

# Reproductive isolation in *Caenorhabditis*: terminal phenotypes of hybrid embryos

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**SUMMARY** Several interspecific combinations of the “elegans” group of *Caenorhabditis* species are cross-fertile. Most F1 hybrids from these crosses arrest during embryogenesis. Developmental defects observed in hybrid embryos include

defects in gastrulation initiation, defects in embryonic compaction, and defects in embryonic elongation. These reproductive barriers have arisen multiple times in the evolution of *Caenorhabditis*.

## INTRODUCTION

Reproductive isolation refers to all genetic mechanisms that prevent or limit gene flow between populations (Mayr 1963; Dobzhansky 1970). These mechanisms are important as the advent of reproductive isolation can result in speciation and/or enforce existing species boundaries. Relatively few genes involved in the implementation of reproductive isolation have been well-characterized (see Wu and Palopoli 1994). This is primarily because the reproductive barriers erected by these genes usually preclude their characterization by traditional genetic methodologies. Thus, while a limited number of reproductively isolating genes in *Drosophila* have been well-mapped and well-characterized (e.g., Hutter and Ashburner 1987; Hutter et al. 1990; Perez et al. 1993; Hutter and Karch 1994; Perez and Wu 1995; Sawamura et al. 1995; Davis and Wu 1996), most remain obscure. In species combinations in which introgression studies are not possible, three approaches are available for the identification of reproductive isolating genes. First, genes can be identified in variant strains that exhibit qualitative differences in reproductive isolation (e.g., Watanabe 1979; Davis et al. 1996); second, genes can be identified by cellular and biochemical studies of reproductive barriers (e.g., Wittbrodt et al. 1989; Glabe and Clark 1991; Wittbrodt et al. 1992); and third, candidate genes can be identified by comparing hybrid terminal phenotypes with nonhybrid mutant phenotypes (e.g., Orr et al. 1997). This last approach requires detailed study of development in hybrid animals.

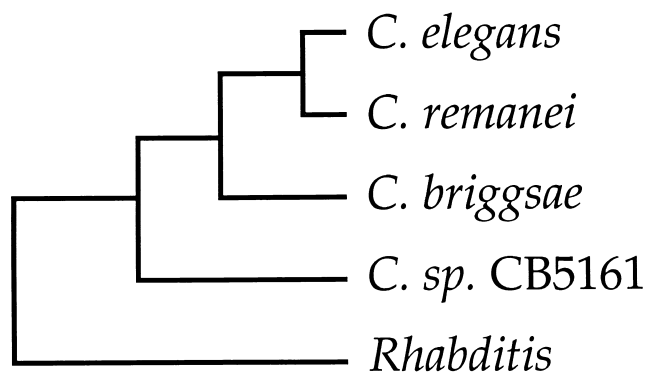
In the nematode genus of *Caenorhabditis*, mechanisms that isolate four species, *C. elegans*, *C. briggsae*, *C. remanei*, and *C. sp.* strain CB5161, have been described (Baird et al. 1992). These species all are members of the “elegans” group of *Caenorhabditis* and are considered to be close relatives of

each other (Sudhaus and Kiontke 1996). Among these four species, no mate preferences have been observed. The phylogeny of *C. elegans*, *C. briggsae*, *C. remanei*, and *C. sp.* strain CB5161, as inferred from 18s rDNA and RNA polymerase II sequences, is shown in Fig. 1 (Baldwin et al. 1997). Reproductive isolating mechanisms that act among *C. elegans*, *C. briggsae*, *C. remanei*, and *C. sp.* strain CB5161 include assortive fertilization, hybrid lethality, and hybrid sterility (Baird et al. 1992). Unfortunately, introgression studies in these species combinations are not possible. Thus, the identification of reproductive isolating genes in *Caenorhabditis* currently must rely upon studies of variant strains, on cellular and biochemical studies of reproductive barriers, and/or on comparisons of hybrid terminal phenotypes to nonhybrid mutant phenotypes. Among the cross-fertile combinations of *Caenorhabditis* are crosses of *C. elegans*, *C. remanei*, and *C. briggsae* males to *C. sp.* strain CB5161 females (Baird et al. 1992). Hybrids that result from these crosses arrest during embryogenesis. In this report, terminal phenotypes in hybrid embryos are described. It is hoped that these descriptions will facilitate the identification of genes that implement these reproductive barriers.

## MATERIALS AND METHODS

### Nematode strains and strain maintenance

Strains used in this study included: *C. elegans* JK577 = *fog-2(q71)*; *C. sp.* CB5161; *C. briggsae* AF16; and *C. remanei* SB146. The *fog-2(q71)* mutation converts *C. elegans* hermaphrodites into females (Schedl and Kimble 1988). In Baird et al. (1992), CB5161 was identified as *C. remanei*. With the collection of *C. remanei* strain SB146 from the type location, this identification has been shown to be in-



**Fig. 1.** *C. elegans*, *C. remanei*, *C. briggsae*, and *C. sp.* strain CB5161 belong to the *elegans* group of *Caenorhabditis* (Sudhaus and Kiontke 1996). A partial phylogeny of this group is shown. Modified from Baldwin et al. (1997).

correct (Sudhaus and Kiontke 1996). Thus, CB5161 represents a currently undescribed species of *Caenorhabditis*. Moreover, strain EM464 [*Caenorhabditis* species v (Baird et al. 1992) and *Caenorhabditis vulgaris* (Baird et al. 1994)] have been shown to be an isolate of *C. remanei* (Sudhaus and Kiontke 1996). Thus, *C. vulgaris* is a junior synonym of *C. remanei* and results described for *C. sp. v* in Baird et al. (1992) should be equated with results reported herein for *C. remanei*. *Caenorhabditis* strains were maintained on 60-mm agar plates seeded with *Escherichia coli* strain OP50 (Brenner 1974). These strains all are available from the *Caenorhabditis* Genetics Center (Theresa Stiernagle; Department of Genetics and Cell Biology, University of Minnesota, St. Paul MN 55108, USA).

### Interspecific crosses

Interspecific crosses were initiated by placing five L4 males and three L4 females on agar plates seeded with ~1-cm circles of *E. coli* strain OP50 (Hodgkin 1983; Baird et al. 1992). These worms were allowed to mature and mate overnight at 25°C. Gravid females were placed in 20  $\mu$ l of M9 buffer on a cover slip and cut in half to liberate hybrid embryos (Edgar 1995). These cover slips were then inverted and mounted on 2.5% agarose pads on microscope slides. Hybrid embryos were observed immediately for cell lineage analysis or were allowed to mature overnight in a humid chamber at 25°C for analysis of terminal phenotypes. Overnight incubation is sufficient time for *C. elegans* and CB5161 embryos to complete embryogenesis and hatch as L1 larvae (Sulston et al. 1983; data not shown).

### Microscopy

Observations for cell lineage analysis and for analysis of terminal phenotypes were made by differential interference contrast (DIC) microscopy (magnification 400–1,000 $\times$ ). Gut granules were visualized by fluorescence microscopy. Micrographic images were digitally captured using a Spot Camera and Software (Diagnostic Instruments, Inc., Sterling Heights MI, USA).

### Statistical methods

Hybrid arrest profiles were compared by chi-squared analyses.

## RESULTS

### Embryogenesis in CB5161

Embryogenesis in CB5161 is essentially identical to embryogenesis in *C. elegans* (Fig. 2; Sulston et al. 1983). Following fertilization, a “fountainhead” pattern of cytoplasmic streaming toward the sperm pronucleus can be observed (data not shown). Presumably, this pattern of cytoplasmic streaming establishes embryonic polarity as it does in other species of *Caenorhabditis* (Goldstein and Hird 1996). As in *C. elegans*, the first division in CB5161 embryos is asymmetric and occurs along the anteroposterior axis (Fig. 2B). Subsequent divisions through the 28-cell stage occur with the same timing and orientation as in *C. elegans* (Fig. 2, data not shown).

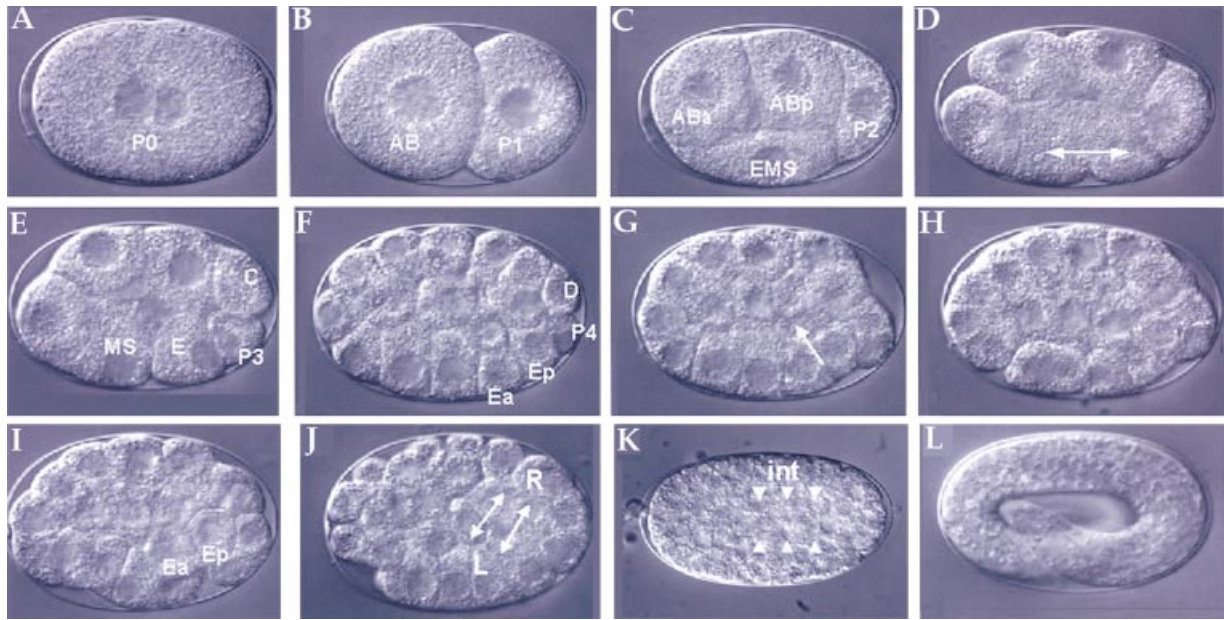
Also as in *C. elegans*, the early embryonic cell lineage of CB5161 results in the formation of five somatic founder cells—AB, MS, E, C, and D—and one germ-line precursor, P4 (Figs. 2B–2F; Fig. 3; Sulston et al. 1983). In *C. elegans*, E gives rise exclusively to intestinal cells (Sulston et al. 1983). These cells are readily identified by the presence of tissue-specific gut granules that can be detected by polarized light or fluorescence microscopy (Siddiqui and Babu 1980; Figs. 4A, 4B). Gut granule autofluorescence can be detected in E cell descendants in CB5161 embryos (Figs. 4C, 4D). Thus, E in CB5161 also is an intestinal precursor cell.

Finally, as in *C. elegans*, gastrulation in CB5161 is initiated at the 28-cell stage by the internalization of the intestinal precursor cells, E.a and E.p (Fig. 2F–2J). These cells begin to migrate from the ventral surface to the center of the embryo shortly before they divide. During this inward migration, E.a and E.p divide along a dorsoventral/left–right axis (Fig. 2J). The descendants of E.a and E.p continue to divide following gastrulation, resulting in the internal development of the anterior and posterior regions of the intestine, respectively (Fig. 2K).

Cell proliferation in CB5161 embryos continues until approximately halfway through embryogenesis. At this point there is a brief period of embryonic compaction that is immediately followed by embryonic elongation. Elongation results in a tripling of the embryo’s length along with a concomitant decrease in width (Fig. 2L). Hatching occurs at approximately 12 h postfertilization.

### Terminal phenotypes of *C. elegans*/CB5161 hybrid embryos

Crosses between *C. elegans* males and CB5161 females result in hybrid progeny that arrest during embryogenesis (Baird et al. 1992). Terminal phenotypes were determined for 122 of these hybrids (Fig. 5; Table 1). Most hybrids failed to gastrulate. These arrested either at the onset of gastrulation or at approximately the 100-cell stage (Figs. 5A and 5B). Hybrids that did gastrulate arrested following the completion or near completion of embryonic cell proliferation (at approximately the 550-cell



**Fig. 2.** Selected embryonic stages of CB5161. In all micrographs, anterior is to the left and dorsal is up. Double-headed arrows indicate axes of division. The single-headed arrow in panel G indicates the direction of E.a and E.p cell migrations. Arrowheads in panel K indicate the position of the developing intestine. Cells are named following the convention of Sulston et al. (1983). (A) Zygote (P0) just prior to first cleavage. Maternal and paternal pronuclei are visible at the center of the cell. (B) Two-cell embryo with a large anterior AB cell and a smaller posterior P1 cell. (C) A four-cell embryo. (D) Anteroposterior division of EMS, to give rise to the intestinal founder cell E and the muscle founder cell MS. (E) Eight-cell embryo with the MS, E, and C founder cells present. (F) An embryo at the onset of gastrulation. The positions of the intestinal precursor cells, E.a and E.p, and the D and P4 founder cells are indicated. (G–I) Embryos at various stages of gastrulation showing the inward migrations of E.a and E.p. (J) A gastrula stage embryo in which E.a and E.p have divided along a dorsoleft and anteroright axis. (K) A postgastrula embryo in which the position of the developing intestine (int) is indicated. (L) A three-fold embryo just before hatching.

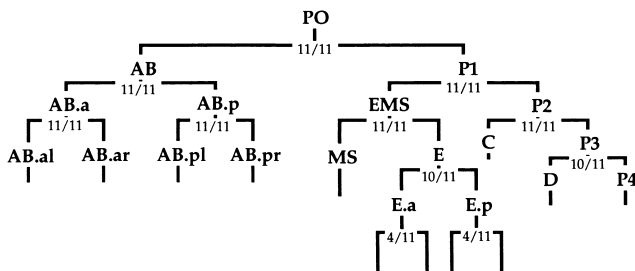
stage). These hybrids arrested either during embryonic compaction or at the two-fold stage (Figs. 5C and 5D). Hybrids that arrested at the two-fold stage were paralyzed. This is in contrast to nonhybrid *Caenorhabditis* embryos in which muscular activity commences at this stage (Sulston et al. 1983; Williams and Waterston 1994). Failure to gastrulate was correlated with defects

in intestinal cell lineages of hybrid embryos. Cell lineages through the 28-cell stage were determined for 11 *C. elegans*/CB5161 hybrids (Fig. 3). In four hybrids, no cell lineage defects were observed. These embryos gastrulated normally and subsequently arrested either at embryonic compaction or at the two-fold stage. In the remaining seven hybrids, defects were observed in the E cell lineage. In one hybrid, E failed to divide; in three hybrids, E.a and E.p failed to divide; and in three hybrids, E.a and E.p divided aberrantly along an anteroposterior axis rather than along a dorsoventral axis as is normal. No inward migrations of E cell descendants were observed in these seven embryos. Hence, these hybrids all failed to gastrulate.

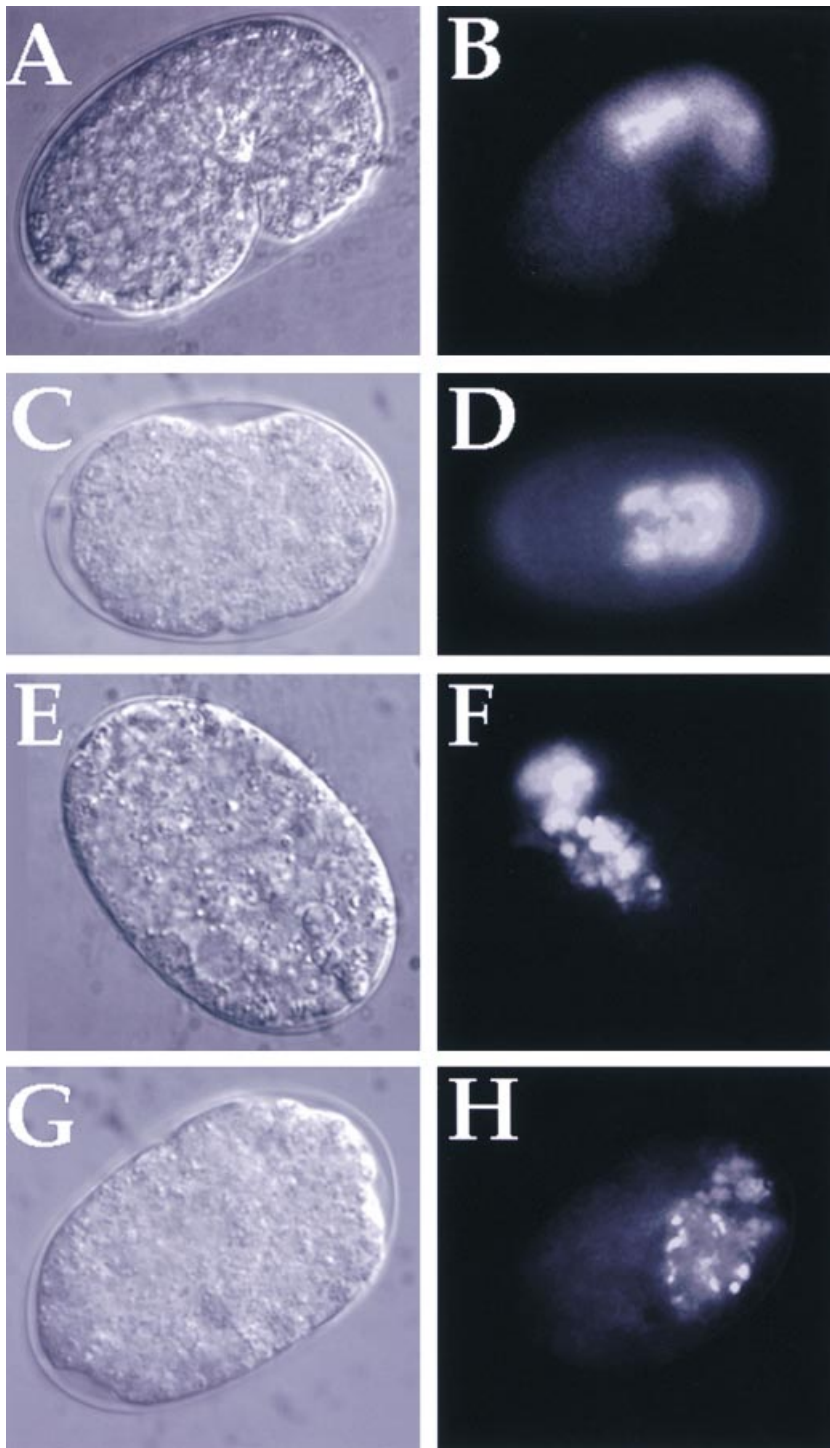
Because of their failure to migrate, E cell descendants remained on the ventral surface in gastrulation-defective hybrid embryos. Despite this ectopic location, gut granule autofluorescence was observed in these cells (Figs. 4E, 4F). Thus, even in gastrulation-defective hybrids, some aspects on intestinal differentiation occurred normally.

**Terminal phenotypes in other combinations of *Caenorhabditis* species**

Crosses of *C. briggsae* and *C. remanei* males to CB5161 females and of *C. briggsae* males to *C. remanei* females also



**Fig. 3.** The early embryonic cell lineage of CB5161 as determined from direct observations of two CB5161 embryos. Vertical lines represent individual cells, horizontal lines represent cell divisions. P0 is the fertilized zygote. Founder cells are named following the *C. elegans* convention of Sulston et al. (1983). Numbers at each cell division indicate the number of *C. elegans*/CB5161 hybrid embryos (out of 11 embryos scored) in which these divisions occurred normally.

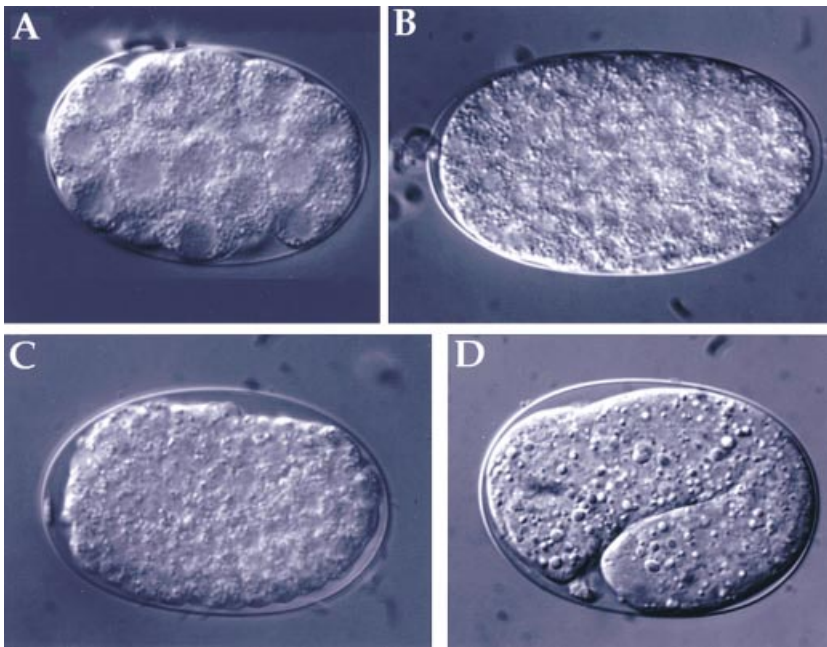


**Fig. 4.** Gut-granule fluorescence in *Caenorhabditis* embryos. DIC and autofluorescence images of *Caenorhabditis* embryos. (A and B) *C. elegans*, comma stage, lateral view. (C and D) CB5161, comma stage, ventral view. (E and F) *C. elegans*/CB5161 gastrulation-defective hybrid, terminal phenotype. (G and H) *C. elegans gad-1* mutant embryo, terminal phenotype.

have resulted in hybrid progeny that arrested during embryogenesis (Baird et al. 1992). These crosses were reexamined to determine arrest profiles of *C. remanei*/CB5161, *C. briggsae*/CB5161, and *C. briggsae*/*C. remanei* hybrids. From these crosses, a similar array of gastrulation-defective and postgastrulation terminal phenotypes were observed (Table 1). The

only qualitative difference between these crosses and crosses of *C. elegans* males to CB5161 females were the infrequent occurrence of L1-arrested larvae in crosses of *C. briggsae* males to CB5161 and *C. remanei* females.

While *C. remanei*/CB5161, *C. briggsae*/CB5161, and *C. briggsae*/*C. remanei* hybrids exhibited the same terminal



**Fig. 5.** Terminal phenotypes of *C. elegans*/CB5161 hybrids. *C. elegans*/CB5161 hybrids exhibited four distinct terminal phenotypes: (A) arrest at the onset of gastrulation; (B) arrest at the approximately 100-cell stage in gastrulation defective hybrids; (C) arrest at the onset of embryonic elongation; and (D) arrest at the two-fold stage.

phenotypes as *C. elegans*/CB5161 hybrids, the distribution of arrested embryos among these phenotypes did differ. In general, *C. remanei*/CB5161, *C. briggsae*/CB5161, and *C. briggsae*/*C. remanei* hybrids were more likely to gastrulate than were *C. elegans*/CB5161 hybrids. This difference was highly significant in all pairwise comparisons of the arrest profile of *C. elegans*/CB5161 hybrids to arrest profiles of other hybrid classes ( $P < 0.001$ ). The arrest profile of *C. briggsae*/*C. remanei* hybrids differed significantly from these arrest profiles of *C. briggsae*/CB5161 and *C. remanei*/CB5161 hybrids ( $P < 0.001$  and  $0.05$ , respectively). These differences were accounted for mostly by the distribution of terminal phenotypes in postgastrula hybrids. The arrest profiles of *C. briggsae*/CB5161 and *C. remanei*/CB5161 hybrids were not significantly different ( $P \approx 0.05$ ).

**Table 1. Arrest profiles of hybrid embryos<sup>a</sup>**

Term. phenotype <sup>b</sup>	Interspecific hybrid			
	Ce/CB5161	Cb/CB5161	Cr/CB5161	Cb/Cr
Gastr. defective				
28-cell stage	47 (0.39)	4 (0.07)	6 (0.12)	3 (0.11)
100-cell stage	40 (0.33)	15 (0.25)	17 (0.33)	15 (0.36)
Post-gastrula				
compaction	28 (0.22)	37 (0.63)	24 (0.47)	13 (0.31)
two-fold	8 (0.06)	2 (0.03)	4 (0.08)	10 (0.24)
L1	—	1 (0.02)	—	1 (0.02)
N	122	59	51	42

<sup>a</sup>Ce = *C. elegans*, Cb = *C. briggsae*, Cr = *C. remanei*.

<sup>b</sup>Values reported are the number and (fraction) of hybrid embryos observed at each arrest stage.

As in *C. elegans*/CB5161 hybrids, failure to gastrulate in *C. remanei*/CB5161, *C. briggsae*/CB5161, and *C. briggsae*/*C. remanei* hybrids was independent of intestinal differentiation. In all of these hybrid classes, superficial gut granules were observed in gastrulation-defective embryos (data not shown).

## DISCUSSION

Terminal phenotypes can be used to infer developmental processes and genetic pathways that are dysgenic in hybrid animals. From the terminal phenotypes described herein, three dysgenic developmental processes can be identified: gastrulation, embryonic compaction, and embryonic elongation. All of these processes have been characterized in some detail in *C. elegans*. These characterizations provide insights into genetic and cellular mechanisms that may underlie these reproductive barriers.

Gastrulation defects in *C. elegans*/CB5161 hybrid embryos are identical to those observed in *C. elegans* *emb-5*, *emb-13*, *emb-16*, *emb-23*, *emb-31*, and *gad-1* embryos (Figs. 3G, 3H; Denich et al. 1984; Nishiwaki et al. 1993; Knight and Wood 1998). These genes are candidate reproductive isolating genes in this combination. However, *emb-5*, *emb-13*, *emb-16*, *emb-23*, *emb-31*, and *gad-1* all are maternal-effect genes. Thus, it is the CB5161 homologs of these genes that may be dysgenic in combination with zygotic genes of *C. elegans*. Although zygotic genes required for gastrulation initiation in *C. elegans* have not been identified, there is evidence that at least one such gene exists (Powell-Coffman et al. 1996).

In *C. elegans*, embryonic elongation is dependent upon the formation of circumferential actin microfilaments (Priess and Hirsh 1986). The formation of these microfilaments is coincidental with the embryonic compaction. Disruption of actin microfilaments at this stage blocks embryonic elongation. Arrest of hybrid embryos at embryonic compaction may be the result of dysgenic interactions affecting the structure or regulation of the actin cytoskeleton. No *C. elegans* genes have been identified that exhibit arrest at embryonic compaction as a mutant terminal phenotype and hence candidate genes involved in this reproductive barrier cannot be identified at this time.

Paralysis and arrest at the two-fold stage in *C. elegans* is correlated with defects in muscle development (Williams and Waterston 1994). A defective musculature may also be the basis of this terminal phenotype in *C. elegans*/CB5161 hybrids. In this case, genes that exhibit paralysis and arrest at the two-fold stage as a terminal phenotype will be candidate genes for this reproductive barrier. Sixteen such genes have been identified in *C. elegans* (Williams and Waterston 1994).

Terminal phenotypes observed in *C. briggsae*/CB5161, *C. remanei*/CB5161, and *C. briggsae*/*C. remanei* hybrids were qualitatively similar to those observed in *C. elegans*/CB5161 hybrids. These hybrid classes did differ in the frequencies with which each terminal phenotype was observed. One caveat to these results is that the *C. briggsae*/*C. remanei* arrest profile reported here is different from the corresponding arrest profile reported by Baird et al. (1992). This reflects a difference in *C. remanei* strains used in this and in the previous study (SB146 vs. EM464). This strain-specific difference is under investigation.

The prevalence of defective gastrulation, embryonic compaction, and embryonic elongation as reproductive barriers in *Caenorhabditis* hybrids is striking. This is especially true since these barriers have arisen multiple times during the evolution of *Caenorhabditis*. A single speciation event may account for their presence in *C. elegans*/CB5161, *C. briggsae*/CB5161, and *C. remanei*/CB5161 hybrids (see Fig. 1 and Baldwin et al. 1997). However, the gastrulation, compaction, and elongation barriers in *C. briggsae*/*C. remanei* hybrids must have arisen following an independent speciation event. Despite the presence of gastrulation, compaction, and elongation defects in all *Caenorhabditis* hybrid classes that have been examined, it is not clear what role these barriers play in speciation. In *Drosophila*, pre- and postzygotic reproductive barriers evolve at equal rates in allopatric pairs of species (Coyne and Orr 1989). If this is true in *Caenorhabditis*, defects in gastrulation, compaction, and elongation may provide the initial barriers to gene flow between incipient species. It then would be expected that natural selection would reinforce the evolution of pre-mating reproductive barriers (Coyne and Orr 1989; Howard 1993; Kelly and Noor 1996).

The apparent absence of pre-mating reproductive barriers among these *Caenorhabditis* species (Baird et al. 1992; S. E.

Baird, unpublished observation) may be explained in several ways. First, pre-mating mechanisms of reproductive isolation may exist that are not detectable in laboratory crosses. Little is known about the natural history of most *Caenorhabditis* species (Sudhaus and Kiontke 1996) and no studies of reproductive isolation have been conducted in wild populations. Second, they may not be sympatric and hence not subject to reinforcement. *C. elegans*, *C. briggsae*, and *C. remanei* are cosmopolitan but *C. sp.* CB5161 is known only from Trinidad (Sudhaus and Kiontke 1996). No sympatric populations of these *Caenorhabditis* species have been reported. Third, *C. elegans*, *C. briggsae*, *C. remanei*, and *C. sp.* CB5161 may be ecologically isolated. Ecological isolation would reduce the selective pressure for reinforcement of other pre-mating mechanisms of reproductive isolation. *C. remanei* is found most frequently in commensal associations with terrestrial isopods and snails (Baird et al. 1994; Sudhaus and Kiontke 1996; Baird 1999). *C. elegans* and *C. briggsae* have been observed in such associations but the prevalence of these associations is not well documented (S. E. Baird, unpublished observation). No potential hosts have been identified for *C. sp.* CB5161. Finally, *C. elegans* and *C. briggsae* are hermaphroditic species that can reproduce by self-fertilization. In populations of these species, males are rare (Hedgcock 1976). The paucity of males and prevalence of self-fertilization in these species also would reduce the selective pressure for reinforcement of pre-mating isolation.

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