

Reevaluation of the Role of the *med-1* and *med-2* Genes in Specifying the *Caenorhabditis elegans* Endoderm

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ABSTRACT

The *med-1* and *med-2* genes encode a pair of essentially identical GATA factor-related transcription factors that have been proposed to be necessary for specification of the *C. elegans* endoderm (intestine or E lineage) as well as part of the *C. elegans* mesoderm. *med-1* and *med-2* are proposed to be the direct downstream targets and the principal effectors of the maternally provided SKN-1 transcription factor; *med-1* and *med-2* would thus occupy the pivotal interface between maternal and zygotic control of gene expression. The conclusion that *med-1* and *med-2* are necessary for *C. elegans* endoderm specification was based on a partially penetrant (~50%) loss of endoderm markers produced by RNA-mediated interference (RNAi). To determine whether this partial penetrance reflects: (i) inefficient RNAi against early zygotic transcripts, (ii) experimental uncertainty in the expected level of endoderm loss in *skn-1* nulls, or (iii) additional redundancy in the pathway of endoderm specification, we constructed worm strains that segregate embryos lacking both the *med-1* gene (because of a gene-specific deletion) and the *med-2* gene (using either of two chromosomal deficiencies). Contrary to expectations, we observe that only ~3–20% of *med-2(-); med-1(-)* embryos do *not* express markers of endoderm differentiation. Furthermore, we found no evidence for a maternal contribution of the *med* genes to endoderm specification. We conclude that the major pathway(s) for endoderm specification in *C. elegans* must be independent of the *med-1* and *med-2* genes.

THE *Caenorhabditis elegans* endoderm (intestine or E lineage) is clonally derived from a single cell, the E cell, in the eight-cell embryo (SULSTON *et al.* 1983). The early endoderm is one of the few *C. elegans* lineages for which a plausible specification pathway has been proposed in molecular detail, beginning with maternally provided transcription factors, progressing through several waves of zygotically produced transcription factors, and ending with gene products that function in the terminally differentiated intestine (see review by MADURO and ROTHMAN 2002; see also BAUGH *et al.* 2003, 2005; ROBERTSON *et al.* 2004).

Figure 1 summarizes the regulatory cascade proposed for specification of the *C. elegans* endoderm. The maternally provided b-ZIP-like transcription factor SKN-1 is essential for correct specification of the fate of the EMS blastomere of the four-cell embryo (BOWERMAN *et al.* 1992, 1993). Within the EMS cell, SKN-1 is proposed to directly activate the zygotic expression of two genes called *med-1* and *med-2*, which encode zinc-finger proteins related to GATA-type transcription factors but with atypical binding sites (MADURO *et al.* 2001; MADURO and ROTHMAN 2002; BROITMAN-MADURO *et al.* 2005).

These two small intronless genes are 98% identical and, for convenience, are often referred to simply as the *med* genes (*mesendoderm-determining*); they are the principal subjects of this article. The *med* genes are proposed to specify both the *C. elegans* endoderm and that portion of the *C. elegans* mesoderm deriving from the MS blastomere (Figure 1). To specify the endoderm, the MED-1 and MED-2 factors are proposed to directly activate the zygotic expression of a redundant pair of genes called *end-1* and *end-3*, which also encode GATA-type factors (ZHU *et al.* 1997, 1998; MADURO *et al.* 2001; MADURO and ROTHMAN 2002; BROITMAN-MADURO *et al.* 2005). This endoderm specification step takes place in the E cell, the clonal progenitor of the intestine, within a permissive environment associated with lowered nuclear levels of the HMG protein POP-1 (LIN *et al.* 1995, 1998; ROCHELEAU *et al.* 1997; THORPE *et al.* 1997; LO *et al.* 2004). The END-1/END-3 pair of GATA factors is proposed to directly activate expression of the *elt-2* gene, which encodes a GATA factor that may be the principal transcription factor directing subsequent intestinal differentiation (HAWKINS and MCGHEE 1995; FUKUSHIGE *et al.* 1998, 2005).

The properties of the *med* genes have generated substantial interest for at least two reasons: (i) they are proposed to occupy the important interface between maternal and zygotic control of gene expression (Figure 1), and (ii) their proposed involvement in specifying

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both MS mesoderm and E endoderm has been used as evidence for an ancient “mesendoderm” region of the embryo, specified by a transcription factor network conserved in all bilateral metazoons (MADURO *et al.* 2001; RODAWAY and PATIENT 2001; MADURO and ROTHMAN 2002; BROITMAN-MADURO *et al.* 2005). MADURO *et al.* (2001, p. 481) have proposed that “the meds are activated by, and function downstream of, SKN-1 in the EMS lineage and are essential to specify E and MS fates in any context.” In this article, we test only part of this assertion, that is, whether the *med* genes are indeed necessary to specify the *C. elegans* endoderm. To be clear about the expectations of our experiments, it is important to realize that loss of endoderm caused by loss of maternal *skn-1* function is not fully penetrant (BOWERMAN *et al.* 1992). Furthermore, the penetrance of this endoderm loss is temperature dependent, such that at 15° and 25°, respectively, 54–81% of embryos produced by *skn-1(zu67)* (strong loss-of-function allele) mothers do not produce endoderm (BOWERMAN *et al.* 1992). MADURO *et al.* (2001) performed RNA-mediated interference (RNAi) to target the *med* genes and found that, among the fraction of embryos that arrested and that were collected from 7 to 9 hr following injection, 52% (75/143) failed to express endoderm markers; this proportion is lower than but possibly within experimental error of the 60–80% of nonexpressing embryos expected to be produced by a *skn-1* null under their experimental conditions. The necessity of the *med* genes for endoderm specification can now be tested, without relying on RNAi, by combining a recently available gene-specific deletion of the *med-1* gene (see below) with a chromosomal deficiency that removes the *med-2* gene. As described in this study, the unexpected result is that only a minority (3–20%), not the expected majority, of embryos lacking both the *med-1* and the *med-2* gene do not express markers diagnostic for endoderm, suggesting that there must be a significant pathway for endoderm specification that does not require the *med-1* and *med-2* genes.

MATERIALS AND METHODS

Strains and alleles: *C. elegans* was maintained and manipulated by standard procedures (BRENNER 1974; WOOD 1988). The strain RB930 containing the *med-1(ok804)* allele was obtained from the *C. elegans* Genome Knockout Consortium and outcrossed three times to wild type, following the *med-1(ok804)* allele by PCR (see below) and choosing a strain of high viability; for convenience, we retain the original strain designation RB930 [*med-1(ok804)*]. The deletion was validated by both Southern blotting and PCR. Southern blotting was by standard methods (SAMBROOK and RUSSELL 2001), using a 1-kb probe amplified and cloned from the *med-2(+)* gene (see Figure 2B); final washing was at 55° and Na⁺ concentration of 0.2 M to allow for simultaneous detection of both the *med-2* gene and the highly similar *med-1* gene. The hybridization pattern was consistent with the complete removal of the *med-1* sequence, as depicted in Figure 2B; specifically, there was no

evidence of an “extra” band, suggesting an unsuspected duplication of the *med-1* gene elsewhere in the genome (that could possibly have occurred during the mutagenesis producing the *ok804* deletion). The absence of an extra *med-1* copy elsewhere in the genome was also verified by PCR using several different sets of primers. One particular PCR search for an extra *med-1* sequence used two primers (oJM306, TCTTTGA CAATACCCAGCAAC, and oJM307, CACCATTTCGTTTCCTG TACC, which anneal near the 5'- and 3'-ends, respectively, of the coding regions of both *med-1* and *med-2*) to amplify a 474-bp band, using template DNA purified from either wild-type N2 worms or strain RB930 [*med-1(ok804)*]. The two amplified fragments were gel purified and sequenced on both strands. Sequence traces obtained with the fragment amplified from wild-type DNA clearly showed the multiple sequence ambiguities expected for a mixture of *med-1* and *med-2* DNA (MADURO *et al.* 2001); in contrast, sequence traces obtained with the fragment amplified from RB930 DNA showed only a single sequence, corresponding to *med-2(+)* (data not shown). The most definitive (but still negative) PCR search for an extra copy of *med-1* followed this same strategy but used two primers that hybridize within the DNA-binding domain of both *med-1* and *med-2* (oJM308, TGAAACAATTTCGTTGGAGGA; oJM309, TGTTAACGGCAGTG-ACTGGA). The expected *med-1/med-2* polymorphism within the zinc-finger sequence (MADURO *et al.* 2001) could be detected in the sequence traces when N2 DNA was used as template but only the *med-2* sequence could be detected when RB930 [*med-1(ok804)*] DNA was used as template. We conclude that any extra copy of the *med-1* gene can neither be intact nor have a DNA-binding domain.

To produce a strain in which the *med-2* gene was removed by the chromosomal deficiency *sDf127*, RB930 [*med-1(ok804)*] males were crossed to hermaphrodites of strain BC4638 [*dpy-17(e164) sDf127(s2428) unc-32(e189) III; sDp3(III,f)*] and Unc Non-Dpy F₂ hermaphrodites that segregated dead eggs were selected. The status of the *med-1* gene in populations

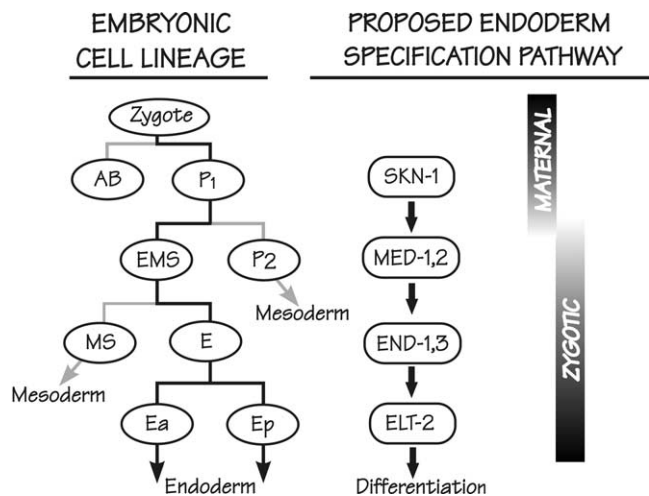


FIGURE 1.—Cell lineage of the early *C. elegans* embryo (left), aligned with the proposed transcription factor cascade that leads to specification of the *C. elegans* endoderm (right). Lineages that lead to the intestine are solid; other lineages are shaded. Only transcription factors that are on the proposed endoderm specification pathway are shown; in particular, roles for SKN-1 and MED-1,2 in specification of the MS lineage are not shown. The proposed activation by SKN-1 of the *med-1* and *med-2* genes marks the transition from maternal to zygotic control of gene expression. This figure was redrawn from Figure 4 of MADURO and ROTHMAN (2002).

segregating from these hermaphrodites was determined by PCR to produce the two final strains: JM133 [*dpy-17 sDf127 unc-32 III*; *sDp3 (III,f)*; *med-1(+)* X] and JM134 [*dpy-17 sDf127 unc-32 III*; *sDp3 (III,f)*; *med-1(ok804)* X]. The presence of the *sDf127* deficiency was confirmed by crossing JM133 and JM134 hermaphrodites to *sma-3(e491)/+* males and observing Sma males in the progeny; the *sma-3* gene is 20–25 kb upstream of *med-2*. The strains were also validated by PCR, as described in the text.

To produce a strain in which the *med-2* gene was removed by the chromosomal deficiency *nDf16*, RB930 [*med-1(ok804)*] males were crossed to hermaphrodites from strain CB1918 [*lon-1(e185) unc-32(e189) III*]; wild-type F₁ hermaphrodites were picked and allowed to self and a progeny strain that was homozygous *lon-1 unc-32* and also homozygous *med-1(ok804)* was identified by PCR. Hermaphrodites from this intermediate strain were crossed to N2 males, F₁ males were crossed to hermaphrodites from strain CX2914 [*nDf16/dpy-17(e164) unc-32(e189) III*] and a wild-type F₁ hermaphrodite [*nDf16/lon-1 unc-32 III*; *med-1(ok804)/+* X] that segregates dead eggs, Lon Unc worms, and (slow-growing) wild types was identified. Individual populations derived from single hermaphrodites were then screened by PCR to identify two strains: JM135 [*nDf16/lon-1 unc-32 III*; *med-1(+)* X] and JM136 [*nDf16/lon-1 unc-32 III*; *med-1(ok804)* X]. The presence of *nDf16* was verified by crossing JM135 and JM136 hermaphrodites to *sma-3(e491)/+* males and observing Sma males in the F₁ progeny.

Individual arrested embryos were picked (from plates incubated in parallel to the slides used for the birefringence assays, using a fresh capillary for each embryo) and digested with proteinase K, essentially as described (WILLIAMS *et al.* 1992). The following primers (see Figure 2, B and C) were used to detect *med-1* and *med-2* alleles in individual arrested embryos: oJM296, AGGTATGAAGCAGGCGTAGGC; oJM297, ACAGAGGTGCAAGGTGGTCC; oJM304, GTTTCACACTT TTTGCTGTGG; and oJM305, CAAAATAGGCTTGCTTTTA CGG. Amplification conditions were as follows: initial denaturation at 94° for 2 min, followed by 30 cycles of 94° for 30 sec, 62° for 30 sec, and 72° for 30 sec, ending with a final extension at 72° for 7 min. Identities of the amplified fragments were verified by restriction enzyme digestions (data not shown). Hermaphrodite mothers removed from the assay slides were frequently allowed to produce a further set of embryos, which were then genotyped by PCR to verify the hermaphrodite genotype and to ensure that deficiency chromosomes remained balanced.

Double-stranded RNAi: Double-stranded RNAi corresponding to the genomic *med-1*, *glp-1*, *skn-1*, and *let-413* loci (KAMATH *et al.* 2003) as well as to the coding sequence of green fluorescent protein (GFP) were produced, purified, and dissolved at a concentration of 1 mg/ml, as previously described (FUKUSHIGE *et al.* 2005). Double-stranded RNA was also produced corresponding to the *med-1* coding region (using as template plasmid pMM239 and primer sequences T7MED1A, taatcagctactataggaggCCTACCCTTACCCCG, and T7MED1C, taatcagctactataggaggAAATCTTGAGTTATGAT, kindly provided by M. Maduro; lowercase letters correspond to the added sequence of the T7 promoter) and, as an additional control, to a portion of the coding sequence of *lacZ* (using as template plasmid pPD16.43 and primer sequences oJM333, taatcagctactataggaggTAATCACGACGCGCTGTATC, and oJM334, taatcagctactataggaggCGGATAAACGGAAGTGGAAA); the latter two double-stranded RNAs (dsRNAs) were purified and dissolved at a final concentration of 2 mg/ml.

In our standard protocol, RNA was injected once into a gonad arm and once into the intestine cytoplasm/body cavity of young wild-type hermaphrodites; if worms are judged to be unhealthy or fragile (*e.g.*, the deficiency-containing strains), hermaphrodites were injected only once into the intestine/

body cavity. Injected worms were allowed to recover for 8–18 hr and then transferred to the birefringence assay slides (see below) at regular intervals over the next 2–3 days. All incubations were at 20 ± 0.1°. RNA injections were also performed according to a protocol provided by M. Maduro: young adult hermaphrodites (usually zero to five embryos in the uterus) were injected in both gonad arms, such that the (nondehydrated) gonad volume was estimated to increase by 20–50%. At least 80% of the worms were successfully injected in both gonads; the remaining 20% of the worms were injected in at least one gonad arm but when the second gonad injection proved difficult or ambiguous, the intestine/body cavity was also injected. Injected worms were incubated at 20 ± 0.1° for 7 hr and then transferred to the birefringence assay slides (see below) for 2 hr for embryo collection; injected hermaphrodites were often transferred to fresh slides to collect later embryos. To ensure maximal effectiveness of the RNAi, we aimed to produce ~20% lethality in the injected hermaphrodites. We routinely verified, using gel electrophoresis, that the dsRNA aliquot used for the injection remained completely undegraded; in half of our injection series, excess uninjected dsRNA was recovered from the injection needle and verified to be intact. In all *med* RNAi experiments, control injections (*lacZ* or GFP dsRNA) were performed in exact parallel and were also performed “blind”; *i.e.*, the person injecting and scoring the phenotype did not know the identity of the RNA. At the conclusion of the injections, wild-type worms were verified to indeed be RNAi sensitive by injection of *skn-1* dsRNA.

Assays for endoderm differentiation: To assay for gut-granule expression with minimal embryo manipulation, 25–50 gravid hermaphrodites of the various strains were placed on thin (~1 mm) NGM-agar (WOOD 1988) pads poured directly onto microscope slides and lightly seeded with *Escherichia coli* strain OP50. After 7 ± 2 hr of egg laying, adults were removed and the slides were incubated in a tightly sealed humidified chamber; all incubations were at 20 ± 0.1° for times ranging from 17 to 46 hr (to demonstrate that the results are essentially independent of incubation time and are not influenced by any slow-developing embryos). Following incubation, a small volume (50–100 µl) of egg salts (EDGAR and MCGHEE 1986) was added to the agar pad, which was then overlaid with a 22 × 50-mm coverslip, prior to viewing arrested embryos with birefringence optics. Images were obtained using a Zeiss Axioplan 2i microscope and a Hamamatsu (Bridgewater, NJ) OrcaER digital camera, using identical exposure settings for comparisons of the *med-1(-)* and *med-1(+)* strains; images were processed using only the default “best-fit” ($\gamma = 1$) setting of the Axiovision software.

The histochemical assay for GES-1 activity was performed essentially as described (EDGAR and MCGHEE 1986); a detailed protocol is available upon request. Immunohistochemical staining of arrested embryos was performed essentially as described (BOSSINGER *et al.* 2004), using monoclonal antibody MH33 (1/50–1/100 dilution of a hybridoma supernatant). Labeled secondary antibodies were obtained from Molecular Probes (Eugene, OR) and used at a dilution of 1/500.

RESULTS

Background and expected results: As explained in the Introduction, loss of maternal *skn-1* function produces an impenetrant loss of endoderm, somewhere in the range of 50–80% on the basis of the original description of the *skn-1(zu67)* strong loss-of-function mutant and depending on the incubation temperature (BOWERMAN *et al.* 1992). To be more precise about experimental

expectations, we injected dsRNA corresponding to the *skn-1* gene into wild-type worms, incubated them under the same experimental conditions used for other endoderm assays performed in this study ($20 \pm 0.1^\circ$ with hermaphrodites brooded on seeded microscope slides), and observed that 70% of the arrested embryos do not express gut granules (136/216, 416/630, and 366/472 gut-granule-negative embryos/total embryos counted at the peak of the RNAi effect following three independent injection series with a total of 36 injected hermaphrodites). Thus the expectation is that, if the zygotic *med* genes are the sole downstream effectors of SKN-1 in the pathway of endoderm specification as shown in Figure 1, $\sim 70\%$ of *med-2(-)*; *med-1(-)* embryos should not produce endoderm.

Our attempts to perform RNAi by standard protocols that are effective against both zygotic and maternal genes (MONTGOMERY *et al.* 1998; SHI and MELLO 1998; see MATERIALS AND METHODS) failed to detect significant differences among embryos produced by mothers injected with *med-1* dsRNA or with control dsRNA corresponding to GFP. We also note that no *med-1* or *med-2* RNAi effects have been reported by others when the dsRNA is administered by either feeding (KAMATH *et al.* 2003) or injection (SONNICHSEN *et al.* 2005). We also collected embryos in the time window from 7 to 9 hr post-injection, as described by MADURO *et al.* (2001). From a total of 75 injected hermaphrodites in six independent injection series, an average of $31 \pm 20\%$ (all reported uncertainties are standard deviations) embryos produced by *med* RNA-injected mothers arrested development and, of these arrested embryos, $24 \pm 21\%$ did not express gut granules; for comparison, $12 \pm 9\%$ of embryos whose mothers had been injected with either dsGFP or *dslacZ* RNA arrested and, of these, $25 \pm 26\%$ were gut-granule negative. Among the arrested embryos in two sets of injections, we observed a subset of partially elongated embryos that resembled the phenotype described by MADURO *et al.* (2001) and, just as they reported, these partially elongated embryos could be either gut-granule positive or gut-granule negative. However, in our judgment, the most important overall conclusion that should be drawn from the above injections is the small size of the experimental signal: hermaphrodites injected with *dsmed* RNA produce an average of 0.4 gut-granule-negative embryos per injected hermaphrodite; injections with dsGFP or *dslacZ* RNA produce an average of 0.2 gut-granule-negative embryos per injected hermaphrodite. We think that this experimental signal is so small that the specificity of such an effect would be difficult to establish unambiguously.

MADURO *et al.* (2001) also reported that endoderm markers were ablated when *med* RNAi was performed by heat-shock overexpression of sense and antisense cDNAs from a transgenic array, according to the method of TAVERNARAKIS *et al.* (2000). This heat-shock-induced

med RNAi could provide an alternative method for producing large numbers of *med* RNAi-arrested embryos, avoiding any limitations of our injection technique. However, when we performed this protocol on N2 control worms (collecting embryos 11–14 hr at 20° after a heat shock of 35° for 4 hr), heat-shocked L4 worms (as in the protocol) were completely sterile; heat-shocked adults produced an average of 0.6 embryo per hermaphrodite, roughly half of which were gut-granule negative because they arrested early in embryogenesis (data not shown). Overall, we believe that it is worthwhile to investigate whether the *med* genes are necessary for endoderm specification using a technique other than RNAi.

RNAi-independent strategy to test whether zygotic expression of the *med-1* and *med-2* genes is necessary for endoderm specification: Figure 2A shows the location of the *med-2* gene near the center of chromosome III (MADURO *et al.* 2001), together with the other genetic markers and chromosomal rearrangements used in this study. The *med-1* gene is located on the right arm of the X chromosome (MADURO *et al.* 2001). The central experiment of this study was to investigate endoderm formation in embryos segregating from a strain homozygous for a gene-specific *med-1* knockout allele (see below) and heterozygous for a chromosomal deficiency that removes the *med-2* gene. As shown in Figure 2A, *med-2* can be removed by either of two overlapping chromosomal deficiencies, *sDf127* (JANKE *et al.* 1997) or *nDf16* (THOMAS *et al.* 1990), together with ~ 100 and ~ 300 neighboring genes, respectively, with several dozen genes in addition to the *meds* being in common. Those embryos lacking both copies of both *med-1* and *med-2* will arrest development because the chromosomal deficiency removes essential genes in the vicinity of *med-2*. Nonetheless, such arrested embryos remain alive for days and can easily be assayed for endoderm-specific markers. Qualifications and justifications of the use of chromosomal deletions are discussed in another section.

Gene-specific deletion of *med-1*: A *med-1* gene-specific deletion (*ok804*) was provided by the *C. elegans* Genome Knockout Consortium and, as shown in Figure 2B, removes the entire *med-1* coding region. Southern blotting and extensive PCR validated this deletion; in particular, no unsuspected *med-1* gene duplication associated with the mutagenesis could be detected elsewhere in the genome (see MATERIALS AND METHODS). After outcrossing three times to wild type in an attempt to remove extraneous mutations, the strain RB930 containing the *med-1(ok804)* deletion appears quite healthy (occasional protruding vulvae) and fertile (brood size was 269 ± 53 with five total broods counted) but harbors a low level of embryonic lethality (percentage hatching was 92.5 ± 3.2). We do not know whether this low level of lethality is caused by *med-1(ok804)* or by a mutation in an unrelated gene within the strain; in any case, $>98\%$ of

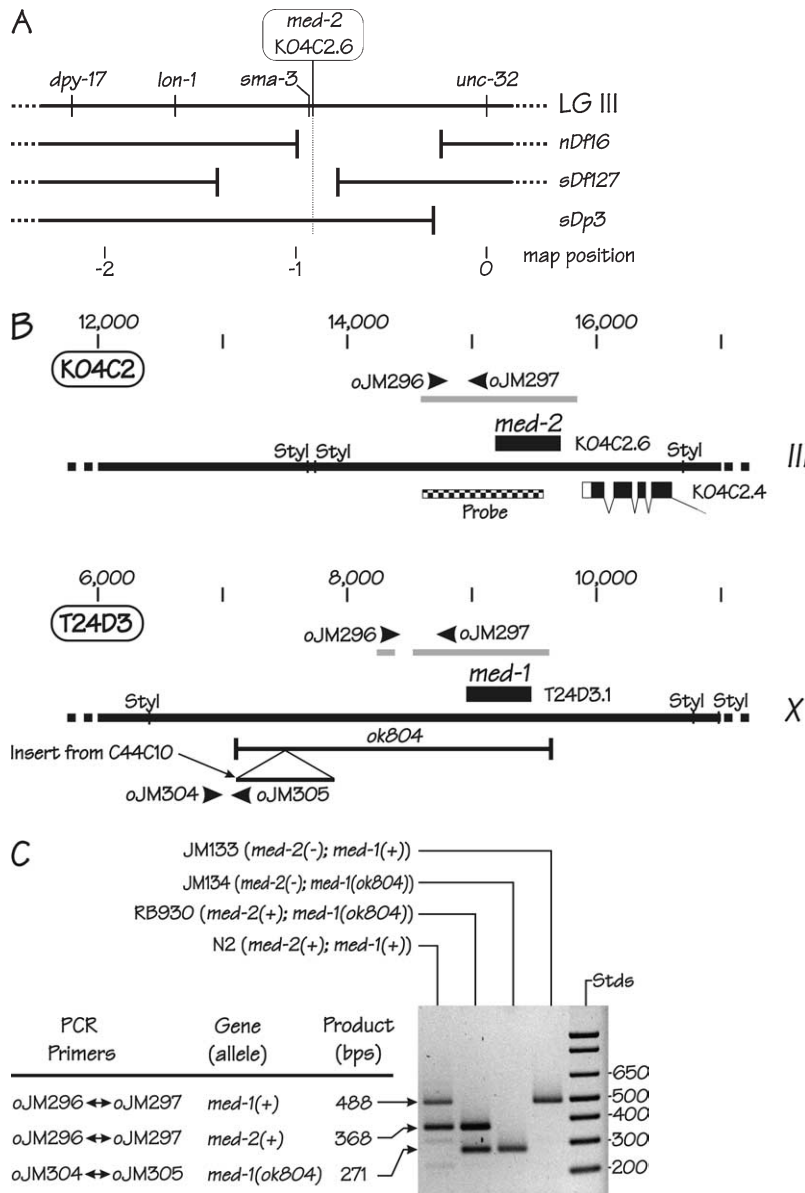


FIGURE 2.—Genetic positions and molecular characteristics of the *med-1* and *med-2* genes. (A) The genetic map of the middle of the *C. elegans* chromosome III. The *med-2* gene is located on cosmid K04C2, which is removed by the chromosomal deficiencies *sDf127* and *nDf16*. As described in the text, the free duplication *sDp3* is used to balance the deficiency *sDf127*; a chromosome marked with *lon-1 unc-32* is used to balance the deficiency *nDf16*. (B) Expanded views of the chromosomal regions surrounding the *med-2* gene (top) and the *med-1* gene (bottom). Coordinates correspond to the cosmid sequences K04C2 and T24D3 for *med-2* and *med-1*, respectively. The intronless ORFs corresponding to *med-1* and *med-2* are indicated by the solid boxes. The shaded bars above the *med-2* and *med-1* ORFs indicate the chromosomal regions that show high sequence similarity between the two genes (see MADURO *et al.* 2001 for details). Because of this region of high sequence similarity, the presence of *med-1* and *med-2* genes can be assayed simultaneously and independently by the same set of primers, oJM296 and oJM297. The extent of the *med-1* deletion allele *ok804* is indicated. We note that an alternative structure of the *med-1* gene (containing a 3'-intron) has previously appeared in the *C. elegans* sequence annotation: the *ok804* allele removes the predicted *med-1* zinc-finger DNA-binding domain and would be predicted to produce a genetic null for either gene model. The *ok804* deletion is associated with a 790-bp insertion of a (non-ORF-containing) sequence from cosmid C44C10, allowing the deletion to be detected by PCR primers oJM304 and oJM305, as indicated. The cross-hatched region beneath the *med-2* gene was amplified by PCR, cloned, and used as a probe to detect both *med-2* and *med-1* sequences by Southern blotting on genomic DNA digested with the restriction enzyme *StyI*. (C) PCR detection of *med-1*(+), *med-1*(*ok804*), and *med-2*(+) alleles in individual arrested embryos produced by strains N2 (wild type), RB930 [*med-1*(*ok804*)], JM134 *med-1*(-), and JM133 *med-1*(+). The deduced status of the *med* genes in the individual embryos are indicated beside the strain names. Locations of PCR primers within the *med-1* and *med-2* genes are shown in B.

the arrested RB930 embryos still produce endoderm (data not shown).

Removal of the *med-2* gene by the chromosomal deficiency *sDf127*: *Strain characterization:* The strain JM134 [*dpy-17 sDf127 unc-32 III; sDp3(III,f); med-1(ok804) X*] was constructed and validated as described in MATERIALS AND METHODS. *sDp3* is a (single-copy) free duplication that covers most of the left half of chromosome III, including all of *sDf127* (ROSENBLUTH *et al.* 1985). The genetic properties of *sDp3* have been well studied (HEDGECOCK and HERMAN 1995) and it is expected that ~40% of JM134 offspring will not receive the free duplication and will thus arrest because they are homozygous for the *sDf127* deficiency; this number will be verified below. As we will also show below, animals that receive two (or more) copies of the duplication

must be rare; in any case, embryos that retain *sDp3* can easily be identified by PCR. The control strain JM133 [*dpy-17 sDf127 unc-32; sDp3; med-1(+)*] was isolated as a *med-1*(+) segregant from the same cross that generated the *med-1*(-) strain JM134. For convenience, these two strains will be referred to as JM133 *med-1*(+) and JM134 *med-1*(-).

There are few obvious differences between strains JM133 *med-1*(+) and JM134 *med-1*(-); *i.e.*, they seem to survive equally well with, on average, three copies (JM133) or only one copy (JM134) of a *med* gene. As shown in Table 1, brood sizes and hatching rates are comparable. The arrested embryos produced by both strains look similar, have >400 nuclei (Table 1), and appear typical of aneuploid "monsters" (see below). The large majority (>98%) of the arrested embryos of

TABLE 1
Comparison of properties of strains JM133 *med-1(+)* and JM134 *med-1(-)*

	JM133 <i>dpy-17 sDf127 unc-32; sDp3; med-1(+)</i>			JM134 <i>dpy-17 sDf127 unc-32; sDp3; med-1(ok804)</i>		
	Average	SD	<i>n</i>	Average	SD	<i>n</i>
Brood size	125	31	8 broods	122	41	7 broods
% arrest	45.2	6.4	8 broods	49.4	2.0	7 broods
No. nuclei	444	50	9 embryos	421	48	10 embryos

both genotypes continue to exclude the vital dye Trypan Blue after 4 days incubation at 20° (data not shown).

Measuring the proportion of arrested embryos that lack both med-1 and med-2 genes: Before inspecting the arrested embryos for gut marker expression, we first demonstrate that the majority (~80%) of the arrested embryos produced by strain JM134 *med-1(-)* are indeed deficiency homozygotes lacking *sDp3* and hence lacking both the *med-1* and *med-2* genes. The two pairs of PCR primers shown in Figure 2B allow detection of the status of both *med-2* and *med-1* genes in individual arrested embryos (Figure 2C) and have been designed to produce at least one positive band per reaction to verify successful PCR. Using this set of primers, 83.5% (76/91) of the arrested embryos produced by JM134 *med-1(-)* were found to have lost the *sDp3* balancer and are thus *med-2(-); med-1(-)* homozygotes. The remaining 16.5% (15/91) contain a *med-2(+)* allele and must arrest for some other reason, perhaps because of haplo-insufficiency or because they have received multiple copies of *sDp3*.

An independent estimate of the proportion of deficiency homozygotes among the arrested JM134 *med-1(-)* embryos can be made from progeny phenotypes. The average proportion of arrested embryos produced by strain JM134 *med-1(-)* is 49.1% [the mean of 49.4 ± 2.0% estimated using individual total broods (Table 1) and 48.8% calculated from 262 unhatched embryos counted among 537 embryos produced in several hours by 50 adult JM134 hermaphrodites]. From the fraction of Dpy offspring produced by a *dpy-17 unc-32 III; sDp3* control strain (five total broods), we estimate that 38.1 ± 4.9% of JM134 “*med-1(-)*” F₁ progeny have lost the *sDp3* duplication. In other words, 38.1/49.1 = 77.6% of arrested JM134 “*med-1(-)*” embryos should be *med-2(-); med-1(-)*, in acceptable agreement with the 83.5% estimated by PCR. We note that the estimated frequency of *sDp3* loss from the *dpy-17 unc-32; sDp3* control strain (38 ± 5%) agrees well with the loss frequency (~40%) estimated previously by HEDGECOCK and HERMAN (1995) using a different strain. We also note that the *dpy-17 unc-32; sDp3* strain produces 4.2% unhatched embryos, thereby providing an upper limit to the frequency of arrested embryos receiving multiple copies of *sDp3*.

Gut marker expression in embryos lacking both med-1 and med-2 genes: The arrested embryos produced by JM133

med-1(+) and JM134 *med-1(-)* were assayed for three biochemically independent markers of endoderm differentiation: (i) gut granules, (ii) histochemical staining for the gut-specific GES-1 esterase, and (iii) immunohistochemical staining using the monoclonal antibody MH33 to detect the intestine-specific IFB-2 intermediate filament protein. All assays in this study were performed “blind”; *i.e.*, the person manipulating the embryos and/or scoring the differentiation marker did not know the strain identity; experiments were repeated at least five times for each genotype, as described in the legends for Figures 3 and 4:

- The most convenient (and foolproof) assay for gut differentiation is birefringent gut granules (CHITWOOD and CHITWOOD 1974; LAUFER *et al.* 1980), which can first be detected when the embryonic intestine has approximately eight cells and which depend on zygotic transcription soon after endoderm specification (EDGAR and MCGHEE 1988). To eliminate the possibility of selective loss of any particular class of embryos during manipulation, gravid hermaphrodites were placed on NGM-agar pads poured directly onto microscope slides and lightly seeded with bacterial food; after 7 ± 2 hr of egg laying, adults were removed and the slide incubated at 20° to allow viable embryos to hatch. Embryos that failed to hatch (after a wide range of incubation times; see MATERIALS AND METHODS) were then inspected with polarized light. Examples of arrested embryos produced by strains JM133 *med-1(+)* and JM134 *med-1(-)* are shown in Figure 3, A and B, and Figure 3, C and D, respectively. It is evident that the majority of arrested embryos produced by either strain still express gut granules. However, there is a small fraction of arrested embryos produced by JM134 *med-1(-)* in which gut granules cannot be detected (see arrows in Figure 3, C and D). Overall, 13.4 ± 3.7% of the arrested embryos produced by strain JM134 *med-1(-)* do not express gut granules, compared to 0.2% of the arrested embryos produced by the control strain JM133 *med-1(+)*. Correcting for the proportion (measured in the previous section) of arrested JM134 *med-1(-)* embryos that are not deficiency homozygotes (and assuming that all embryos in this class are marker positive), we estimate that 16.0–17.3% of *med-2(-)*;

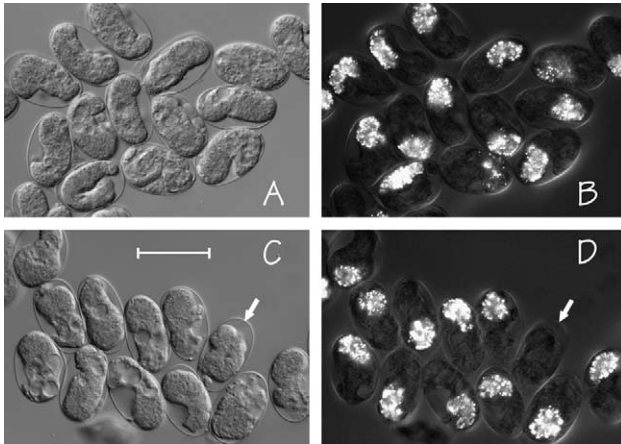


FIGURE 3.—Microscopic images of typical arrested embryos produced by control strain JM133 *med-1*(+) (A and B) and by strain JM134 *med-1*(-) (C and D), as seen by differential interference contrast (A and C) or by birefringence optics (B and D). As described in the text, the majority of the arrested embryos produced by either strain still express birefringent gut granules. The arrows in C and D indicate one arrested embryo produced by strain JM134 *med-1*(-) that does not express gut granules. Bar, 50 μ m. This experiment was repeated six times for each genotype; the total number of scored embryos was 671 and 690 for strains JM133 *med-1*(+) and JM134 *med-1*(-), respectively.

med-1(-) embryos still express gut granules. On the basis of the penetrance of gut-granule-negative embryos produced by *skn-1*(null) mothers (measured above), if the *meds* were solely responsible for *skn-1*-specified endoderm, we would have expected 70% of the arrested embryos to be gut-granule-negative, rather than our observed 16–18%.

- ii. The arrested embryos were stained histochemically for activity of the gut-specific GES-1 esterase (EDGAR and MCGHEE 1986). Because the assay for GES-1 activity is more technically demanding than the simple birefringence assay for gut granules (in particular, the pressure permeabilization-fixation step in GES-1 staining is prone to variability), the intensity of GES-1 staining was scored blind and semiquantitatively as 0, +, ++, or ++++. Figure 4A shows a histogram summarizing the staining results, together with typical images of arrested embryos [from strain JM134 *med-1*(-)] representing each of the four classes of staining intensity. The majority of arrested embryos from both strains stain intensely for GES-1 activity (scored as ++++); nonstaining JM134 *med-1*(-) embryos are only slightly more prevalent (~6%) than nonstaining JM133 *med-1*(+) control embryos. Indeed, it is doubtful that any difference in GES-1 staining can be reliably detected between the two sets of embryos (see Figure 4 legend).
- iii. Arrested embryos were stained immunochemically using the monoclonal antibody MH33 (FRANCIS and WATERSTON 1985), which reacts with IFB-2,

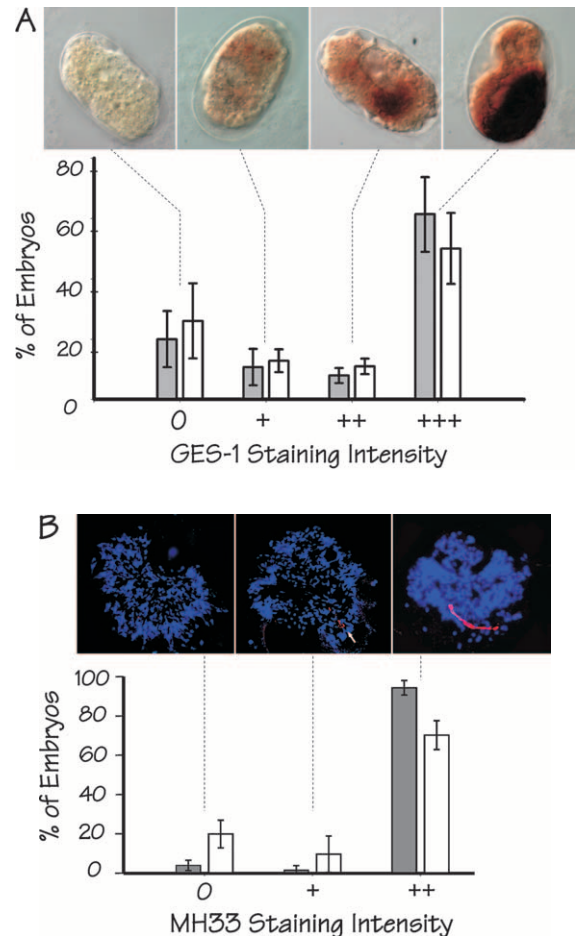


FIGURE 4.—Expression of additional markers of endoderm differentiation in arrested embryos produced by strains JM133 *med-1*(+) and JM134 *med-1*(-). (A) Histogram showing the distribution of GES-1 staining intensity (classified as 0, +, ++, or ++++) within arrested embryos produced by strain JM134 *med-1*(-) (open bars) or by control strain JM133 *med-1*(+) (shaded bars). Typical images of each class of stained embryo [from strain JM134 *med-1*(-)] are shown above the corresponding histogram bar. The experiment was repeated a total of five times for each genotype, scoring a total of 830 and 872 individual arrested embryos produced by strains JM133 *med-1*(+) and JM134 *med-1*(-), respectively; error bars are standard deviations. The apparent difference between the JM133 *med-1*(+) and JM134 *med-1*(-) histograms is due entirely to one particular pair of slides (corresponding to the longest incubation time) in which some embryos appeared to have degenerated and the staining appeared erratic. If this set of data is excluded from the analysis, the behaviors of JM133 *med-1*(+) and JM134 *med-1*(-) are essentially indistinguishable (data not shown). (B) Histogram showing the distribution of MH33 staining intensity (classified as 0, +, ++, or ++++) within arrested embryos produced by strain JM134 *med-1*(-) (open bars) or control strain JM133 *med-1*(+) (shaded bars). Images [from strain JM134 *med-1*(-)] represent Z-projections of deconvolved image stacks taken for each embryo; blue, DAPI staining; red, MH33 immunofluorescence. A well-formed endotube is obvious in embryos classified as “++”; a rudimentary endotube can be detected in embryos classified as “+” (see arrow). This experiment was repeated five times, scoring a total of 305 and 386 arrested embryos for strains JM133 *med-1*(+) and JM134 *med-1*(-), respectively; error bars represent standard deviations. DAPI-stained images from these experiments were used to estimate the total number of nuclei in the arrested embryos (Table 1).

an intestine-specific intermediate filament protein (KARABINOS *et al.* 2001; BOSSINGER *et al.* 2004); the intensity of staining was scored blind and semi-quantitatively as 0, +, or ++. As shown in Figure 4B, the majority of arrested embryos from either JM133 *med-1(+)* or JM134 *med-1(-)* stain intensely with MH33 (scored as ++) and exhibit an obvious “endotube” [the subluminal structure containing IFB-2 (BOSSINGER *et al.* 2004)]. After correcting for nonstaining JM133 *med-1(+)* control embryos, we estimate that ~16% of all arrested embryos produced by strain JM134 *med-1(-)* or ~20% of the *med-2(-); med-1(-)* embryos do not express the endoderm-specific *ifb-2* gene.

Removal of the *med-2* gene by the chromosomal deficiency *nDf16*: In an attempt to rule out possible confounding influences associated with the particular chromosomal deficiency (for example, if *sDf127* were to remove a repressor of an alternative endoderm-specification pathway), the analysis was repeated using the overlapping deficiency *nDf16* (see Figure 2A); *nDf16* is a larger deficiency than *sDf127* and removes ~300 genes surrounding *med-2*; several dozen or so of these genes are also removed by *sDf127*. We constructed two new strains: JM136 [*nDf16/lon-1 unc-32 III; med-1(ok804) X*] and the control strain JM135 [*nDf16/lon-1 unc-32 III; med-1(+)* X]; again, for convenience, these strains will be referred to as JM135 *med-1(+)* and JM136 *med-1(-)*.

For both strains JM135 *med-1(+)* and JM136 *med-1(-)*, it is expected that 25% of the F₁ progeny should arrest because they are homozygous for *nDf16*. The control strain JM135 *med-1(+)* shows a slight degree of excess lethality above the expected 25%: 31% (62/200) of F₁ progeny arrest. Thus, ~6% of the total F₁ progeny or 19% of the arrested JM135 *med-1(+)* embryos fail to hatch for reasons other than *nDf16* homozygosity, *e.g.*, haplo-insufficiency. In contrast, strain JM136 *med-1(-)* shows a substantial degree of excess lethality: ~66% (428/648) of F₁ progeny produced by strain JM136 *med-1(-)* failed to hatch; that is, only 25/66 = 38% of JM136 arrested embryos are expected to be *nDf16* homozygotes and hence *med-2(-); med-1(-)*. In acceptable agreement with this estimate, the direct PCR assay found that 41.5% (39/94) of arrested JM136 embryos are indeed *med-2(-); med-1(-)* (data not shown). Although this excess lethality is intriguing, it is irrelevant for interpreting the current experiment because 98.8% (2136/2162) of the arrested embryos produced by strain JM136 *med-1(-)* strongly express birefringent gut granules. For comparison, 97.3% (733/753) of arrested embryos produced by the control strain JM135 *med-1(+)* are also gut-granule positive. Typical birefringent images of arrested embryos produced by strains JM135 *med-1(+)* and JM136 *med-1(-)* are shown in Figure 5, A and B, respectively. Correcting for the fraction of JM136 *med-1(-)* arrested

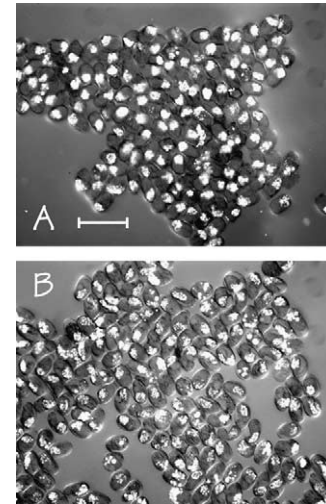


FIGURE 5.—Microscopic images (birefringence optics) of typical arrested embryos produced by (A) strain JM135 *med-1(+)* and (B) strain JM136 *med-1(-)*. This experiment was repeated a total of eight times for each genotype, scoring a total of 753 and 2162 arrested embryos for strain JM135 *med-1(+)* and strain JM136 *med-1(-)*, respectively. As described in the text, >97% of arrested embryos produced by either strain still express birefringent gut granules. Bar, 100 μ m.

embryos that are not homozygous for *nDf16*, we estimate that only 3% of *med-2(-); med-1(-)* embryos produced by JM136 *med-1(-)* do not express gut granules, essentially the same low proportion found in the *med-2(-); med-1(+)* control embryos.

Do *med-1* and *med-2* genes exhibit a maternal effect?

We consider whether the differences between our results and those of MADURO *et al.* (2001) can be explained by the *med* genes specifying endoderm by both a maternal function and a zygotic function. In particular, we test the simplest “maternal *med*” model: if a particular embryo does not express endoderm markers, it must lack both maternal and zygotic contributions from the *med* genes; *i.e.*, either maternal or zygotic *med* function is sufficient to specify endoderm.

A first test of the “maternal *med*” model is based on the argument that, if a putative *med-2* maternal product is necessary to specify embryonic endoderm in the absence of zygotic *med* genes, then the 15–20% of arrested JM134 *med-2(-); med-1(-)* embryos that do not express endoderm markers must have been produced by individual mothers who had lost the *sDp3* in their germline. Although the rarity of such animals (HEDGECOCK and HERMAN 1995) would argue against this possibility, we nonetheless inspected arrested embryos produced by 20 individual JM134 *med-1(-)* mothers; all 20 broods contained roughly the expected fraction of arrested embryos that do not express gut granules, arguing that the gut-granule-negative embryos do not arise from infrequent mothers lacking *sDp3* in their germlines.

As a second test of the “maternal *med*” model, double-stranded RNA corresponding to the *med-1* gene was injected into the sensitized JM134 *med-1(-)* mothers and the population of arrested F₁ embryos was inspected for birefringence. As described in the MATERIALS AND METHODS, these injections were performed by standard protocols found to be effective against maternal transcripts (FIRE *et al.* 1998; SHI and MELLO 1998), injecting into the intestine/body cavity and monitoring F₁ embryos over the next few days. The proportion of *med-1* [and therefore, because of high sequence similarity (MADURO *et al.* 2001), also *med-2*] RNAi JM134 *med-1(-)*-arrested embryos that do not express gut granules was only marginally greater ($4.1 \pm 5.2\%$) than that found in arrested JM134 *med-1(-)* embryos whose mothers had been injected with double-stranded RNA corresponding to GFP (experiment repeated three times, injecting 10–20 hermaphrodites per repeat). In addition, RNAi to *med-1* did not obviously enhance the level of embryonic arrest. As a control on technique and on the RNAi sensitivity of strain JM134, the same injection protocol using double-stranded *skn-1* RNA caused 100% arrest of embryos produced by JM134 *med-1(-)* mothers. In summary, these two different experimental approaches provide no evidence for a model in which either zygotic or maternal expression of the *med* genes is necessary (or sufficient) to specify endoderm.

DISCUSSION

In this study, we have tested whether the *med-1* and *med-2* genes are necessary for specification of the *C. elegans* endoderm. If the *med-1* and *med-2* genes are the major effectors downstream of the maternal SKN-1 factor and are responsible for specifying the *C. elegans* endoderm (MADURO *et al.* 2001), then 70% of *med-2(-)*; *med-1(-)* embryos should not produce endoderm. Our results do not agree with this expectation; instead, we find that only ~3% (~0% if corrected for the background observed with control embryos) or 15–20% of *med-2(-)*; *med-1(-)* embryos do not express gut markers, depending on which chromosomal deficiency (*nDf16* or *sDf127*, respectively) was used to remove the *med-2* gene. Either result indicates that the *C. elegans* endoderm can be specified with high efficiency in the complete zygotic absence of the *med* genes. We emphasize that our experiments have addressed only *necessary* roles of *med-1* and *med-2* in endoderm specification and have not addressed other proposed roles for the *med* genes, such as sufficiency for endoderm marker expression, necessity and sufficiency for specification of the MS lineage, or restriction of the fate of the neighboring C blastomere (MADURO *et al.* 2001; MADURO and ROTHMAN 2002; BROITMAN-MADURO *et al.* 2005).

Two different experimental approaches failed to provide evidence for a maternal contribution of the *med* genes to endoderm specification: (i) an unsuccessful

attempt to identify individual JM134 broods showing a penetrant loss of endoderm in the arrested embryos and (ii) an unsuccessful attempt to perform *med* RNAi in hermaphrodites that segregate the *med-2(-)*; *med-1(-)* embryos, using a standard injection protocol that has generally been found to be effective against maternal transcripts (FIRE *et al.* 1998; SHI and MELLO 1998). However, it would be difficult to verify that the *med-1* RNAi has indeed been effective because, using either oocyte or early embryo RNA, any putative maternal *med* transcripts are below the detection level of Affymetrix microarrays (BAUGH *et al.* 2003, 2005). Finally, we point out that any maternally provided *med* contribution (proteins or transcripts) that can rescue the loss of zygotic *med* function would be inconsistent with the endoderm loss caused by loss of maternal *skn-1* (BOWERMAN *et al.* 1992).

A secondary issue is to resolve why our estimates of possible *med*-dependent endoderm vary between the two deficiencies: 15–20% using *sDf127* and ~3% using *nDf16*. If the *sDf127* results are valid, one possibility to explain the *nDf16* results is that the 1–2% of the genome included in *nDf16* but not in *sDf127* contains a repressor of the *end-1* or *end-3* genes or perhaps a repressor of an alternative pathway to specify endoderm. Alternatively, if the *nDf16* results are valid, then the *sDf127* results could possibly be explained by increased embryo fragility, a slightly earlier stage of embryo arrest and/or non-specific loss of endoderm marker expression, all of which could be exacerbated by the fact that *sDf127* homozygotes are also homozygous *unc-32*; null mutations in *unc-32* are known to cause embryonic arrest with vacuolated intestines (PUJOL *et al.* 2001) and we found that *unc-32* RNAi greatly decreases gut-granule intensity in the arrested embryos, such that ~20% would be classified as negative by our scoring criteria (data not shown). However, we really have no reason for choosing between these two possibilities and the important point remains that the results obtained with either deficiency are incompatible with the model being tested.

A potential weakness of the present analysis is our use of multigene chromosomal deficiencies to remove the *med-2* gene, raising the theoretical possibility of generating an “endoderm permissive environment” within the deficiency embryos. Although such a possibility is difficult to rule out, several considerations suggest that it is unlikely. The perfect candidate for a gene whose removal would generate an “endoderm permissive environment” is the *pop-1* gene (LIN *et al.* 1995, 1998; ROCHELEAU *et al.* 1997, 1999) but *pop-1* is not removed by either *sDf127* or *nDf16*. Other known endoderm-specifying genes (*e.g.*, *skn-1*, *lit-1*, *mom-1,2,4,5*, etc.) are not removed by either deficiency and, in any case, loss of function of these genes inhibits endoderm formation. Furthermore, genes involved in endoderm specification (up to the *med* genes) are expected to be maternally provided and thus the homozygous deficiency embryos

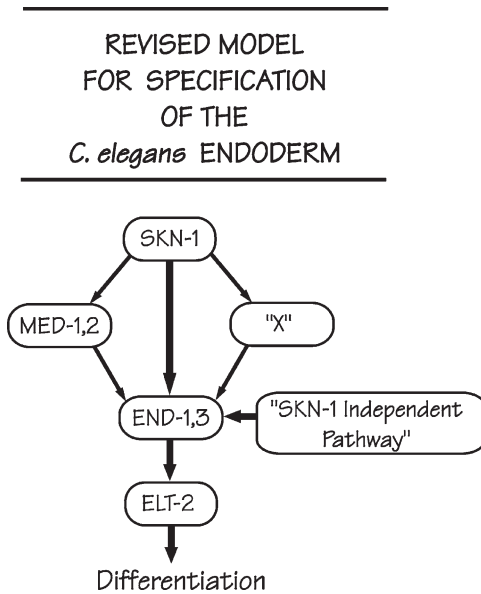


FIGURE 6.—Revised proposal for the transcription factor cascade responsible for specifying the *C. elegans* endoderm. As described in the text, we suggest that the SKN-1-activated *med-1* and *med-2* genes may play a relatively minor role in specifying the *C. elegans* endoderm. We suggest that the major pathway for endoderm specification could involve the SKN-1 transcription factor itself directly activating the *end-1* and *end-3* genes, as originally proposed by ZHU *et al.* (1997) or acting through an unknown factor, depicted as X. Finally, there must be a significant SKN-1 independent pathway to specify endoderm (~30% under the current experimental conditions).

receive at least a haploid dose of maternal product. As a final defense of the use of chromosomal deficiencies, we suggest that poor embryonic health caused by aneuploidy is more likely to reduce than enhance endoderm gene expression. If only for this final reason, we suggest that the current experimental system should, if anything, overestimate the importance of the *med-1* and *med-2* genes in endoderm specification.

If the *med-1* and *med-2* genes are not required for (most) *C. elegans* embryos to produce endoderm, then what is the major endoderm activator? We suggest that, as has been previously proposed and as depicted in Figure 6, the maternal SKN-1 factor may still be the best candidate for initiating the endoderm pathway, for example, by direct activation of the *end-1* and *end-3* genes within the endoderm precursor E cell (ZHU *et al.* 1997), apparently in direct conjunction with transcriptional cofactors such as CBP-1 (SHI and MELLO 1998; WALKER *et al.* 2000) and in the permissive environment provided by low nuclear concentrations of the POP-1 protein (LIN *et al.* 1995, 1998; ROCHELEAU *et al.* 1997; THORPE *et al.* 1997; LO *et al.* 2004). It has recently been reported that ablation of MED-binding sites from the 5'-flanking region of the *end-1* genes abolishes expression of an *end-1* reporter (BROITMAN-MADURO *et al.* 2005). However, this alteration was performed in the context

of a short promoter fragment (<250 bp) and it had previously been pointed out that six potential SKN-1-binding sites lie within the next 1 kb upstream (ZHU *et al.* 1997). There is, of course, the possibility that SKN-1 specifies endoderm by acting through a factor other than MED-1/2, and this unknown factor is designated by the traditional X shown in Figure 6. Finally, Figure 6 draws attention to the significant SKN-1 independent pathway for endoderm specification.

Finally, we point out a limitation in the interpretation of our results that is a common feature of studies of early *C. elegans* development, namely the analysis of phenotypes in terms of penetrance rather than in terms of expressivity. It is possible that the MED factors could be participating in many acts of transcription that lead to endoderm specification; the present experiments would have detected this participation only if the consequences of *med* removal were changes in penetrance or substantial changes in expressivity. That is, a 20% lower penetrance of endoderm in the population of arrested embryos was obvious but it is doubtful that a 20% lowering of the intensity of marker expression in the *med-2(-); med-1(-)* embryos would have been detected. Likewise, the *med* genes could be intimately involved in endoderm specification but be redundant with some other set of endoderm-specifying genes. These two possible characteristics could, in principle, explain the reported sufficiency of *med-1* and *med-2* for endoderm marker expression (MADURO *et al.* 2001; MADURO and ROTHMAN 2002; BROITMAN-MADURO *et al.* 2005).

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Note added in proof: Laurent Segalat (Université Lyon Claude Bernard) has recently identified a Mos insertion within the *med-2* gene, thereby producing a likely *med-2* null allele (*cxP9744*). Our preliminary analysis of gut-granule production by *med-2 (cxP9744); med-1(ok804)* embryos confirms the results obtained with chromosomal deficiencies as described in this article: 16% (18/112) of *med-2 (cxP9744); med-1(ok804)* embryos do not express gut granules. A more complete analysis of endoderm formation in these embryos will be reported once the strains have been sufficiently outcrossed.

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