entire broods from non-Unc animals and found that 1155 of 1762 (66%) progeny were non-Unc. The frequency of non-Unc animals may differ slightly from the transmission frequency, since some of the Unc animals may be genetic mosaics and display an Unc phenotype even though they inherited the extrachromosomal array. Conversely, some of the parental animals may have carried multiple copies of the extrachromosomal array and transmitted the array to a higher fraction of progeny than parents with a single copy. The transmission frequency correlates with the frequency with which genetic mosaics are generated because loss of an array (or free duplication) in the germline is primarily due to loss during the mitotic divisions in the soma that generates the germline or to the mitotic divisions in the germline that generate the gametes (HERMAN 1995; A. VILLENEUVE, personal communication). For these experiments, we found a transmission frequency of ~66% to be satisfactory for mosaic analysis; it is high enough that genetic mosaics can be found and low enough that the majority of mosaics at the 28-cell stage have lost the extrachromosomal array only once. Transmission frequencies of 40, 44, 57, and 59% have also proven to be satisfactory for mosaic analysis (LACKNER *et al.* 1994; L. M. MILLER, unpublished observations; J. SIMSKE, personal communication).

The mitotic loss rate for *gaEx25* was calculated as follows. Of the cells shown in Figure 1, loss of the extrachromosomal array at any one of 24 cell divisions would generate a mosaic animal with at least two Ncl cells. We found four such losses in the 50 animals that were scored. In those four mosaic animals, 29 cell divisions were excluded since the mother cell had previously lost the extrachromosomal array. Thus, the mitotic loss rate is $4/1171 = 3.4 \times 10^{-3}$ losses per cell division.

Identification of mosaic animals: Nomarski optics were used to screen 2406 non-Unc animals (20–25/slide) from SD167 for mosaic animals in which some, but not all, cells were Ncl. This screen yielded 41 mosaic animals that had lost the array before the 28-cell stage and thus were suitable for use in the *lin-31* and *unc-29* mosaic analysis experiments. Most of these non-Unc animals (34) had lost the array in the AB blastomere or in one of its descendants, since losses at most other points in the early cell lineage generates semi-Unc or Unc mosaic animals (Figure 2). A few animals (seven) had lost the array in one of the descendants of MS and were non-Unc, presumably because only a small fraction of body muscle

cells lacked *unc-29*(+) activity. A dissecting microscope was used to screen an additional 2045 Unc animals (lethargic movement on the petri plate) for those with residual movement when placed in liquid (thrashed in M9 buffer, whereas Unc-29 mutant animals do not thrash in liquid); this phenotype is referred to as semi-Unc. Sixty-nine of the 2045 animals (3.4%) were semi-Unc. The Ncl phenotypes of individual cells in these semi-Unc animals were determined using Nomarski optics (see below), and all animals were found to be genetic mosaics. Twenty-nine (42%) of these genetic mosaics appeared to have lost the array at a single point in the lineage, applying the stringent criteria that all of the observed Ncl cells were derived from one point in the lineage and no Ncl cells were observed from other points in the lineage. All 29 of these mosaics had lost the array in cells derived from P_1, as would be expected for *unc-29* mosaic animals with a site-of-action in the body muscle cells (which are predominantly derived from P_1). Nine of the single loss mosaics had lost the array at some point in the lineage leading to the anchor cell and were analyzed in the *unc-29* and *lin-31* mosaic analysis experiments (Figures 2 and 3). Several hundred Unc animals from SD167 were also screened for genetic mosaics by directly observing the Ncl phenotypes of individual cells using Nomarski microscopy, and none were found.

The following specific patterns of Ncl cells were used to assign mosaic loss at each of the different cell divisions. All mosaic animals analyzed in this paper contained multiple Ncl cells in a pattern consistent with loss of the extrachromosomal array at a single point in the cell lineage. Pharyngeal neurons (I1L, I1R, I2L, I2R, I5, NSML, NSMR, M2L, M2R, M3L, M3R, MCL, MCR, and MI) (ALBERTSON and THOMSON 1976), ring neurons ALA and RID, and amphid neurons ASKL and ADLL were used to identify loss of the array at AB (all ring neurons Ncl) or AB.a (half of the ring neurons Ncl). Postdeirids, the excretory cell, and the Pn.a-derived neurons were used to identify loss of the array at AB.p. Ring neurons other than ALA and RID were scored as a group; a Ncl phenotype for all ring neurons indicates loss at AB, and a Ncl phenotype for approximately half of the ring neurons was used as one criterion to assign loss at AB.a or AB.p. The excretory cell, the left postdeirid, and half of the Pn.a cells identified AB.pl losses; the right postdeirid, hyp10, and one half of the Pn.a cells identified AB.pr mosaics. hyp10 is a syncytium containing two nuclei, both of which must be mutant to display the Ncl mutant phenotype (HEDGECOCK and HERMAN 1995). Pharyngeal neurons (I3, I4, I6, M1, M4, and M5) (ALBERTSON and THOMSON 1976), both distal tip cells, the anchor cell, and all body muscle cells in the head were used to identify loss of the array at EMS or MS. EMS and MS losses could not be distinguished because intestinal cells normally have enlarged nucleoli, and loss of *ncl-1* activity does not change their nucleolar phenotype. Losses in a descendant of MS exhibited a Ncl phenotype in some but not all of the MS-derived cells and were divided into two groups based on whether any body muscle cells in the head were Ncl. Although there could be exceptions, mosaics in which some head muscle cells were Ncl would typically include losses of the extrachromosomal array at an earlier cell division than mosaics in which none of the head muscle cells were Ncl. A specific pattern of body muscle cells posterior to the head was used to identify loss of the extrachromosomal array in the C blastomere (SULSTON *et al.* 1983; AUSTIN and KIMBLE 1987).

RESULTS

Rescue of the *ncl-1* mutant phenotype in germline transformation experiments: *ncl-1* is an excellent cell lineage marker that can provide single cell resolution in mosaic analysis experiments (HEDGECOCK and HERMAN 1995). To use *ncl-1* for our mosaic analysis experiments, we needed to identify a cosmid clone that contains the *ncl-1* gene to link it to our gene of interest, *lin-31*, in an extrachromosomal array. *ncl-1* is located in the small genetic interval (0.1 map unit) between *lin-39* and *mab-5* on chromosome *III*. Molecular cloning experiments have established the positions of both *lin-39* and *mab-5* on the *C. elegans* physical map (COSTA *et al.* 1988; CLARK *et al.* 1993; WANG *et al.* 1993), and the region between these two genes is spanned by nine overlapping cosmids, covering a maximum of 360 kb (COULSON *et al.* 1986). We found that one of these nine cosmids (C33C3) was able to rescue the phenotype of *ncl-1* mutants in germline transformation experiments, strongly suggesting that it contains the *ncl-1* gene (see MATERIALS AND METHODS).

Mosaic analysis using an extrachromosomal array: We generated a strain (SD167) that has an extrachromosomal array (*gaEx25*) containing wild-type copies of a transformation marker (*unc-29*), a mosaic marker (*ncl-1*), and our gene of interest (*lin-31*), and in which the chromosomal copies of these three genes are mutant (see MATERIALS AND METHODS). To determine if this strain was suitable for use in mosaic analysis experiments, we characterized three aspects of the genetic behavior of the extrachromosomal array in SD167.

First, we needed to determine if SD167 generates mosaic animals. We scored the Ncl and non-Ncl phenotypes of specific individual cells in 50 animals with a non-Unc-29 phenotype that were picked at random from the SD167 strain and found that 20 had both Ncl and non-Ncl cells (Figure 1). We then showed that the Ncl phenotypes of individual cells in those animals are likely due to mitotic loss of the extrachromosomal array, rather than to insufficient gene expression from the array. Four animals displayed simple Ncl patterns in which all of the Ncl mutant cells were derived from a single progenitor cell and all of the scored cells derived from that progenitor cell displayed the Ncl mutant phenotype. These simple Ncl patterns strongly suggest that the Ncl mutant phenotypes in these animals were caused by spontaneous loss of the array at a single point in the cell lineage during development. Twelve animals exhibited a single Ncl mutant cell (of those cells that were scored). For those 12 animals, one possibility is that each has lost the extrachromosomal array at a late point in the cell lineage, such that only one of the scored cells lacked the array. Because only one scored cell in these 12 animals is Ncl, we cannot rule out the possibility that the Ncl phenotype of that one cell is due to variable expression of the array. Four animals showed more complex Ncl patterns, in which Ncl mutant cells were derived from multiple points in the cell lineage. These complex Ncl patterns could be due to loss of the array at multiple points in the cell lineage

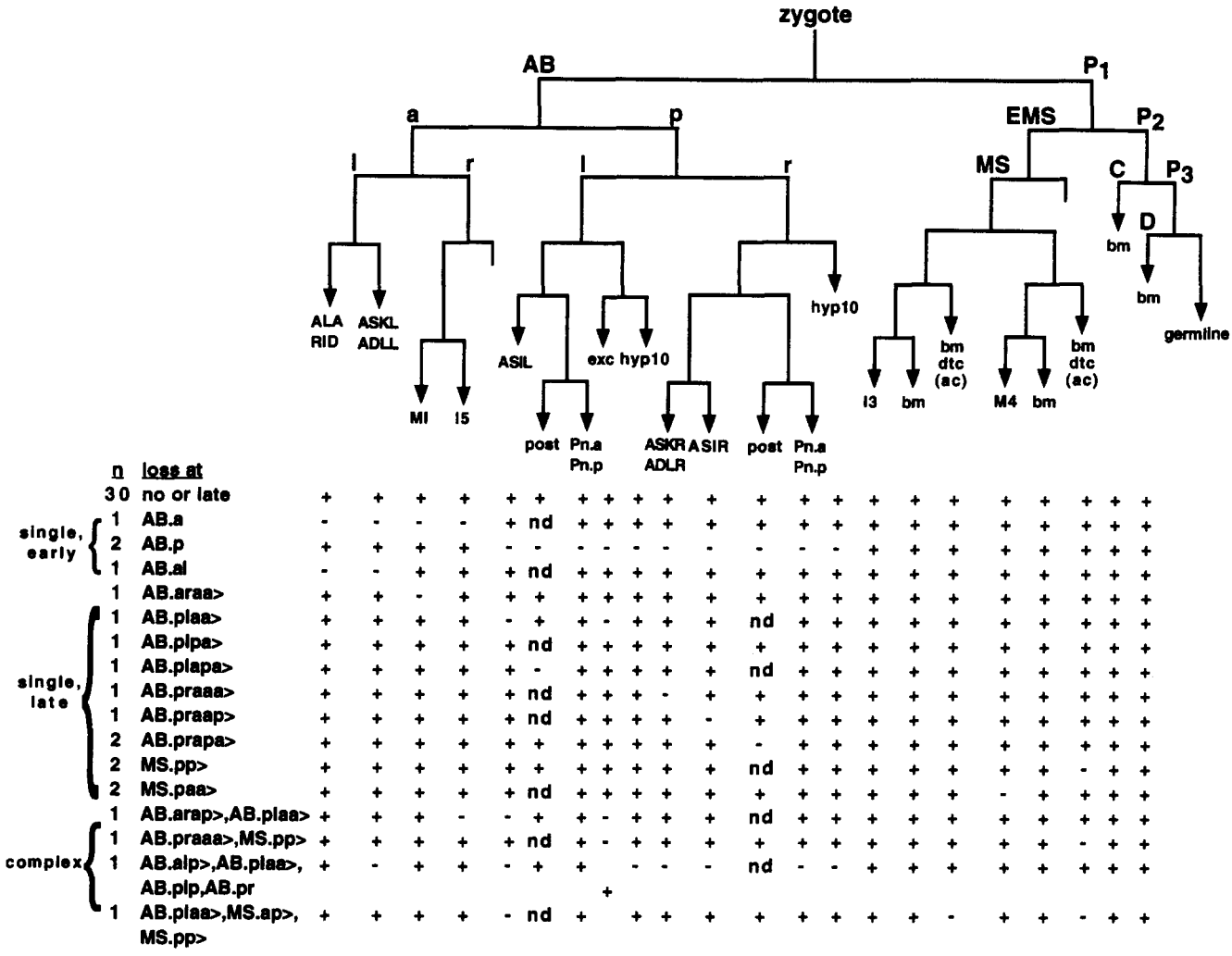

FIGURE 1.—Genetic mosaic animals. Ncl phenotypes of specific cells in 50 non-Unc animals from SD167 are shown. + and − indicate non-Ncl and Ncl mutant phenotypes, respectively. Vertical lines represent cells. Horizontal lines represent cell divisions. Arrows represent many cell divisions that lead to the generation of the cells that can be scored for the Ncl phenotype (shown). a, p, l, and r refer to anterior, posterior, left, and right, respectively. ALA and RID are ring neurons. ASKL, ADLL, ASIL, ASKR, ADLR, and ASIR are amphid neurons. MI, I5, I3, and M4 are pharyngeal neurons. exc, excretory cell; post, postdeirid; Pn.a, P(3–8).a; Pn.p, P(3–8).p; hyp10, hyp10 syncytial hypodermis; bm, body muscles; dtc, distal tip cell; ac, anchor cell (generated from either MS.ap or MS.pp). A subset of these cells (MI, I5, post, Pn.a, Pn.p, exc, I3, bm, dtc, ac, and M4) were used to identify the mosaic animals represented in Figures 2–6. nd, Ncl phenotype was not determined; *n*, number of animals; >, the extrachromosomal array was lost in the named cell or in one of its descendants. In most cases, only the right or left postdeirid was scored in any one particular animal.

or, alternatively, they could be the result of variable levels of *ncl-1*(+) gene expression from the extrachromosomal array in different cells. Finally, the remaining 30 animals did not appear to be genetic mosaics (or had lost the extrachromosomal array at a late point in the cell lineage), because all of the cells that were scored exhibited a non-Ncl phenotype. Thus, although the Ncl patterns of some animals carrying the extrachromosomal array could be interpreted as the result of variable expression of the array, several animals displayed a Ncl pattern that strongly supported loss of the array at a single point in the cell lineage.

Another argument supporting array loss instead of variable expression is that we were able to show that individual cells or tissues appear to coordinately express the Ncl, Unc-29, and Lin-31 phenotypes. If these phenotypes were not coordinately expressed (due to variable expression from the array), then cells could exhibit a mutant phenotype for one gene and a wild-type phenotype for another. For example, Pn.p cells could be Ncl and non-Lin-31, or they could be non-Ncl and Lin-31. Instead, as discussed below, we found that animals in which all of the Pn.p cells were Ncl exhibited a Lin-31 phenotype as strongly as *lin-31* mutants, and that animals in which all the Pn.p cells were non-Ncl exhibited a non-Lin-31 phenotype. This result suggests that *lin-31* acts in the Pn.p cells and that the Ncl and Lin-31 phenotypes are coordinately expressed. Similarly, results presented below indicate that the body muscle cells appeared to coordinately express the Ncl and the

Unc-29 phenotypes. Coordinate expression of mutant phenotypes is most likely explained by mitotic loss of the extrachromosomal array leading to simultaneous loss of *ncl-1*(+), *unc-29*(+) and *lin-31*(+).

Second, because an extrachromosomal array typically consists of many tandem copies of each coinjected DNA clone (STINCHCOMB *et al.* 1985), it is possible that perdurance of overexpressed RNA or protein products contributed by the last cell containing the array could prevent the observation of mutant phenotypes in cellular descendants that lack the array. However, we found Ncl mosaic animals at a relatively high frequency, indicating that at least in these cases, the Ncl phenotype of a cell that had lost the extrachromosomal array was not masked by perdurance of *ncl-1* RNA or protein products (Figure 1). For example, the Ncl phenotype of the excretory cell can be observed as few as four cell divisions after loss of the array in AB.p. However, it is possible that in other cases, the Ncl phenotype in individual cells in mosaic animals was not observed due to perdurance of *ncl-1* RNA or protein. For genes other than *ncl-1*, it is possible that perdurance of gene products could mask the mutant phenotype of cells that have recently lost the extrachromosomal array.

Third, our results showed that this array was lost at a frequency appropriate for use in mosaic analysis experiments. We found that 8% of the non-Unc animals (four out of 50 total) were genetic mosaic animals in which the array had been lost before the 28-cell stage (Figure 1). These early-loss mosaics were useful in the *unc-29* and *lin-31* mosaic analysis experiments presented below, since at an early stage of development, most or all of the cells of a particular tissue (*e.g.*, body muscles, nerves, ventral ectoderm, gonad, hypodermis) are derived from a single progenitor cell, and just a single loss of the extrachromosomal array is required to remove wild-type gene activity from the entire tissue. Since ~8% of the non-Unc animals in this strain are early-loss genetic mosaics, mosaic animals can be directly identified by using Normarski optics to score the Ncl phenotype of individual cells. When the frequency of mosaic animals is much lower, early-loss genetic mosaic animals can only be easily identified by using a dissecting microscope to screen a large number of animals for a visible mosaic phenotype, such as semi-Unc-29.

The acetylcholine receptor subunit gene *unc-29* acts in the body muscle cells: While using *unc-29* as a cotransformation marker, we were fortuitously able to show that the site-of-action for *unc-29* is likely to be in the body muscle cells. *unc-29* encodes a protein similar to a subunit of the acetylcholine receptor (FLEMING *et al.* 1993), which causes muscle cells to contract in vertebrates. In these experiments, we picked non-Unc and semi-Unc animals, and then scored the Ncl and non-Ncl phenotypes of individual cells to see if they were genetic mosaics and to determine the point in the cell lineage at which the array had been lost (see MATERIALS AND METHODS).

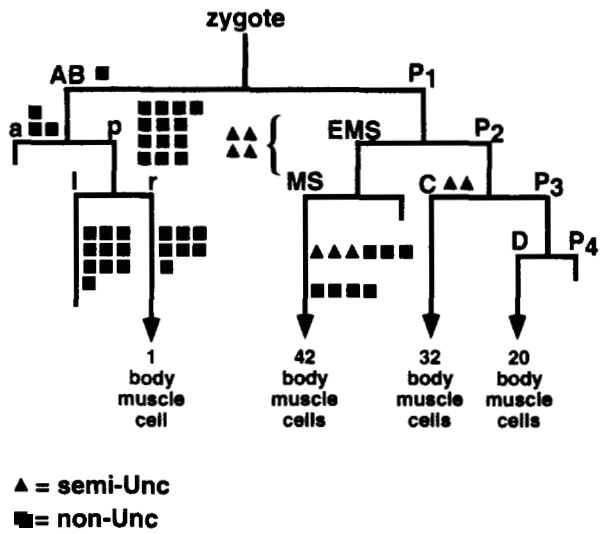

FIGURE 2.—*unc-29* acts in the body muscle cells. non-Unc (■) or semi-Unc (▲) mosaic animals are shown. The position of each symbol indicates the point in the lineage at which the array was lost. Symbols next to an arrow indicate mosaic animals in which the loss occurred in one of the descendants of the last named cell. Brackets indicate that losses at MS and EMS cannot be distinguished from each other. The origin of each of the 95 body muscle cells within the cell lineage is shown.

First, we found that mosaics in which a significant fraction of the body muscle cells lacked *unc-29*(+) have a semi-Unc phenotype, indicating that *unc-29*(+) activity is required in body muscles. We found two animals that lost the extrachromosomal array in the C blastomere, which generates 32 out of 95 (34%) of the body muscle cells, and four animals that lost the array in either the EMS or MS cell, which generate 42 out of 95 (44%) of the body muscle cells; all six of these animals were semi-Unc (Figure 2). We also found six animals in which the extrachromosomal array was lost in a cell derived from MS. In these mosaic animals, between one and 27 body muscle cells from MS were Ncl (1–28% of total body muscle cells), but multiple other cells derived from MS (such as other body muscle cells and/or a distal tip cell) were non-Ncl. Because we did not score each body muscle cell separately, we cannot precisely identify the point in the cell lineage at which the array was lost in these animals. Three of these six mosaic animals were semi-Unc and the remaining three animals were non-Unc.

Next, we found that mosaics in which almost all body muscle cells contain the extrachromosomal array display a non-Unc phenotype. We found 34 mosaic animals that had lost the array in AB or in a cell derived from AB (AB.a, AB.p, or a descendant of AB.p), and all were non-Unc, indicating that *unc-29*(+) activity is not required in cells derived from AB (Figure 2). AB generates 662 of the 959 (69%) somatic cells in an adult

hermaphrodite (including 97% of the neurons) but only a single body muscle cell (SULSTON and HORVITZ 1977; SULSTON *et al.* 1983). We found four mosaic animals in which several cells derived from MS (such as the distal tip cells, pharyngeal cells, and the anchor cell) were Ncl but none of the 18 muscle cells in the head that are derived from MS were Ncl, indicating that the extrachromosomal array was lost in a descendant of MS that does not give rise to any of the body muscle cells in the head. Although we did not score the Ncl and non-Ncl phenotypes of the remaining 24 MS-derived body muscle cells in these animals, the pattern of cell divisions in the MS cell lineage indicates that a single loss of the extrachromosomal array could affect at most six body muscle cells (6% of the total body muscle cells) without affecting any muscle cells in the head. All four of these mosaics were non-Unc.

Finally, we did not obtain any mosaic animals that had lost the array in P_1, which would result in the absence of *unc-29*(+) activity in 94 out of 95 body muscle cells. This class of mosaic animal presumably would have exhibited an Unc phenotype and would not have been picked as a non-Unc or semi-Unc animal in these experiments. We attempted to identify a P_1 mosaic animal by using Nomarski optics to directly screen animals for those with an appropriate pattern of Ncl cells but did not find any and so were unable to unequivocally determine the phenotype of animals in which almost all of the body muscle cells lacked *unc-29*(+) activity. In summary, these results suggest that *unc-29*(+) is required in the body muscle cells and that the semi-Unc phenotype can be used to identify mosaic animals that have lost an extrachromosomal array in some of the body muscle cells.

***lin-31* acts in the Pn.p cells:** Vulval induction is regulated by the anchor cell signal, a lateral signal from other Pn.p cells, and an inhibitory signal from the hyp7 syncytial hypodermis. *lin-31* mutations could affect the choice of vulval cell fates by causing defects in any of the cells or tissues that send signals, or in the Pn.p cells that respond to these signals. We first addressed this issue by determining if *lin-31* acts in the Pn.p cells, rather than in the anchor cell or hyp7. We scored the Lin-31 phenotypes of the same mosaic animals that were previously identified in the *unc-29* mosaic analysis (Figure 3). Mosaic animals that had lost the extrachromosomal array in cells other than the Pn.p cells exhibited wild-type vulval development, including 14 animals that had lost the array in the anchor cell (loss of the array in EMS, MS, or a descendant of MS), three that had lost the array in most of hyp7 (75 of 132 total hyp7 nuclei due to loss in AB.a) and two that had lost the array in some of hyp7 (12 of 132 total hyp7 nuclei due to loss in C). These results suggest that *lin-31*(+) activity is not required in the anchor cell or the hyp7 syncytial hypodermis.

Twenty-six of the 31 (84%) mosaic animals that had lost the extrachromosomal array in some or all of the Pn.p cells as well as some of hyp7 (up to 45 nuclei) displayed a multivulva (Muv) phenotype (loss in AB, AB.p, AB.pl, AB.pr, or the descendants of AB.pl or AB.pr). However, analysis of the AB.a and C mosaic animals described above suggest that *lin-31* does not act in hyp7, suggesting that *lin-31*(+) activity is required in the Pn.p cells rather than hyp7. The fraction of these mosaic animals that exhibit a Muv phenotype (84%) is similar to that of nonmosaic *lin-31*(*n1053*) animals (77%) (MILLER *et al.* 1993), suggesting that loss of *lin-31*(+) in the Pn.p cells can completely account for the Lin-31 vulval phenotype.

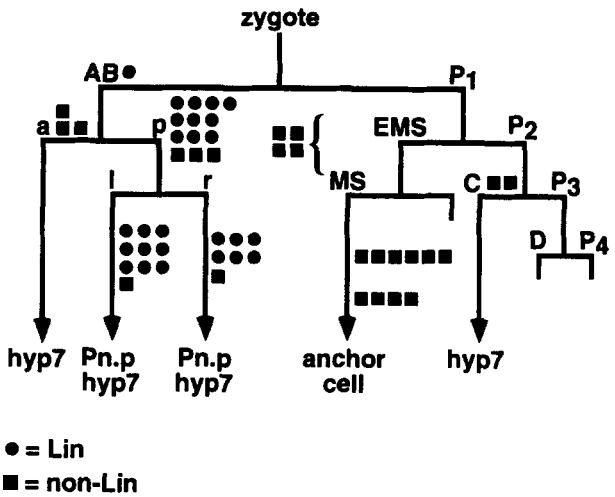

FIGURE 3.—*lin-31* acts in the Pn.p cells. Mosaic animals that displayed a wild-type vulval phenotype (■) or a Lin-31 vulval phenotype (●) are shown. Cell lineages, cell names and placement of symbols for each mosaic animal are as in Figure 2. Animals represented in this figure are the same mosaic animals represented in Figure 2. hyp7, hyp7 syncytial hypodermis.

***lin-31* function within individual Pn.p cells:** To investigate *lin-31* function within individual Pn.p cells, we identified mosaic animals that had lost the array in either AB.pl or AB.pr, because this type of mosaic animal contains Pn.p cells that lack *lin-31*(+) activity that are adjacent to Pn.p cells that contain *lin-31*(+) activity. The single ventral row of six Pn.p cells is generated from three cells derived from AB.pl and three from AB.pr. One cell from each pair of cells (P3.p/P4.p, P5.p/P6.p, P7.p/P8.p) is derived from AB.pl and the other is derived from AB.pr, so that one cell out of each pair lacks *lin-31*(+) activity when the array is lost in either AB.pl or AB.pr. We picked non-Unc animals and used the Ncl phenotype of individual cells to identify 10 mosaic animals in which the array was lost in AB.pl or AB.pr (or their descendants) (see MATERIALS AND METHODS). We then used Nomarski microscopy to directly observe the pattern of Pn.p cell divisions to score two different Lin-31 phenotypes in individual Pn.p cells.

The first phenotype caused by *lin-31* mutations is that ~22% of the Pn.p cells undergo a cell division during

mosaic	loss at	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
wild-type	no loss	+	+	+	+	+	+
1	AB.pla-AB.plapp	−	+	+	−	+	−
2	AB.pra-AB.prapp	+	(−)	(−)	+	(−)	+
3	AB.pl	−	+	+	(−)	+	−
4	AB.pl	−	+	(−)	+	+	−
5	AB.pl	−	+	+	(−)	+	−
6	AB.pl	+	−	+	−	+	−
7	AB.pr-AB.pra	+	−	+	(−)	+	−
8	AB.pl	(−)	+	−	+	(−)	+
9	AB.pr-AB.pra	+	−	+	(−)	−	+
10	AB.pl	+	(−)	+	(−)	+	(−)

FIGURE 4.—*lin-31*(+) acts cell-autonomously to prevent Pn.p cell division during the L2 larval stage. Each line shows one mosaic animal that lost the extrachromosomal array in AB.pl, AB.pr or in one of their descendants. (+) and (−) refer to non-Ncl and Ncl cells, respectively. Pn.p cells that divided during the L2 larval stage are shown as a dividing cell. These mosaic animals are a subset of the animals shown in Figures 2 and 3.

the second larval stage (L2), generating a vulval precursor cell and either another vulval precursor cell or a Pn.a-like neuroblast (MILLER *et al.* 1993). The Pn.p cells do not divide at this time in the wild type. In the 10 mosaic animals described above, there were 30 Pn.p cells that were Ncl and thus lacked *lin-31*(+) activity; 13 of these cells (43%) underwent an extra cell division (Figure 4). There were also 30 Pn.p cells that were non-Ncl and thus contained *lin-31*(+); none of these cells divided during the L2 larval stage. Thus, *lin-31* acts cell-autonomously within each Pn.p cell to prevent an extra cell division during the L2 larval stage.

The second phenotype caused by *lin-31* mutations is that vulval cell fates become deregulated, such that each of the six Pn.p cells can adopt any of the three vulval cell fates (MILLER *et al.* 1993). To study the role of *lin-31* in the specification of each cell fate, we studied the effect of losing *lin-31*(+) in each individual Pn.p cell. We identified mosaic animals in which an individual Pn.p cell lacked *lin-31*(+) but was surrounded by cells and tissues that contained *lin-31*(+), and then determined whether loss of *lin-31*(+) in that cell resulted in a cell fate transformation (Figure 5). In this group of mosaics, the individual Pn.p cell is expected to receive intercellular signals as in the wild type since most of its neighbors are genotypically wild type. Therefore, expression of cell fates different from those normally expressed in the wild type indicate a cell-autonomous requirement for *lin-31* activity in that Pn.p cell. These mosaic animals are discussed in three groups, according to which Pn.p cell(s) lacked *lin-31*(+). The first group contains mosaics in which *lin-31*(+) is missing in P6.p, which is closest to the anchor cell and normally expresses the 1° cell fate. The second group contains mosaics in which *lin-31*(+) is missing in either P5.p or P7.p, which normally express the 2° cell fate, and the third group contains mosaics in which *lin-31*(+) is missing in P3.p, P4.p or P8.p, which normally express the 3° cell fate (Figure 6). In these experiments, loss of *lin-31*(+) in an individual Pn.p cell sometimes caused that cell to divide during the L2 stage, and we grouped the resulting daughter cells along with their mother cell (*e.g.*, P6.pa and P6.pp were grouped with P6.p).

In the first group, P6.p lacked *lin-31*(+) but the anchor cell and most of hyp7 contained *lin-31*(+); this group includes mosaics 1, 3, 5, 7, 9 and 10 (Figures 5 and 6A). P6.p (or descendants of P6.p if an L2 division had occurred) expressed either an uninduced 3° cell fate (three cells in mosaics 5 and 10), a partially induced cell fate (three cells in mosaics 3 and 7) or the 1° cell fate (four cells in mosaics 1, 5, 7, and 9). Thus, in this first group of mosaic animals, P6.p expressed an inappropriate cell fate six out of 10 times. Furthermore, in at least two cases (mosaics 3 and 10), loss of *lin-31*(+) in P6.p not only affected the 1° cell division pattern (less than eight vulval descendants were generated) but also likely prevented the expression of the lateral signal. In these mosaics, we infer that P6.p did not express the lateral signal because P7.p was genotypically wild type but was not induced to express the 2° cell fate. These results indicate that *lin-31* acts in P6.p, and that loss of *lin-31*(+) activity can sometimes prevent P6.p from undergoing the 1° pattern of cell divisions and expressing the lateral signal.

In the second group, either P5.p or P7.p (or their daughters) lacked *lin-31*(+) but P6.p, the anchor cell and most of hyp7 contained *lin-31*(+) (Figure 5). In these mosaics, P6.p was genotypically wild type and should have expressed the lateral signal, and we determined whether loss of *lin-31*(+) in either of the adjacent vulval precursor cells could prevent expression of the 2° cell fate. Within this group, three cells were directly adjacent to P6.p: the posterior P5.p daughter in mosaic 4, P5.p in mosaic 8, and the anterior P7.p daughter in mosaic 8. Two cells adopted the 2° cell fate, and one cell expressed the uninduced, 3° cell fate. Since only one cell fate transformation was observed in this group, additional examples are required to draw conclusions regarding the role of *lin-31* in 2° cell fate specification in these cells.

In the third group, either P3.p, P4.p or P8.p lacked *lin-31*(+) but adjacent Pn.p cells, the anchor cell and most of hyp7 contained *lin-31*(+) (Figures 5 and 6B). In this group, either P3.p or P4.p, but not both, lacked *lin-31*(+). P3.p, P4.p or P8.p should not receive ectopic inductive signals from the surrounding tissue since

mosaic	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	vulva
WT	SS/U 3°	SS 3°	LLTN 2°	TTTT 1°	NTLL 2°	SS 3°	WT
1	NS P	3°	2°	OOTT ~1°	2°	3°	Muv
3	3°	3°	2°	DDDD DDDD 1°/2° 1°/2°	3°	NN P	Pvl, Egl
4	SN P	TLLL ~2°	NNNN P 2°	OOOO ~1°	2°	3°	Muv
5	3°	3°	NNTN ~2°	OOOO 3° ~1°	2°	3°	Pvl
7	U	NNNN P	2°	NNNN P 1°	2°	3° 3°	Pvl
8	U U	3°	LLLN ~2°	OOOO ~1°	3° 3°	3°	ND
9	U	2°	2°	nb 1°	3°	3°	Muv
10	3°	NNNN P 3°	1°	3° 3°	3°	NS P 3°	Muv, Pvl

FIGURE 5.—Mosaic analysis of *lin-31* function in individual Pn.p cells. Each line shows the Pn.p cell lineages of one mosaic animal that lost the extrachromosomal array in AB.pl, AB.pr or in one of their descendants. Cell lineages of the Pn.p cells were determined using Nomarski optics. Cells that contain or lack *lin-31*(+) are shown in bold or outline, respectively. S, N, U, D, L, T, O refer to syncytial cell, nondividing cell (does not usually divide), undivided cell (fate of a cell that can sometimes divide), divided cell (division axis not determined), longitudinal cell division, transverse cell division, and oblique cell division, respectively. Underline indicates that the cell division generated daughter cells that attached to the cuticle. 1° (TTTT), 2° (LLTN or NTLL), 3° (SS or U) or neuroblast (nb) cell fates are shown below each cell lineage. P, ~1°, ~2° refer to partially induced, primary-like and secondary-like, respectively. NS and NN are referred to as partially induced because the N cells did not attach to the cuticle and fuse with the hyp7 syncytium as would be expected for cells from the 3° lineage. Brackets indicate a cell division during the L2 larval stage. The vulval phenotype of each mosaic animal is indicated. WT, Muv, Pvl, Egl and ND refer to wild-type, multivulva, protruding vulva, egg-laying defective, and not determined, respectively. The cell lineage pattern in wild type is shown on the first line for comparison. These animals are a subset of those shown in Figure 4.

most of the surrounding cells were genotypically wild type, and thus this group tests whether loss of *lin-31*(+) in P3.p, P4.p or P8.p can result in the expression of induced cell fates. Of the 18 cases in this group, there were 11 cells that adopted the 3° cell fate: P3.p in mosaics 3 and 5, both P3.p daughters in mosaic 8, P8.p in mosaics 1, 4 and 5, both P8.p daughters in mosaic 7, a P4.p daughter and a P8.p daughter in mosaic 10. The

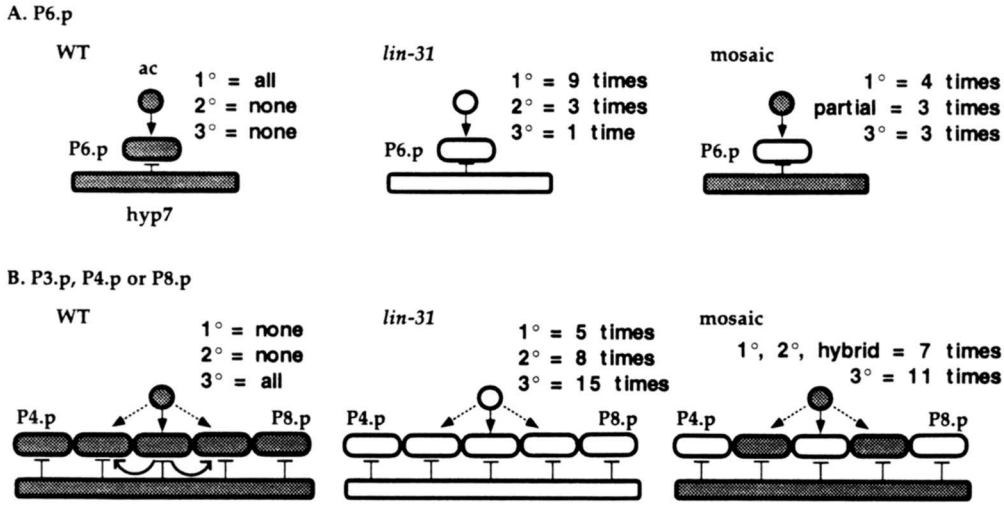

FIGURE 6.—Summary of individual Pn.p cell mosaics. Tissues or cells that either contain (shaded) or lack (clear) *lin-31*(+) are shown. Cell fates expressed in wild-type (left), nonmosaic *lin-31*(*n301*) or *lin-31*(*n1053*) null mutants (middle, data from Miller *et al.* 1993) or mosaic animals from Figure 5 (right) are shown. Straight arrows indicate the anchor cell signal, curved arrows indicate the lateral signal and lines with bars indicate the hyp7 inhibitory signal. Each group shows the fates expressed by the named Pn.p cells. (A) P6.p mosaics. The other Pn.p cells may or may not contain *lin-31*(+). (B) P3.p, P4.p or P8.p mosaics. In these mosaics, at least one of these three Pn.p cells lacked *lin-31*(+), and adjacent Pn.p cells contained *lin-31*(+). The genotype of Pn.p cells two or more cells distant is not specified; for example, P6.p may or may not contain *lin-31*(+) in this group. The last group of mosaics (P5.p or P7.p mosaics) is not shown.

remaining seven cells adopted induced (1°, 2°, or hybrid) cell fates: P3.p in mosaics 1 and 4, P4.p in mosaics 7 and 9, P8.p in mosaic 3, a P4.p daughter and a P8.p daughter in mosaic 10. In one case (mosaic 4), loss of *lin-31*(+) caused P3.p and P5.p to divide to generate vulval cells and may also have resulted in ectopic expression of the lateral signal, since P4.p (which was genotypically wild-type) expressed an ~2° cell fate. These results indicate that *lin-31*(+) acts in P3.p, P4.p, and P8.p and is normally required for the expression of the 3° cell fate.

DISCUSSION

Mosaic analysis using a *ncl-1* cosmid on an extrachromosomal array: We have identified a cosmid clone that can rescue the Ncl mutant phenotype in germline transformation experiments and have used an extrachromosomal array containing this *ncl-1*(+) clone for mosaic analysis. We characterized the genetic behavior of an extrachromosomal array and found that it could be used in mosaic analysis experiments to show that the acetylcholine receptor gene *unc-29* likely acts in the body muscles and that the *HNF3/fork head* gene *lin-31* likely acts in the Pn.p cells.

Previously, many mosaic analysis experiments in *C. elegans* used free duplications to generate mosaic animals. In those experiments, the free duplication contained wild-type copies of a gene of interest and a cell lineage marker gene. The *ncl-1* gene is an excellent cell lineage marker: it is required in a cell-autonomous fashion for the normal appearance of nucleoli and does not appear to be required for other cellular processes, the Ncl phenotype can be scored in most cells and in animals of various ages, and the Ncl mutant phenotype appears shortly after mitotic loss of the duplication carrying *ncl-1*(+) (HEDGECOCK and HERMAN 1995). To use *ncl-1,* previous mosaic analysis experiments required that the free duplication contain both *ncl-1* and the gene of interest (as well as any other desirable genetic markers). As free duplications are derived from chromosomes, only those genes linked to *ncl-1* on linkage group III (~10% of the total genome) are also linked to *ncl-1* on any free duplication (HODGKIN *et al.* 1995b). Thus, previous mosaic analysis experiments have preferentially been performed on the minority of genes linked to *ncl-1*(+), due to the desire to use *ncl-1* as a cell lineage marker.

To determine the site-of-action for genes that are not linked to *ncl-1* on LGIII, at least three groups have created free duplications in which their gene of interest is artificially linked to *ncl-1.* HUNTER and WOOD (1992) and HEDGECOCK and HERMAN (1995) accomplished this by screening for animals in which two free duplications had been linked together following X-ray irradiation, creating one large free duplication. LEUNG-HAGENSTEIJN *et al.* (1992) isolated animals in which an extrachromosomal array containing the *unc-5* gene was linked to the free duplication *sDp3,* which contains *ncl-1.* In each of these cases, one drawback is the need to screen for animals containing a fusion event in which either two free duplications or a free duplication and an extrachromosomal array are joined. Another drawback is that fused duplications are more mitotically stable than either of the starting duplications or the extrachromosomal array, and this added stability causes mosaic animals to appear at a lower frequency (although mitotic loss frequencies can be increased by a *him-10* mutation) (HEDGECOCK and HERMAN 1995).

In this work, we used germline transformation to artificially link our gene of interest to the *ncl-1* cell lineage marker gene on an extrachromosomal array. We injected DNA containing *lin-31*(+), a cosmid carrying *ncl-1*(+), and a cosmid carrying the *unc-29*(+) cotransformation marker into the germlines of *unc-29; lin-31; ncl-1* mutant hermaphrodites, resulting in the formation of an extrachromosomal array carrying wild-type copies of all three genes. The genetic behavior of the extrachromosomal array was similar to a free duplication, and our gene of interest was quickly and easily linked to the *ncl-1* cell lineage marker. The same germline transformation procedure typically generates several strains containing independently derived extrachromosomal arrays. The extrachromosomal arrays have different transmission frequencies, and it is likely that at least one array will have a mitotic loss rate appropriate for mosaic analysis. Also, while our approach requires a DNA clone that can rescue the mutant phenotype of the gene of interest, this requirement is becoming easier to fulfill due to the near completion of the physical genome map, improvements in molecular genetic techniques, and the resulting molecular cloning of more genetic loci (HODGKIN *et al.* 1995a).

Several points are worth noting when using extrachromosomal arrays in genetic mosaic experiments. First, coinjection of cloned DNAs in germline transformation experiments is a straightforward method to artificially link genes into one extrachromosomal array. Second, we found that the Ncl phenotype of individual cells in potential genetic mosaic animals is likely due to mitotic loss of the array rather than to variable expression from the array. We picked non-Unc animals at random and found that 8% were early-loss mosaic animals that exhibited a simple Ncl pattern in which all of the observed Ncl mutant cells were lineally derived from a single progenitor cell. These simple Ncl patterns are best explained by spontaneous loss of the array at a single point in the cell lineage during development. The remaining animals were not useful for mosaic analysis because they either contained no scored Ncl cells (60%), only one scored Ncl cell (24%), or displayed a complex Ncl pattern in which Ncl mutant cells were derived from cells with independent lineal origins (8%). The Ncl phenotype of the last class could be

explained by multiple mitotic losses of the extrachromosomal array during development or from variable expression of the *ncl-1* gene. In addition to the pattern of Ncl cells, coordinate expression of the Ncl-1, Unc-29, and Lin-31 mutant phenotypes in mosaic animals indicates that these phenotypes are caused by mitotic loss of the extrachromosomal array leading to simultaneous loss of *ncl-1*(+), *unc-29*(+) and *lin-31*(+) activities. Third, the rate of mitotic loss of many extrachromosomal arrays is satisfactory to generate mosaic animals at a desirable frequency for mosaic analysis. We observed that the *gaEx25* extrachromosomal array was lost at a rate of 3.4×10^{-3} per mitotic cell division, which is very similar to the mitotic loss rates previously reported for several free duplications (0.1×10^{-3} to 5.3×10^{-3}) (see MATERIALS AND METHODS; HEDGECOCK and HERMAN 1995). We could isolate mosaic animals by direct observation of the Ncl phenotype of individual cells using Nomarski optics since 8% of randomly picked non-Unc animals were genetic mosaics that had lost the array before the 28-cell stage. Fourth, the ability to obtain genetic mosaics itself argues that strong perdurance of *ncl-1*(+) RNA or protein derived from the progenitor cell and inherited by its cellular descendants does not prevent expression of the Ncl-1 mutant phenotype to a large extent, which would interfere with mosaic analysis experiments. Fifth, in addition to the results presented here, other work reveals that mosaic analysis can be successful using *ncl-1*(+) on an extrachromosomal array, as this approach has been used to determine the site-of-action of at least six *C. elegans* genes: *lin-31* (this paper), *mpk-1* (LACKNER *et al.* 1994), *let-23* (KOGA and OHSHIMA 1995; SIMSKE and KIM 1995), *lin-7* (SIMSKE and KIM 1995; J. SIMSKE, personal communication), *lin-44* (HERMAN *et al.* 1995), and *lin-2* (HOSKINS *et al.* 1995).

In addition to *ncl-1,* we found that the *unc-29* gene is a useful marker for mosaic analysis. Genetic mosaic animals that have lost the extrachromosomal array in the EMS, MS, or C blastomeres can be readily identified by picking semi-Unc-29 animals. These blastomeres generate a significant fraction of the body muscle cells, and loss of *unc-29*(+) activity in some but not all of the body muscle cells results in a semi-Unc-29 phenotype.

***lin-31* acts in the Pn.p cells:** During vulval induction, multiple intercellular signals (the anchor cell signal, the lateral signal and an inhibitory signal) specify the fates (1°, 2° or 3°) of the Pn.p cells. *lin-31* is involved in Pn.p cell fate specification, since it is required for the expression of appropriate vulval cell fates. *lin-31* encodes a putative transcription factor similar to mammalian HNF3 and Drosophila fork head, suggesting that *lin-31* may specify cell fates by regulating gene expression. The results presented here indicate that *lin-31*(+) acts in each Pn.p cell to prevent an extra cell division during the L2 stage. Later, during the L3 stage, *lin-31*(+) activity is required in the Pn.p cells but not the anchor cell or hyp7 for the proper selection of cell fates during vulval induction.

To investigate the role of *lin-31*(+) in specifying each of the three cell fates, we examined mosaic animals in which an individual Pn.p cell lacked *lin-31*(+) but surrounding cells or tissues (the Pn.p cells adjacent to the mutant Pn.p cell, the anchor cell and hyp7) contained *lin-31*(+). In these mosaics, the Pn.p cell lacking *lin-31*(+) should receive intercellular signals (the anchor cell signal, the lateral signal or the inhibitory signal) as it would in wild-type, so that expression of a mutant cell fate indicates a cell-autonomous requirement for *lin-31* function.

In one group of mosaics, *lin-31*(+) was missing in P6.p but not in adjacent Pn.p cells, most of hyp7 or the anchor cell. In these mosaics, P6.p expressed a mutant cell fate (uninduced or partially induced) in six out of 10 cases. This result indicates that *lin-31* acts in P6.p, and that loss of *lin-31*(+) activity can sometimes prevent the expression of the 1° cell fate. These results are consistent with a role for *lin-31*(+) in mediating the response of P6.p to the anchor cell signal, as *lin-31* encodes a putative transcription factor in the HNF3/fork head family, and genetic epistasis experiments suggest that *lin-31* is likely to act after the signal transduction genes in the *let-23 receptor tyrosine kinase/let-60 ras/mpk-1 MAP kinase* pathway (MILLER 1993; M. LACKNER and P. TAN, personal communication).

In another group of mosaics, *lin-31*(+) activity was missing in P3.p, P4.p or P8.p but not in adjacent Pn.p cells, most of hyp7 or the anchor cell. Loss of *lin-31*(+) in these cells allowed inappropriate expression of induced cell fates. These results indicate that *lin-31* acts in these three Pn.p cells, and that *lin-31*(+) is required for the expression of the 3° cell fate.

Comparison of the results from the two mosaic groups mentioned above indicates that loss of *lin-31*(+) in P6.p results in less induction than in the wild type, but loss of *lin-31*(+) in P3.p, P4.p or P8.p results in excess vulval induction. Thus, *lin-31* may have two functions during vulval induction. *lin-31* may be required for the proper expression of the 1° cell fate and also for expression of the 3° uninduced cell fate.

The 1° cell fate is comprised of two separate aspects: (1) a specific cell division pattern resulting in the generation of eight vulval descendants and (2) expression of the lateral signal that induces neighboring Pn.p cells to adopt the 2° cell fate. Results from the individual Pn.p cell mosaics suggest that *lin-31*(+) is required for both aspects of the 1° cell fate. For example, there were two cases (mosaics 3 and 10) in which loss of *lin-31*(+) affected both the 1° cell division pattern and lateral signal expression by P6.p, because this cell generated less than eight descendants and P7.p was genotypically wild type but did not express the 2° cell fate. Conversely, there was one case (mosaic 4) in which loss of *lin-31*(+) activity resulted in the generation of excess vulval cells

and may have caused ectopic appearance of the lateral signal; in this mosaic, P3.p and P5.p divided to generate vulval descendants and likely caused P4.p (which was genotypically wild type) to express the 2° cell fate.

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LITERATURE CITED

ALBERTSON, D. G., and J. N. THOMSON, 1976 The pharynx of *Caenorhabditis elegans*. Phil. Trans. Roy. Soc. London Ser. B **275:** 299–325.

AUSTIN, J., and J. KIMBLE, 1987 *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. Cell **51:** 589–599.

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

CLARK, S. G., A. D. CHISHOLM and H. R. HORVITZ, 1993 Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. Cell **74:** 43–55.

COSTA, M., M. WEIR, A. COULSON, J. SULSTON and C. KENYON, 1988 Posterior pattern formation in *C. elegans* involves position-specific expression of a gene containing a homeobox. Cell **55:** 747–756.

COULSON, A., J. SULSTON, S. BRENNER and J. KARN, 1986 Toward a physical map of the genome of the nematode *C. elegans*. Proc. Natl. Acad. Sci. USA **83:** 7821–7825.

EISENMANN, D. M., and S. K. KIM, 1994 Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. Curr. Opin. Genet. Dev. **4:** 508–516.

FERGUSON, E. L., and H. R. HORVITZ, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. Genetics **110:** 17–72.

FIRE, A., 1986 Integrative transformation of *Caenorhabditis elegans*. EMBO J. **5:** 2673–2681.

FLEMING, J. T., C. TORNOE, H. A. RIINA, J. COADWELL, J. A. LEWIS *et al.*, 1993 Acetylcholine receptor molecules of the nematode *Caenorhabditis elegans*. Exs **63:** 65–80.

HEDGECOCK, E. M., and R. K. HERMAN, 1995 The *ncl-1* gene and genetic mosaics of *C. elegans*. Genetics **141:** 989–1006.

HERMAN, M. A., L. L. VASSILIEVA, H. R. HORVITZ, J. E. SHAW and R. K. HERMAN, 1995 The *C. elegans* gene *lin-44*, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. Cell **83:** 101–110.

HERMAN, R. K., 1984 Analysis of genetic mosaics of the nematode *Caneorhabditis elegans*. Genetics **108:** 165–180.

HERMAN, R. K., 1989 Mosaic analysis in the nematode *Caenorhabditis elegans*. J. Neurogenet. **5:** 1–24.

HERMAN, R. K., 1995 Mosaic analysis. Methods Cell Biol. **48:** 123–146.

HERMAN, R. K., and E. M. HEDGECOCK, 1990 Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in surrounding hypodermis. Nature **348:** 169–171.

HILL, R. J., and P. W. STERNBERG, 1992 The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. Nature **358:** 470–476.

HODGKIN, J., R. H. A. PLASTERK and R. H. WATERSTON, 1995a The nematode *Caenorhabditis elegans* and its genome. Science **270:** 410–414.

HODGKIN, J., R. DURBIN and S. MARTINELLI, 1995b *Genetic Map of Caenorhabditis elegans. Caenorhabditis* Genetics Center.

HORVITZ, H. R., and P. W. STERNBERG, 1991 Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. Nature **351:** 535–541.

HOSKINS, R., F. HAJNAL, S. A. HARP and S. K. KIM, 1996 The *C. elegans* vulval induction gene *lin-2*, encodes a member of the MAGUK family of cell junction proteins. Development **122:** 97–111.

HUNTER, C. P., and W. B. WOOD, 1992 Evidence from mosaic analysis of the masculinizing gene *her-1* for cell interactions in *C. elegans* sex determination. Nature **355:** 551–555.

KATZ, W. S., R. J. HILL, T. R. CLANDININ and P. W. STERNBERG, 1995 Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precursor fates. Cell **82:** 297–308.

KOGA, M., and Y. OHSHIMA, 1995 Mosaic analysis of the *let-23* gene function in vulval induction of *Caenorhabditis elegans*. Development **121:** 2655–2666.

LACKNER, M. R., K. KORNFELD, L. M. MILLER, H. R. HORVITZ and S. K. KIM, 1994 A MAP kinase homolog, *mpk-1*, is involved in *ras*-mediated induction of vulval cell fates in *Caenorhabditis elegans*. Genes Dev. **8:** 160–173.

LEUNG-HAGESTIJN, C., A. M. SPENCE, B. D. STERN, Y. ZHOU, M.-W. SU *et al.*, 1992 UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. Cell **71:** 289–299.

MELLO, C. C., J. M. KRAMER, D. STINCHCOMB and V. AMBROS, 1991 Efficient gene transfer in *C. elegans:* extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10:** 3959–3970.

MILLER, L. M., M. E. GALLEGOS, B. A. MORISSEAU and S. K. KIM, 1993 *lin-31*, a *Caenorhabditis elegans* HNF-3/*fork head* transcription factor homolog, specifies three alternative cell fates in vulval development. Genes Dev. **7:** 933–947.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SIMSKE, J. S., and S. K. KIM, 1995 Sequential signalling during *C. elegans* vulval induction. Nature **375:** 142–145.

STERNBERG, P. W., 1988 Lateral inhibition during vulval induction in *Caenorhabditis elegans*. Nature **335:** 551–554.

STERNBERG, P. W., and H. R. HORVITZ, 1986 Pattern formation during vulval development in *C. elegans*. Cell **44:** 761–772.

STINCHCOMB, D. T., J. E. SHAW, S. H. CARR and D. HIRSH, 1985 Extrachromosomal DNA transformation of *Caenorhabditis elegans*. Mol. Cell. Biol. **5:** 3484–3496.

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.

THOMAS, J. H., M. J. STERN and H. R. HORVITZ, 1990 Cell interactions coordinate the development of the *C. elegans* egg-laying system. Cell **62:** 1041–1052.

WANG, B. B., M. M. MULLER-IMMERGLUCK, J. AUSTIN, N. T. ROBINSON, A. CHISHOLM *et al.*, 1993 A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. Cell **74:** 29–42.

WOOD, W. B. (Editor), 1988 *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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