Analysis of the Constancy of DNA Sequences during Development and Evolution of the Nematode Caenorhabditis elegans

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Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*

(Southern transfer/DNA-DNA hybridization/DNA rearrangements/speciation/molecular evolution)

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ABSTRACT In order to test for the occurrence of rearrangements in DNA during development and to assess the rate of DNA divergence during evolution, we have compared restriction fragments derived from DNA from four sources: sperm cells and somatic tissues of one strain of the nematode *Caenorhabditis elegans,* somatic tissues of a second strain of the same species, and whole animals of a closely related species. Restriction fragments were detected by hybridizing radioactive cloned fragments to restriction digests that had been fractionated by size on agarose gels and transferred to nitrocellulose sheets. In this way, approximately 50 **BamHI** restriction fragments were visualized and compared. Fragments from sperm and somatic DNAs were found to be identical; 15% differed in size between the two strains. Little cross homology was found between the two species. We conclude that, if rearrangements occur in *C. elegans* DNA during development, they must affect fewer than a few percent of the restriction fragments or restriction sites. The difference found between the two strains and the two species is surprisingly great.

It has been generally assumed until recently that the nucleotide sequences present in the DNA of a eukaryotic organism remain unchanged during the development of the organism and the differentiation of its cells. Contrary to this assumption, in a few organisms changes in the primary structure of the DNA, or in the chromosomal content of cells, are known to take place during development. In certain protozoans, the sequences present in the somatic nucleus are a subset of those present in the germ nucleus (1). Similarly, in some nematodes, crustaceans, and insects, a set of germ-line sequences is absent from somatic tissues (2, 3). In polytene chromosomes of dipteran insects, highly repetitive satellite sequences are known to be underrepresented (4, 5). Genes coding for ribosomal RNA are amplified in oocytes of several organisms (6), and certain tissuespecific sequences appear to be amplified during the differentiation of chicken cartilage and neural retina cells (7). In the mouse, somatic recombination results in rearrangement of the genes coding for antibodies during the differentiation of lymphocytes (8). Rearrangements within DNA have been postulated to be central to the mechanism of cellular differentiation (9,10).

We sought direct evidence for the presence or absence of rearrangements in the DNA of the nematode *Caenorhabditis elegans,* an organism currently the subject of extensive genetic and developmental research (11). In studies of hybridization kinetics, Sulston and Brenner (12) detected no loss of germ-line sequences in somatic DNA of *C. elegans*. The ability to detect individual restriction fragments in digests of DNA from whole organisms by hybridization using cloned fragments (13) provides a sensitive method for probing the arrangement of DNA sequences. Using this method, we have compared restriction fragments from DNA of somatic tissues with fragments from DNA of sperm of C. *elegans*. Also, we have compared these to fragments from DNA of a second strain of C. *elegans* and from a closely related species. We find no differences between the sperm and somatic DNAs but a surprising degree of divergence between the two strains and the two species.

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MATERIALS AND METHODS

Nematodes. Two strains of C. *elegans* (14, 15) were used in this work. One was isolated in Bristol, England, and was identified as C. *elegans* by Nigon and others (16). Worms designated C. *elegans* var. *Bristol,* strain N2, are descendants of a single hermaphrodite of this strain (17). The other strain, C. *elegans* var. *Bergerac,* was isolated in France and identified by Nigon (18); it was obtained from J. Brun. *C. briggsae* (15, 19) was obtained from B. Zuckerman. Our stock is descended from the original animal isolated in California (ref. 20; B. Zuckerman, personal communication). Strain E879 is a derivative of N2 carrying a mutation in the *him-1 III* gene, which results in a high frequency of males among the self-progeny of hermaphrodites (21).

Isolation of Eggs with Hypochlorite. Gravid worms were gently shaken at room temperature in 10 vol of fresh 1 % NaOCI (Fisher, laboratory grade, 4-6%)/0.5 M NaOH. After 5-10 min, worm carcasses and other debris dissolved, and eggs, which are resistant to this treatment, were recovered by pelletting and washed several times in M9 buffer (17). These eggs are 50-100% viable.

DNA. Methods for cultivating C. *elegans,* also applicable to *C. briggsae,* have been described (17). DNA was isolated as follows. Worms were washed and suspended in 0.04 M NaCl/ 0.01 M Tris, pH 8.0, frozen in liquid nitrogen, and ground in a mortar and pestle. After thawing, the suspension was brought to a final composition of 0.1 M Tris (pH 8.5), 0.05 M EDTA, 0.2 M NaCl, 1 % sodium dodecyl sulfate, and 200 μg of proteinase K (EM Laboratories) per ml, and the carcasses were dissolved by digestion at 65°C for 15 min. The clear, highly viscous solution was then extracted three times with phenol at 5°C and once with chloroform/isoamyl alcohol, 24:1 (vol/vol), and DNA was isolated by winding from an ethanol precipitate. DNA was further purified from RNA by digestion with RNase A (Worthington), organic extraction, and winding again from an ethanol precipitate. A remaining insoluble opalescent substance was removed by sedimentation at *30,000 X g* for *30* min.

Sperm cells were isolated from males of strain E879. The procedure, which will be described in detail elsewhere, briefly was as follows. Males were purified by screening a mixed population of worms through a nylon screen and were compressed between two Plexiglas plates. This procedure causes the release of sperm cells into the medium, which can then be separated from carcasses and soluble components by filtration and pelletting. Sperm nuclei were released by mild homoge-

nization in a low-salt buffer and pelletted, and the pellets were used for DNA isolation by the procedure used for worms.

Construction of Recombinant Plasmids. Fragments of nematode DNA generated by digestion with the restriction endonuclease *BamHI* were cloned by using the vector plasmid pBR313 (22) and were randomly selected for use as hybridization probes. The nematode DNA used was from first-stage (L1) larvae of strain N2 that had been hatched into sterile buffer from eggs isolated with hypochlorite, in order to exclude the possibility of contamination with bacterial DNA. Plasmid and nematode DNAs were cleaved with *BamHI* (New England Biolabs), joined *in vitro* by using *Escherichia colt* ligase (New England Biolabs), and introduced into E. *colt* strain SF8 (C600 *r*k⁻ *m*k⁺ *recBC` lop-11 lig+,* F. Schachat, obtained from R. Davis) essentially by the methods of Morrow *et al. (23).* Transformed cells were selected by plating primary transformants in 2.5 ml of soft agar onto plates without drug, incubating for 1 hr to allow expression of drug resistance, and then overlaying with an additional 2.5 ml of soft agar containing 450 µg of ampicillin per ml (final concentration in plate, 15 µg/m1 of agar). Cells carrying hybrid plasmids were identified by their sensitivity to tetracycline, and plasmid DNA was isolated from them by the method of Clewell and Helinski (24), after induction with chloramphenicol (25). Plasmid DNA was purified from the cleared lysate by phenol extraction, ethanol precipitation, and banding in a CsCI/ethidium bromide density gradient; the size of the cloned nematode fragment was determined after digestion with Barn HI by comparison with restriction fragments of known length on agarose and polyacrylamide gels.

Work with recombinant DNA was carried out under P2-EK1 containment conditions according to the guidelines of the National Institutes of Health.

Fourteen recombinant plasmids (which are designated pCel, -2, etc.) were selected for use as hybridization probes. The 14 plasmids were present as 13 clones (one cell carried two recombinant plasmids), and 1 plasmid carried two inserted worm fragments. The cloned fragments ranged in size from 450 to 18,000 base pairs and represented in all, 0.07% of the nematode genome.

Southern Hybridizations. Restriction endonuclease digests of DNA were fractionated on a 0.7% agarose gel (Sigma, type II, medium EEO) in 0.04 M Tris/0.02 M NaOAc/2 mM EDTA (pH of X10 stock adjusted to 7.8 with acetic acid) with the horizontal apparatus of McDonell *et al. (26).* DNA was transferred from the gel onto nitrocellulose sheets (Millipore, HAWP) by the method of Southern (13) except that SET buffer (0.15 M NaCl/0.05 M Tris/1 mM EDTA; pH of X20 stock adjusted to 7.9 with HC1) was used instead of SSC. Hybridizations were carried out at 32°C in 50% formamide (Eastman Spectrograde)/0.1 M NaPO4, pH 7.0/X3 SET buffer/0.1% sodium dodecyl sulfate, for 24 hr in sealed plastic bags. The hybridization temperature of 32°C is calculated to be 25°C below the melting temperature of *C. elegans* DNA in a buffer of this composition (12, 27, 28). Plasmid DNA to be used as hybridization probe was labeled by nick translation to greater than 10' cpm/µg with Ia-^{32}P)dATP [New England Nuclear, 100-300 Ci (1 Ci = 3.7 X 10^{10} becquerels)/mmol *I* by the procedure of Maniatis *et al. (29),* except that DNase I (Worthington) at 1 ng = 10^{-9}g/ml was added to the reaction mixture. Probe (0.1-0.5 µg) was denatured at 95°C for 5 min before addition to the hybridization solution. Final hybridization volume was 5 ml for a Millipore sheet 5 *x* 16 cm. After hybridization, the Millipore sheets were washed at 32°C with four changes of hybridization buffer, two changes of X2 SET buffer, dried, and exposed for several days under x-ray film (Kodak XR5). For some exposures, the film was flashed (30) and exposed at -70°C with an intensifying screen (Kodak X-Omatic Regular).

RESULTS

Hybridization of Cloned Fragments to Fractionated Digests of DNA from Worms. Fifteen randomly cloned *BamHI* restriction fragments have been hybridized to *BamHI* restriction digests of DNA from worms. Representative results of these hybridizations are shown in Fig. 1.

We first consider the results of the homologous hybridization-that is, hybridization of the cloned fragment to DNA from *C. elegans* var. *Bristol* L1 larvae (lane b, Fig. 1). In all cases except two, it was possible to show that the recombinant plasmid hybridized to a fragment equal in size to the cloned fragment it carried. For this comparison, a reconstruction consisting of E. *coli* DNA plus a small amount of *BamHI-di*gested recombinant plasmid was included on each nitrocellulose sheet (lane r, Fig. 1). This fragment, which we assume is identical to the cloned fragment in the probe, is called the "primary fragment," and its presence indicates that cloning has been achieved without rearrangement. The two exceptional cases are those in which the cloned fragment was either too small (450 base pairs) or too large (18,000 base pairs) to be visualized by these methods. The reconstruction futher shows that the primary fragment hybridizes roughly to the extent expected if it is present once in the worm genome (see the legend to Fig. 1).

In addition to the primary fragment, most of the recombinant plasmids hybridized to a number of other fragments, which we term "secondary fragments." Secondary fragments are not due to incomplete digestion with restriction enzyme. This can be concluded from the fact that they were not present in four cases (e.g., pCel1, Fig. 1) (a single digest of each nematode DNA was used in all of these hybridizations) and from the fact that a number of them were smaller than the primary fragment in the same lane. We consider below three other possible explanations for the presence of secondary fragments: that the DNA of the population of worms used in these experiments is heterogeneous, that the DNA of single worms is heterogeneous, and that the cloned fragment of the probe carries sequences present more than once in the nematode genome.

Comparison of Restriction Fragments in Different Strains and Different Species. In order to obtain information about the rate of DNA sequence divergence in nematodes, we included DNA from a second strain of *C. elegans (C. elegans* var. *Bergerac)* and a second closely related species (C. *briggsae)* in these experiments (lanes a and d, Fig. 1). Eleven of the cloned (primary) fragments from *C. elegans* var. *Bristol* were found unaltered in size in *Bergerac* DNA; two were of a different size, and two could not be visualized as before. In addition, most secondary fragments were present in *Bergerac* DNA. We conclude from this fact that the secondary fragments are not present because of a heterogeneity of DNA sequences among the worms used in these experiments. The populations of *Bristol* and *Bergerac* worms that we have used are descendants of single individuals and it seems unlikely that rapid rearrangements would result in identical population heterogeneity in the two cases.

Five *Bergerac* restriction fragments did not correspond in size to a *Bristol* fragment (see for example, pCe14, -1, and -5, Fig. 1). In each case, one fragment corresponding to *a Bristol* fragment was absent and one new fragment was present.

In contrast to the similarity in restriction fragments in the two strains of *C. elegans,* the DNA of C. *briggsae* was found to be highly diverged (Fig. 1). Nine of the cloned fragments from C.

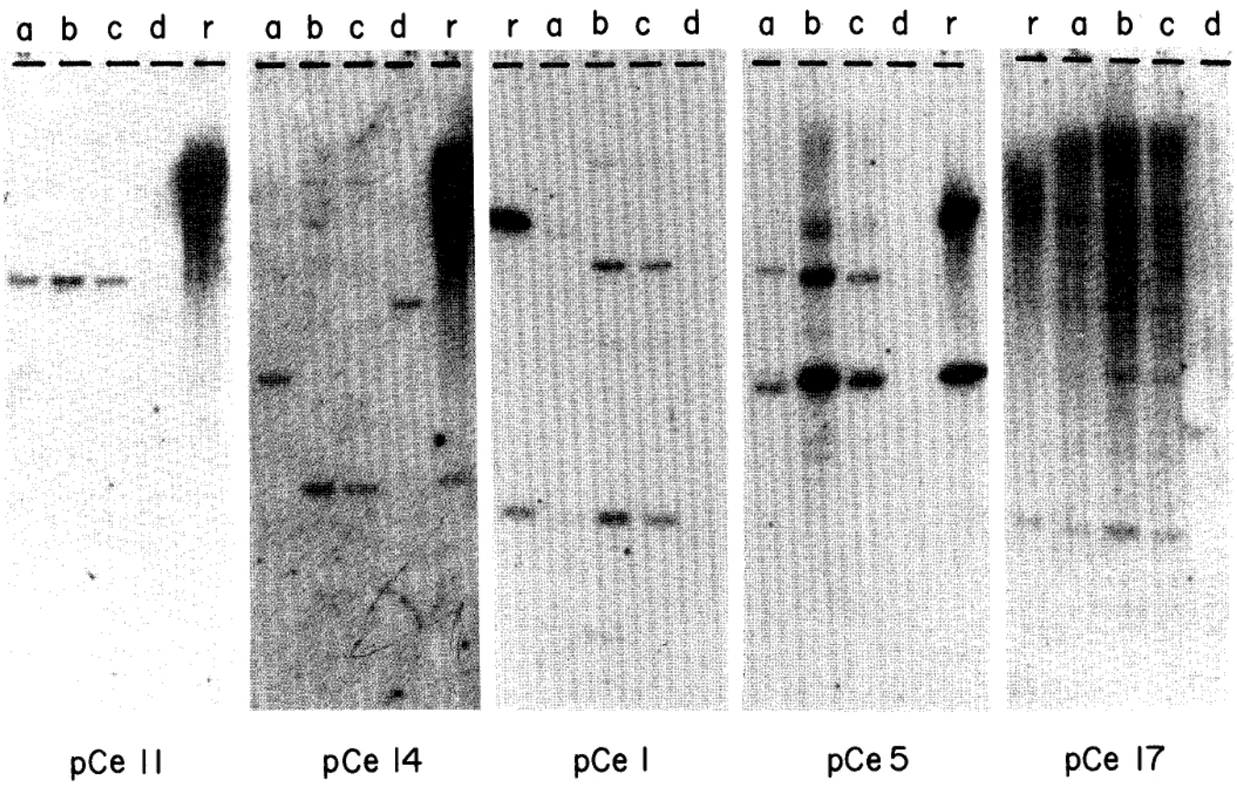

FIG. 1. Results of hybridizing five recombinant plasmids to DNA from Lt larvae of *C. elegans* var. *Bergerac (a),* DNA from Ll larvae of *C. elegans* var. *Bristol (N2) (b),* DNA from young adult hermaphrodites of *C. elegans* var. *Bristol* (N2) (c), mixed worms of *C. briggsae (d),* and a reconstruction to show the size of the fragment used as probe (r). Lanes a-d have approximately 2 μg of DNA from *a BamHI* restriction digest. In each case the reconstruction consisted of 5 μg of sheared *E. coli* DNA plus 0.1 ng of plasmid to be used as probe, cleaved with *BamHI.* Because the *C. elegans* genome consists of 8 X 10^7 base pairs (12), 2 μg of worm DNA will contain 0.1 Dg of a restriction fragment 4000 base pairs long. Hybridization in the reconstruction lane is to the cloned nematode fragment, to the pBR313 vector plasmid, and to the *E. coli* DNA (the last resulting from contamination of the radioactive probe with *E. coli* sequences). The sizes of the cloned fragments are as follows: pCel l, 5300 base pairs; pCe14, 2400 and 450 base pairs (two inserts); pCel, 1800 base pairs; pCe5, 3750 base pairs; pCe17, 1650 base pairs.

elegans had no homologous sequences in C. *briggsae* DNA. Four plasmids hybridized weakly to fragments in C. *briggsae* DNA, and only one hybridized well (pCel4, Fig. 1). None of the fragments in *C. briggsae* DNA corresponded in size to a fragment in *C. elegans* DNA.

Comparison of Restriction Fragments in Germ Cells and Somatic Cells. In order to test directly for the occurrence of rearrangements in the DNA of C. *elegans* during development, we compared the results of hybridizing cloned fragments to DNA of sperm and L1 larvae. L1 larvae isolated as described here have about 550 somatic cells and only 2 germ-line cells (ref. 31; J. Kimble, personal communication); hence, this experiment allows a direct comparison of germ-line and somatic line DNA sequences. Because it is difficult to prepare sperm in large quantities, several cloned fragments were hybridized together in some of these experiments.

The results of these hybridizations are shown in Fig. 2. No differences were found between the patterns of sperm and L1 larvae, either in the sizes of the bands or in their relative intensities. Because all restriction fragments present in the somatic DNA were also present in DNA of sperm, none has appeared as a result of rearrangements during development. This conclusion is reinforced by the identity of the bands from DNA of N2 LI larvae and N2 young adult hermaphrodites (Fig. 1, lanes b and c). Approximately one-half of the DNA of young adult hermaphrodites is expected to be germ-line DNA from the large gonad.

We conclude that cloned fragments that hybridize to more than one restriction fragment in DNA from worms must carry sequences present in more than one copy per genome. Because most of the cloned restriction fragments used in these experiments carry such sequences (9 of 13, with 2 additional fragments uncertain), we conclude that the DNA of *C. elegans,* like that of other eukaryotic organisms, is interspersed with repetitive sequences. Furthermore, the interspersion is at relatively short intervals because even our shortest cloned fragments (three of five fragments of less than 2000 base pairs) carried repetitive sequences.

DISCUSSION

Approximately 50 restriction fragments (representing 0.3% of the nematode genome) have been visualized in this work and found to be present in DNA from both somatic tissues and sperm. We conclude that, if changes take place in the DNA of *C. elegans* during development of the organism, they must affect less than a few percent of the restriction fragments or restriction sites. Repetitive sequences, which are present on most of our cloned fragments, are arranged identically in germ-line and somatic line DNAs; they do not arise as a result of insertion of germ-line sequences into new sites in the DNA during development. Tissue-specific rearrangements could have been detected because L1 larvae of C. *elegans* have only four major cell types (hypodermis, muscle, nerve, and intestine). We would not have detected large rearrangements, rearrangements within

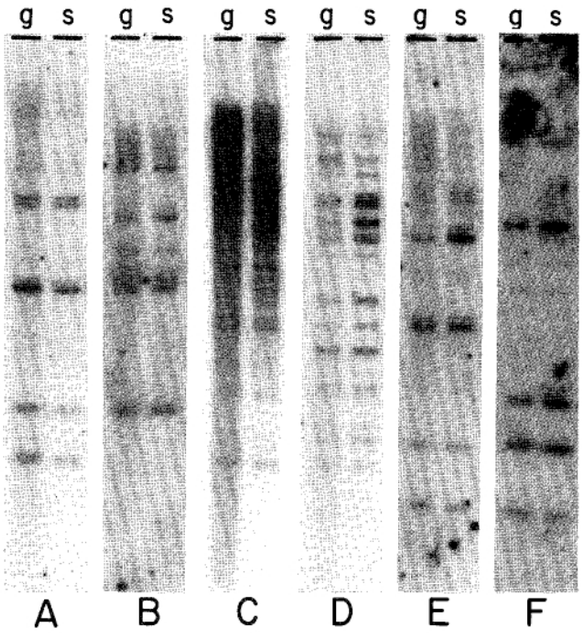

FIG. 2. Results of hybridizing cloned fragments to sperm DNA (lanes labeled g) and DNA from N2 Li larvae (lanes labeled s). Each lane carried 4 μg of a *Bam* HI restriction digest. The probes used were: A, pCel, pCe4, pCe5; B, pCe2, pCe3, pCe5; C, pCe17; D, pCel8a, pCel8b; E, pCel, pCelO, pCe14; and F, pCe5, pCel3, pCe19.

restriction fragments, or changes such as small additions or deletions (<100 base pairs) that did not significantly alter the size of restriction fragments. We would also not have detected changes taking place in only a small number of cells (less than 10%). Our experiments are not a sensitive test for chromatin diminution in *C. elegans* because the fragments used as hybridization probes were from somatic DNA. Our results are similar to results in *Drosophila* which have shown an identity of restriction fragments in embryo and adult DNAs (32, 33).

The presence of repetitive sequences on most of the cloned fragments studied here suggests that the DNA of C. *elegans is* interspersed with such sequences at relatively short intervals. The opposite conclusion has been drawn from studies of hybridization kinetics (ref. 34; unpublished data) in which most fragments of greater than 2000 base pairs reanneal as if they consist entirely of unique DNA. A possible explanation for this discrepancy is provided by the low repetition number of the repeated sequences we have observed. Most of our cloned fragments that carry repeated sequences hybridize to fewer than 10 other fragments. An acceleration in reannealing rate due to interspersion of sequences with such a low repetition number might not have been detected.

Rearrangements in DNA have been postulated to play an important role in the evolution of genetic systems (e.g., see refs. 35 and 36), and such rearrangements might explain the differences we have observed between *Bristol* and *Bergerac* DNAs. On the other hand, if all the differences (5 fragments differing of 37 that can be visualized in the *Bergerac* patterns) are due to single base changes, then *Bristol* and *Bergerac* DNAs differ in approximately 1% of their nucleotides (37). This is a high degree of divergence, almost as great as has been observed between distinct species (38, 39). Yet these two strains are morphologically indistinguishable and completely cross-fertile. Because they are cross-fertile, it may be possible to use the nucleotide differences observed after digestion with restriction enzymes as phenotypic markers to map restriction fragments genetically.

Similarly, the difference we find between the DNAs of C. *elegans* and C. *briggsae* is surprisingly great. An estimate of the degree of difference can perhaps be made by assuming that the amount of hybrid formed on the filter is limited by the rate of annealing, an assumption we make because the DNA sequences on the filter are never saturated by the probe. Then, by assuming that a restriction fragment from C. *elegans* that shows no homology with *C. briggsae* DNA is annealing with at most 1/10th the homologous rate, we can calculate that it must differ from any related sequences in *C. briggsae* by at least 20% (40). This is a degree of divergence found in other studies for species separated for tens of millions of years (38, 39, 41, 42). Yet, *C. elegans* and *C. briggsae* are almost identical and have been described as "twin species" (43). Nevertheless they are either rather old species or the evolutionary rate of DNA divergence in these nematodes is greater than in other groups.

We are particularly interested in the fact that *C. elegans* and *C. briggsae* differ so much in their nucleotide sequences al though morphologically they are almost identical. This may be related to the fact that, in a mutational or transcriptional analysis, much of the DNA of eukaryotic organisms appears to be functionally silent (17, 44, 45). The DNA is not uniformly diverged, however; some of our cloned fragments from C. *elegans* do hybridize to C. *briggsae* DNA. Angerer *et al.* (39) similarly observed a conserved portion of DNA sequences between sea urchin species.

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