Caenorhabditis elegans DNA does not contain 5-methylcytosine at any time during development or aging

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ABSTRACT

DNA, isolated from age-synchronous senescent populations of *Caenorhabditis elegans* has been quantitatively and qualitatively analyzed for the presence of 5-methylcytosine. High performance liquid chromatography on two wild-type and several mutant strains of *C. elegans* failed to detect any 5-methylcytosine. The restriction endonuclease isoschizomers, *HpaII* and *MspI*, were used to digest genomic DNA after CsCl purification and failed to detect any 5' cytosine methylation at any age. We conclude that *C. elegans* does not contain detectable (0.01 mole percent) levels of 5-methylcytosine.

INTRODUCTION

The association between cytosine methylation and altered eukaryotic gene expression (1) or X-chromosome inactivation (2), is well documented. The association between cellular (3-5) or organismic senescence (6,7) and cytosine methylation is less clear. Levels of 5-methylcytosine as high as 14 mole percent have been reported in DNA isolated from senescent populations of the nematode, *Caenorhabditis elegans* but not in DNA isolated from the first larval stage of life (8). A generalized hypermethylation of cytosine residues could result in decreased transcription and thereby explain the large decreases in rates of protein biosynthesis reported in senescing populations of *C. elegans* (9), and *Turbatrix aceti* (10).

We report here a detailed analysis of purified DNA from several strains of *Caenorhabditis elegans* both during development and at several times in later life. These studies involved high performance liquid chromatographic (HPLC) analysis of total organismic DNA, as well as analysis by restriction endonuclease digestion. The DNA was incubated with the isoschizomers, *HpaII* and *MspI*, followed by Southern blots and hybridized with a clone of a moderately repeated transposable element, Tc1 (11). We detect no 5-methylcytosine at any time during the life-span of this nematode species.

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MATERIALS AND METHODS

General procedures

The conditions for growth and maintenance of *C. elegans* are standard (12). Methods for determining life-span have been presented earlier (13).

Strains and plasmids

Two laboratory wild-type strains (N2 or Bristol and Bergerac BO) and DH26, a strain carrying *fer-15(b26)*, were obtained from David Hirsh. TJ135 and TJ143 are strains with altered life spans (Table 1) derived from crosses between N2 and Bergerac BO and have been described elsewhere (13-15). MK546 is a long-lived strain obtained from Michael Klass (16). pCe2002 is a plasmid carrying a 1.75 Kb *Sat* insert of Tc1 and was a gift of Scott Emmons (11,17).

DNA isolation and Southern hybridization

DNA was isolated as described by Emmons, et. al. (18). Further purification was performed by collecting main band DNA from CsCl. Salt was removed by dialysis and DNA stored in TE pH 8.0 (19) at 2°C until use. DNA was digested to completion with restriction endonuclease (BRL) following suppliers directions. Double digests were performed similarly except that DNA was ethanol precipitated after *EcoRV* digestion and resuspended in appropriate buffer (19).

Following digestion, DNA was fractionated on 0.8% or 1.2% agarose gels in TAE (0.04 M Tris, pH 7.8; 2 mM EDTA). DNA was transferred to nitrocellulose (Schleicher & Schuell) following the method of Southern (20). Radiolabeled pCe2002 was nick-translated (21), to 5×10^7-2×10^8 dpm/γ, sometimes using a kit (BRL). Salmon sperm DNA, calf thymus DNA and standards were obtained from Sigma.

High performance liquid chromatography

Analyses were performed on an Altex Ultrasphere C (5 micron) column (22). The column was equilibrated with buffer containing 10 mM sodium phosphate, 10 mM hexanesulfonic acid, 5% acetonitrile, pH 2.9. Peaks were eluted from the column isocratically at 2500 psi and flow rate 1 ml/min using the same buffer. Peaks were detected by UV at 254 and 280 nm.

UV scans of peaks of free bases on the HPLC were performed using a Hewlett Packard Model 1040A diode array detector.

Preparation of free bases

Nematode or control DNA was dissolved in 88% formic acid and digested at 120 C for 90 minutes. Samples were evaporated to dryness and then redissolved in column equilibration buffer and aliquots injected into the column.

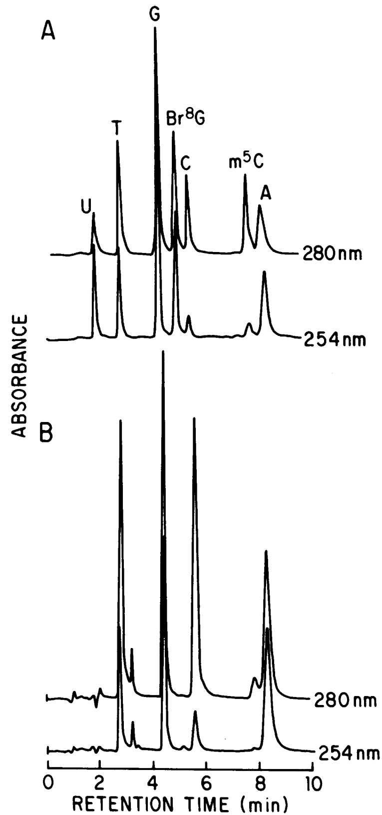

Figure 1. (A) HPLC analysis of authentic standards of free bases. Peaks were detected by UV scan at 254 and 280 nm. Peaks labeled in this chromatogram are: uridine (U), thymine (T), guanine (G), 8-bromoguanine (Br^8G), cytosine (C), 5-methylcytosine (m^5C), and adenine (A). (B) HPLC of formate digests of calf thymus DNA.

RESULTS

Analysis of standards

Chromatographic separation of the free bases: adenine, guanine, cytosine, thymine, uridine, 8-bromoguanine and 5-methylcytosine are shown in Figure 1A. Determinations of the standards at 254 and 280 nm help to insure peak identity because the ratio of peak areas at these wavelengths is characteristic for each compound. All compounds are clearly separated; 5-methylcytosine elutes at 7.6 minutes in this elution profile. Figure 1B is a chromatogram of calf thymus DNA. A peak eluting at the position of 5-methylcytosine is clearly seen even at low sensitivity.

Table 1. Strains analyzed for 5-methylcytosine.

Sample	Age	Mole Percent 5-methylcytosine (Mean ± SD)	Mean Life-span (days)*	References
N2	hatch	≤0.01	19.8	13 - 15
Bergerac BO	hatch	≤0.01	17.9	13 - 15
DH26	hatch	≤0.01	17.1	9, 25
DH26	5 day	≤0.01	17.1	9, 25
DH26	7 day	≤0.01	17.1	9, 25
DH26	10 day	≤0.01	17.1	9, 25
Bergerac BO	10 day	≤0.01	17.9	13 - 15
TJ 135	10 day	≤0.01	18.1	13 - 15
TJ 143	10 day	≤0.01	29.6	13 - 15
DH26	15 day	≤0.01	17.1	9, 25
Bergerac BO	15 day	≤0.01	17.9	13 - 15
MK546	15 day	≤0.01	29.9**	16
TJ143	15 day	≤0.01	29.6	13, 15
TJ143	35 day	≤0.01	29.6	13, 15
Salmon Sperm DNA	-	1.79 ± .45	-	26
Calf Thymus DNA	-	3.61 ± 1.51	-	26

* Mean life-spans determined as described (13).

** Measured at 20°C. We find an average life span of 29.9 days under our conditions as compared to the 14.1 days originally reported by Klass (16) at 25°C.

Limits of sensitivity of the HPLC system were tested by adding known amounts of 5-methylcytosine to digested worm DNA before injection. Approximately 0.01 mole percent 5-methylcytosine can be detected and Figure 2B shows that 0.7 mole percent is easily detected.

HPLC analyses of *C. elegans* DNA

Total DNA from several strains and ages was banded in a CsCl gradient, isolated, dialyzed and concentrated by ethanol precipitation. This isolation strategy was employed because Klass et. al. (8) had not analyzed extensively purified DNA and we reasoned that the copurification of contaminating compounds from *C. elegans* during phenol extraction and ethanol precipitation might be responsible for their observations. Purified DNA was acid-hydrolyzed to yield free bases and analyzed by reverse phase, ion-pairing, HPLC.

In typical chromatograms of *C. elegans* DNA no peaks were observed which eluted near 7.6 minutes (Table 1). A peak occuring near 7.85 minutes was seen in some samples. For example, in DNA isolated from a 15 day old population of the temperature sensitive, fertilization defective strain, DH26, a small unknown peak was observed near the position at which

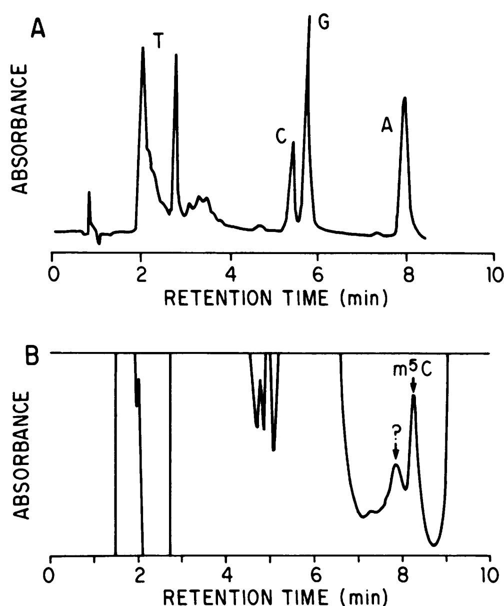

Figure 2. HPLC of formate digests of genomic DNA samples. (A) A low sensitivity chromatogram of free bases isolated from DNA of 15 day old DH26 showing an unknown peak eluting at 7.85 minutes. (B) Exogenous 5-methylcytosine (0.05 nM) was added to the material used in Fig. 2A and the sample was rechromatographed at high sensitivity (m^5C = 5-methylcytosine).

5-methylcytosine was expected to elute (Figure 2A). Figure 2B represents a higher sensitivity determination of the components in this DNA (approximately 100-fold more sensitive than that shown in Figure 1 or 2A). Adding 0.05 nmole of exogenous 5-methylcytosine to this sample resulted in a new peak eluting at the position characteristic of 5-methylcytosine (Figure 2B); this demonstrates that the unknown was not 5-methylcytosine.

Ultraviolet absorbance spectrum of unknown peak

As a final check on the identity of the small peak at 7.85 minutes, we chromatographed 15 day old samples of DNA from DH26 on a Waters HPLC equipped with a Hewlett Packard Model 1040A diode array detector. All peaks from the chromatogram were scanned in the UV range from 200 nm to 400 nm. Figure 3 shows UV profiles of the unknown peak at 7.85 minutes, the authentic 5-methylcytosine peak at 8.25 minutes, as well as the profile of 5-methylcytosine alone from a separate chromatogram. The profiles of the unknown and of 5-methylcytosine are clearly not identical. The UV scan of authentic 5-methylcytosine has a lambda-max at 282 nm and lambda-min at 242

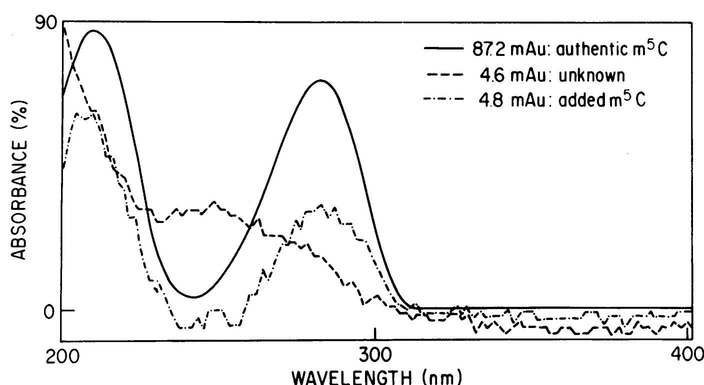

Figure 3. UV scans of authentic 5-methylcytosine (---), the added 5-methylcytosine peak of Figure 2B (-•-) and the unknown peak of Figure 2B (---).

nm. The scan of the unknown peak bears no resemblance to a purine or pyrimidine base and may represent the acid hydrolysate of an unknown compound that copurified with nematode DNA. In 14 strains and ages that were examined by HPLC, no 5-methylcytosine was detected (Table 1).

Analysis by isoschizomer digestion

As a final test for the absence of 5-methylcytosine we isolated high molecular weight genomic DNA from aged *C. elegans* and digested it with the restriction endonuclease isoschizomers *HpaII* and *MspI*. Both enzymes recognize CCGG; *HpaII* but not *MspI* fails to cleave ($C^{m}CGG$). As a chemical test for methylation of genomic DNA, restriction enzyme digestion with *HpaII* and *MspI* is much less sensitive than the total DNA analysis described above. However, limited methylation could occur in some sequences and not be detected by HPLC due to the rarity of these regions in the genome. Digested DNA was probed with radiolabeled, cloned Tc1, (pCe2002), a repeated sequence that structurally resembles a transposable element (23,24) and functions as such in *C. elegans* (11,17). There were no differences in the restriction patterns of DNA completely digested with *HpaII* as compared with *MspI* digestion (Figure 4).

DISCUSSION

We fail to detect 5-methylcytosine in *C. elegans* during either development or senescence. Attempts to detect 5-methylcytosine in three different ways were negative.

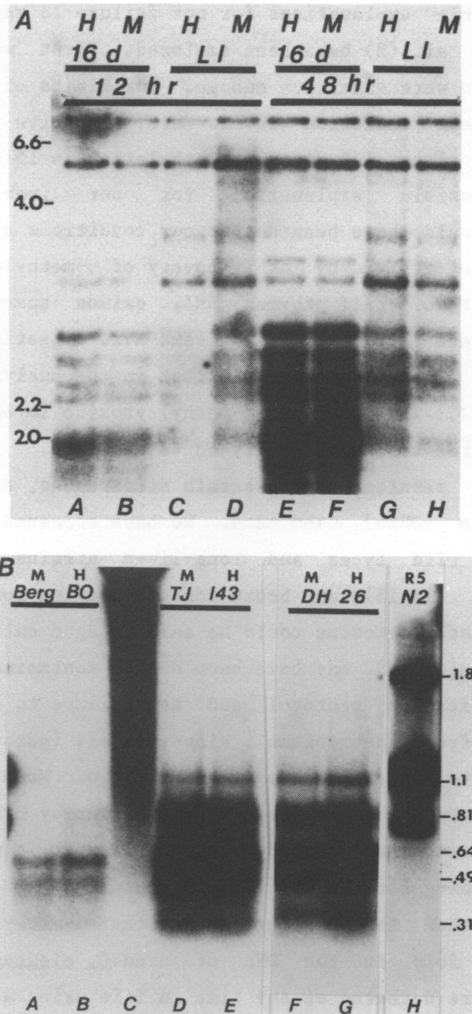

Figure 4. DNA from C. elegans DH26, digested to completion with HpaII or with MspI transferred and probed with labeled pCe2002. (A) Total DNA was isolated from DH26 at 16 days of age (lanes A,B, E, F) or at the first larval stage (lanes C,D, G, H) and digested with either HpaII (lanes A, C, E, G) or MspI (lanes B, D, F, H) for either 12 hour (lanes A-D) or 48 hours (lanes E-H) before fractionation on 0.8.% agarose. (B) Total DNA was digested with EcoRV (yields 1.57 kb fragments of Tc1), and with either HpaII or MspI, fractionated on a 1.2% agarose gel, blotted and hybridized. We blotted and probed undigested DNA from strain DH26, 16 day old, (lane C). Restriction endonuclease digests of DNA from 15 day old worms of strains Berg BO (lanes A and B) and TJ143 (lanes D and E), 16 day old worms of DH26 (lanes F and G), and N2 larvae (lane H). Digestion was with EcoRV alone (lane H) or double digests of EcoRV followed by either MspI (lanes A, D, F) or HpaII (lanes B, E, G).

Several possible explanations for our failure to replicate the earlier data of Klass et al. (8) have been explored. First, we asked whether our limits of detection were sensitive enough. Our limits of detection are 0.01 mole percent. These detection levels are sensitive enough to detect published values of almost 14% cytosine methylation in 15 day old cultures.

A second possible explanation for our inability to detect 5-methylcytosine could have been due to our conditions of acid hydrolysis. As a test of the efficiency of recovery of 5-methylcytosine after acid hydrolysis, samples of calf thymus DNA, salmon sperm DNA and authentic 5-methylcytosine were subjected to the same acid digestion as purified worm DNA samples. No 5-methylcytosine was lost in the analysis of any of these samples (Figure 1B). The levels of 5-methylcytosine in both calf thymus and salmon sperm DNA agree well with published values (26).

Nor were our results due to strain differences, since Klass used the same mutant strain (DH26) used here. We have screened a variety of other strains including wild types and long-lived strains, derived by both selective breeding (13-15) and brute force screening for longevity mutants (16). Again no 5-methylcytosine could be seen in aged cultures (Table 1).

Earlier findings (8) may have been due to contaminants not eliminated in the DNA purification protocol and to failure to chemically identify unknowns eluting from the column. Klass et. al. (personal communication) have since failed to repeat their earlier findings. When using purified DNA they find no evidence for 5-methylcytosine either by HPLC or by HpaII/MspI isoschizomer analysis when probed with cloned MSP1, a moderately repeated genomic sequence coding for the major sperm protein.

We conclude that the earlier report that 5-methylcytosine increased more than 10,000 fold in the DNA of aged *C. elegans* is incorrect. No methylated bases are detected at any time in life using a variety of systems to detect methylation. It appears that in *C. elegans*, as in many other invertebrate species (27-30), neither development nor senescence involves differential methylation of cytosine.

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