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Comparative Biochemistry and Physiology, Part C 144 (2006) 279–285
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Extra-long PCR, an identifier of DNA adducts in single nematodes (*Caenorhabditis elegans*)

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Received 15 June 2006; received in revised form 11 October 2006; accepted 11 October 2006
Available online 21 October 2006

Abstract

DNA adducts are frequently caused by chemical induced changes in DNA. If mis-repaired, they can lead to nucleotide substitutions, deletions or chromosomal rearrangements. Depending on adduct stereochemistry and properties of the DNA target, adducts can inhibit transcriptional mechanisms. Here we demonstrate how this phenomenon can be exploited to detect DNA adducts in individual nematodes (*Caenorhabditis elegans*). An extra-long (XL)-PCR (16,144 bp) target amplicon, the 11 exon spanning *ced-1*, could be amplified reliably from genomic lysate extracted from single nematodes. Amplification efficiency was assessed by means of a second, fully quantitative PCR. Following the normalization with an invariant control gene, adduct formation could be evaluated by the identification of XL-PCR amplifications that were, relative to the control gene, reduced or inhibited by >95%. No DNA adducts could be detected in *C. elegans* maintained under optimal growth conditions (no exposure controls) or nematodes exposed to 20 μg/g copper sulfate (exposure negative control). However, exposure to 5 μg/g benzo[*a*]pyrene induced a stark response, with 40% of nematodes displaying measurable DNA adducts. Similarly, adducts were identified in 10% of nematodes subjected to 3 μg/g fluoranthene or a mixture containing 0.5 μg/g benzo[*a*]pyrene and 1 μg/g fluoranthene.
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Keywords: Benzo[*a*]pyrene; Fluoranthene; Copper; *C. elegans*; qPCR

1. Introduction

Some toxicants, such as electrophilic chemical species, have the capacity to interact with nucleophilic sites and, thereby, modulate the structure of DNA molecules. This phenomenon can ultimately result in the formation of a DNA adduct which, by definition, is a complex formed by the chemical binding of a xenobiotic to DNA.

Several techniques have been developed for detecting DNA adducts, which can be classified broadly into four groups: immunology methods, fluorescence-based approaches, chromatography/mass spectroscopy, and ^{32}P-postlabelled radiology (Qu et al., 1997). The latter is, in particular, a sensitive and selective technique for the quantitative and qualitative detection of DNA adducts, where isolated DNA is digested, subjected to 5-phosphorylation with ^{32}P-ATP, separated by multi-directional thin layer chromatography, detected and quantified by autoradiography and scintillation counting (Randerath et al., 1981; Gupta et al., 1982; Gupta, 1985; Walsh et al., 1995; Qu et al., 1997; Martin et al., 2005). However, given that this approach requires an excess of 20 μg DNA, it is impractical when dealing with precious material or samples that are limited in biomass. A prime example is the microscopic nematode *Caenorhabditis elegans* which, as an individual, is too small in biomass to apply traditional DNA adduct methodologies. However, this hurdle may be circumvented via the use of advanced amplification-based technologies such as XL-PCR and quantitative polymerase chain reaction (qPCR). The approach is based on the assumption that adduct formation in genomic DNA differentially

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doi:10.1016/j.cbpc.2006.10.005

inhibits the transcriptional mechanism via several non-exclusive factors, namely the size, shape or stereochemistry of adduct, the choice of base incorporation during transcription, the specifics of the targeted DNA sequence and the efficiency of the DNA polymerase to accommodate the damaged nucleotide(s) (Scicchitano, 2005). If template DNA is inhibited/blocked by DNA adducts, the yield of any amplified product will be correlated negatively, a concept that has been demonstrated elegantly (Laws et al., 2001). This study aims to demonstrate the feasibility of a molecular genetic detection of DNA adducts in *individual* nematodes and highlights its potential exploitation as a powerful biomarker for DNA mutagenesis.

2. Materials and methods

2.1. Exposures

Caenorhabditis elegans, N2 wild type (obtained from CGC, University of Minnesota, USA) were maintained at 20 °C on agar plates (diameter: 55 mm) containing nematode growth medium (NGM) with *Escherichia coli* OP50 as a food source (Brenner, 1974). Gravid nematodes were bleached to generate a synchronous F1 generation. Single larvae (L2) were isolated from the F1 generation and transferred to petri dishes containing NGM with at least six biological replicates for each treatment and control group. Nematodes were transferred to fresh Petri dishes after each generation to replenish the food source. Analysis was performed following an exposure period of 2 generations.

Copper sulfate ($CuSO_4$) was dissolved in sterile, ultra pure, HPLC filtered water (Sigma, Gillingham, Dorset, UK), and benzo[*a*]pyrene (BaP) and fluoranthene (Fla) in acetone. To avoid the possibility of solvent induced adduct formation, equal amounts of acetone were added to all plates. Both agar and bacteria were dosed. The exposure concentrations were deemed to be non-lethal, however displaying a reduction in reproductive fitness. Specifically, these concentrations were as follows: 3 μg/g Fla; 20 μg/g $CuSO_4$, 5 μg/g BaP and a mixture containing 0.5 μg/g BaP and 1 μg/g Fla.

2.2. Genomic polymerase chain reaction (PCR)

Individual nematodes were transferred to 2.5 μL lysis solution (25 mM KCl, 25 mM Tris–HCl (pH 8.2), 1.25 mM $MgCl_2$, 0.1% (w/v) NP-40, 0.1% (w/v) Tween20, 5% (w/v) Gelatin and 0.25 mg/mL proteinase K), cooled to −80 °C for 30 min, lysed at 65 °C for 1 h followed by a final heating step to 95 °C for 10 min. XL-genomic PCR was conducted using a DNA polymerase with superior 3′ to 5′ exonuclease activity as recommended by the manufacturer (AccuTaq LA™, Sigma), namely an initial denaturation at 98 °C for 30 s followed by 30 cycles of 94 °C for 10 s, anneal at 63 °C for 20 s and extend at 68 °C for 20 min. Standard genomic PCR was performed over 10 cycles (denature: 94 °C for 30 s; anneal: 59 °C for 60 s; extend 72 °C for 30 s) with conventional Taq DNA polymerase (Promega, Southampton, UK). Details of the primer sequences are given in Table 1.

2.3. Quantitative PCR

A standard reference stock of *rpa-1* and *ced-1* was generated by amplification of 1 μL crude genomic DNA lysate (see Table 1 for primer sequences) using either conventional Taq (Promega) for *rpa-1* amplifications or AccuTaq LA™ DNA Polymerase (Sigma) for *ced-1* amplifications as recommended by the manufacturers. The resultant PCR products were separated by 2.5% (for *rpa-1*) and 0.5% (for *ced-1*) agarose gel electrophoresis, extracted using the QIAquick Gel Extraction kit (Qiagen, Crawley West Sussex, UK) and ligated into the pGEM-T Vector (Promega), and transformed into *E. coli* DH5α (Invitrogen, Paisley, UK) as recommended by the manufacturers. Colonies were grown up, purified (Wizard Plus SV Miniprep, Promega), recombinants confirmed by PCR using M13 forward and reverse primers and DNA quantified spectrophotometrically (Utrospec 2100 Pro). Serial dilutions were prepared from a stock solution (1 μg/mL) to a range covering at least five orders of magnitude.

The fluorogenic 5′ nuclease polymerase chain reactions (TaqMan) were performed in semi-skirted 96-well thermo-fast plates (ABgene, Inc., Epsom, UK) on a PE Applied Biosystems ABI Prism 7700 Sequence Detector (Foster City, CA). Fluorescently-labeled probes, labeled with both a reporter dye (6-FAM) and a quencher dye (TAMRA) were synthesized by Eurogentec (Hampshire, UK) (see Table 1 for sequence information) and gene-specific quantifications performed as suggested by the manufacturer and described elsewhere (Galay et al., 2003). Every 96-well TaqMan run contained triplicates of *rpa-1* and *ced-1* standards, DNA samples from individual nematodes and NTCs (No Template Controls). As recommended

Table 1
Primer sequences for the genomic PCRs (gPCR) and primer and probe sequences for the quantitative PCRs (qPCR)

Name	Primer sequence (5′ to 3′)
gPCR *rpa-1* sense	GAAGATCGCTACCCTTCTCA
gPCR *rpa-1* antisense	GTCATCGGATTCCTCCTTTG
qPCR *rpa-1* sense	GGGAGTTGATGTGAAGGTAAGGA
qPCR *rpa-1* antisense	CAGAAGTGATGAGGTTCTGAAAAC
qPCR *rpa-1* probe (sense)	[6-FAM]TCTCATTAAGTATATTCAAGTTTCACCCC [TAMRA-6-FAM]
gPCR *ced-1* sense	AGGTGTACAAATTGTCCTGAGCACGTT
gPCR *ced-1* antisense	TGCGTCTCATTCTCCTTGTGCTACTT
qPCR *ced-1* sense	CGACACGACCGACCCATT
qPCR *ced-1* antisense	TCAGAACCTGGTCTCAAAACGA
qPCR *ced-1* probe (sense)	[6-FAM]TTTTAACGCAATCACCACACATTCATGACA [TAMRA-6-FAM]

Optimized concentrations for *rpa-1* qPCR were 300 nM sense primer, 900 nM antisense primer and 200 nM/μL probe; and for *ced-1* qPCR 900 nM sense primer, 900 nM antisense primer and 200 nM/μL probe.

(Ayala-Torres et al., 2000), routine experiments included a 20% template control, to ensure the linearity of the quantitative measurement.

3. Results and discussion

Quantification of DNA adducts in a single nematode has, to date, not been feasible due to issues related to nematode biomass coupled with the limitations pertinent to detection sensitivity of available methodologies. The underlying concept introduced here, relies on the notion that xenobiotics have the capacity to form a complex with DNA that impairs cell autonomous replication and/or repair mechanisms. Consequently, a DNA polymerase-based amplification of a stretch of DNA containing DNA adducts will be affected proportionally. Although specific DNA hotspots have been reported to be particularly susceptible to adduct formation (Feng et al., 2002; Gaskell et al., 2004), the occurrence is also a simple stochastic function, namely the probability of nucleophilic attack (i.e., resulting in a DNA adduct) is proportional to the quantity of DNA target. Simply, a large stretch of DNA will be targeted, on average, more frequently than a small fragment or, the detection frequency/sensitivity increases the larger the DNA molecule. Conversely, the challenge of amplification by polymerase chain reaction (PCR) increases with amplicon size. DNA adduct detection by means of PCR, therefore, poses a particular challenge due to the above mentioned contradicting forces. Any amplicon too large, will not amplify reliably, but too small will result in the frequency of adducts to be reduced below the detection limit. A compromise is clearly desired, where the amplification is sufficiently robust to allow the identification of adduct-induced PCR inhibition. Of course, as with any amplification protocol, a failed reaction may be the result of inferior DNA preparation, rather than a direct consequence of DNA adducts. To allow the identification of substandard DNA, the same genomic preparation of every individual nematode

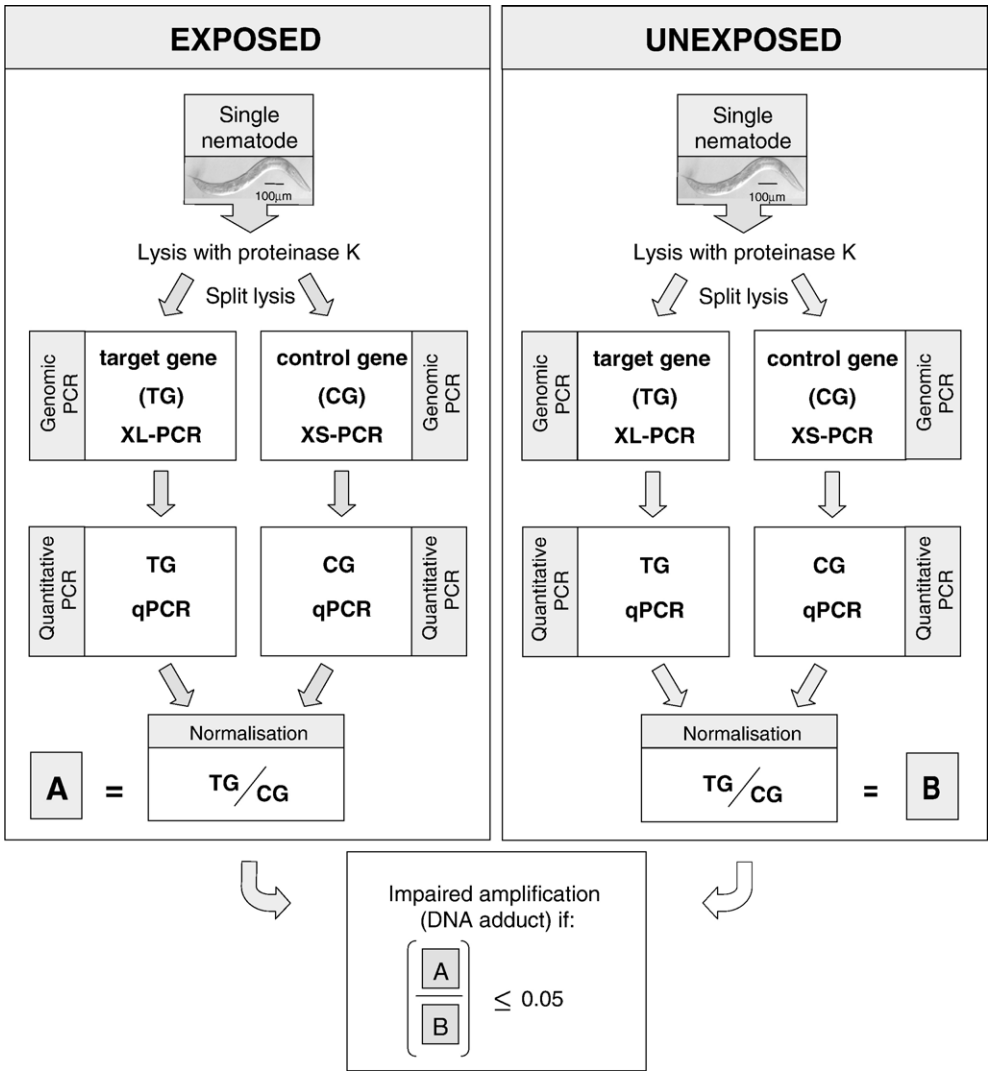

Fig. 1. Schematic diagram depicting the underlying concept of DNA adduct detection in single nematodes. Following the DNA lysis, an extra large (XL) fragment of the target gene (TG) and an extra small (XS) fragment of a control gene (CG) are amplified. Amplicon formation is quantified by respective quantitative PCRs and the ratio TG/CG calculated. Note: amplifications of TG in exposed animals that are impaired by ≥95% (compared to unexposed control animals) are indicative of PCR-inhibiting DNA adducts.

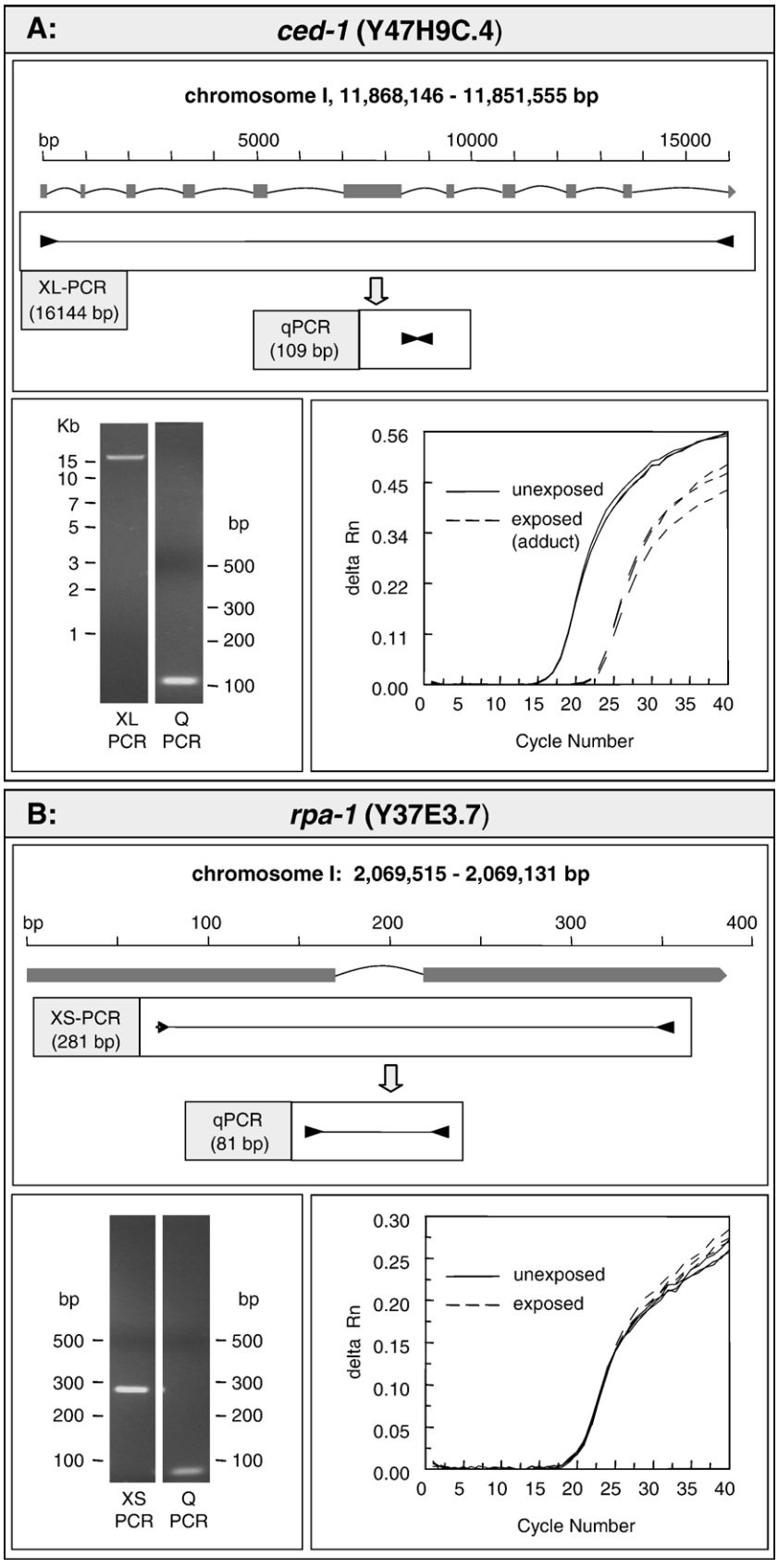

Fig. 2. Genomic location, amplification scheme and representative amplifications (visualized by agarose gel electophoresis or quantitative PCR, see Materials and methods) of the target gene *ced-1* (A) and the control gene *rpa-1* (B). Quantitative amplifications were performed in triplicate on exposed or unexposed single nematodes.

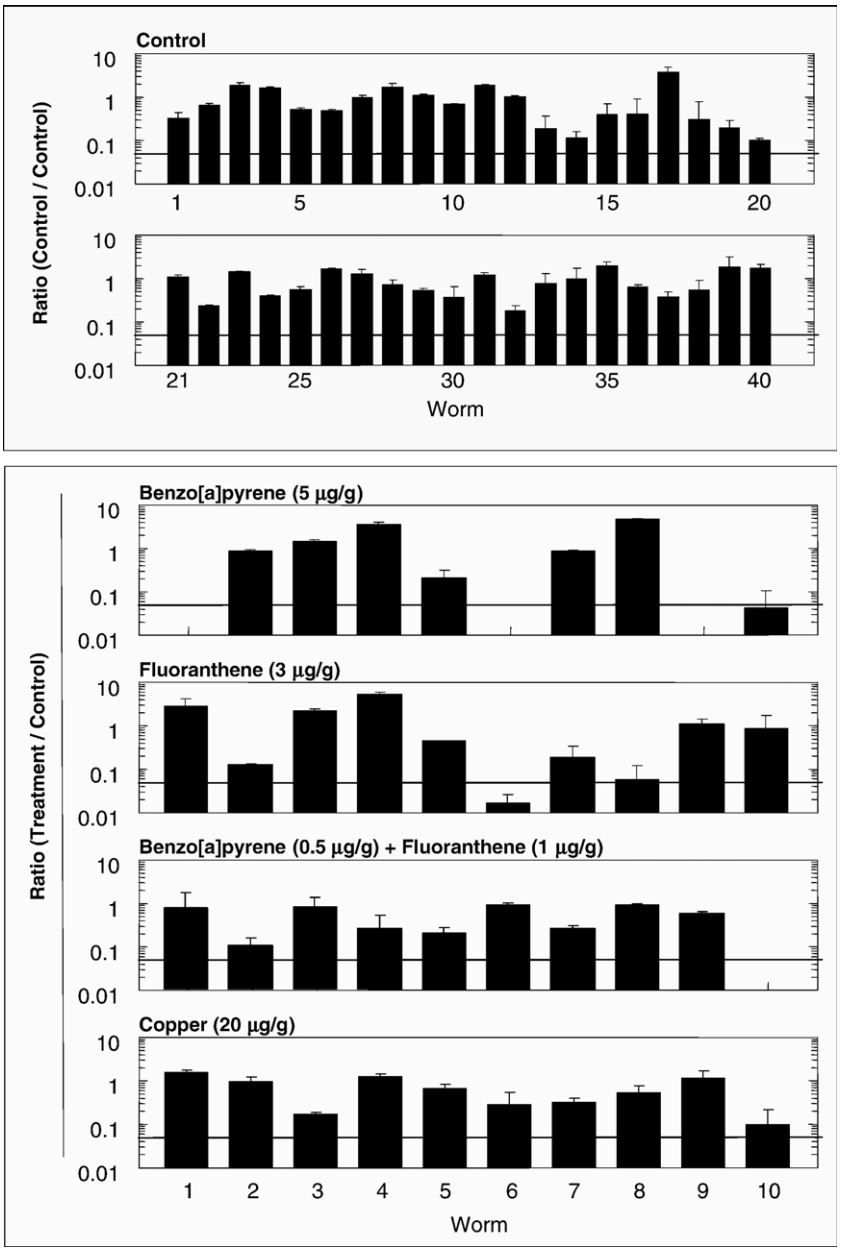

Fig. 3. Ratio of $[(ced\text{-}1/rpa\text{-}1)_{(individual)}/(ced\text{-}1/rpa\text{-}1)_{(control\ mean\ for\ each\ run)}]$ as schematically presented in Fig. 1. Individual nematodes were either maintained under control conditions or subjected to xenobiotics (5 µg/g benzo[*a*]pyrene, 3 µg/g fluoranthene, 0.5 µg/g benzo[*a*]pyrene and 1 µg/g fluoranthene or 20 µg/g copper). All ratios were calculated from means derived from triplicate measurements per nematode. The horizontal line is the threshold that denotes the presence of an amplification-inhibiting DNA adduct.

was split in two equal portions and subjected to two independent PCR reactions, (1) an extra long PCR (XL-PCR) to provide the template for bulky adduct identification and (2) an extra short PCR (XS-PCR) for quality control and signal normalization. Then, product formation can be assessed for both amplicons using a standard quantitative PCR (qPCR) approach.

Ideally, in the absence of any biological and experimental variation, any two nematodes not exposed to DNA adducts should display an equal ratio of XL-PCR over XS-PCR. This equilibrium will reverse if the XL-PCR is impaired or inhibited. Admittedly rather arbitrarily, we defined a quantifiable adduct or strong adduct (which is significantly distinct from any background variation) when ≥95% reduction of signal was observed (Fig. 1).

An initial screen was conducted on 10 different genomic loci (each spanning >6.0 kb) to identify a long stretch of DNA that could be amplified in a reproducible manner as the XL-PCR target gene (data not shown). The 11 exon spanning *ced-1*, located on chromosome I, proved an ideal target as it was possible to amplify routinely and reliably a 16,144 bp amplicon from genomic lysate extracted from individual nematodes (Fig. 2, A). In addition to superior amplification characteristics, *ced-1* was deemed of interest because of its historic involvement in cell recognition, phagocytosis and programmed cell death (Zhou et al., 2001; Reddien and Horvitz, 2004).

To facilitate the means of signal normalization, the acidic ribosomal subunit protein *rpa-1* was chosen as an invariant

housekeeping gene (Swain et al., 2004). Located on the same chromosome as *ced-1*, the genomic *rpa-1* amplification of 281 bp was equally robust (Fig. 2, B). A second, nested PCR using the genomic PCR products as a template yielded strong amplifications of 109 bp and 81 bp for *ced-1* and *rpa-1*, respectively (Fig. 2) which, in combination of gene specific fluorogenic probes, allowed the quantification of product formation. In detail, the primary amplification of the genomic *ced-1* and *rpa-1* PCRs were quantified by qPCR in nematodes maintained under control conditions or exposed to specific concentrations of selected xenobiotics. Adduct formation could be evaluated by the identification of *ced-1* amplifications that were, relative to *rpa-1*, reduced or inhibited (Fig. 2).

Genomic digests, *ced-1* XL-PCR and *rpa-1* XS-PCR followed by respective qPCRs were performed on 80 individual nematodes maintained either under control conditions or subjected to a suite of xenobiotic compounds. As each qPCR measurement represented the mean of three independent quantifications, it was possible to estimate the experimental error, a value that reflects the integration of the variability caused by machine and handling errors and possible differences in PCR efficiency. At 28%, experimental variation was clearly significant and by no means trivial. In addition to the identified experimental variation, inter-nematode differences are intrinsic to the quantitative analysis. Together, both factors resulted in a substantial, but unavoidable, background "noise" as can be seen in the measurements obtained from control nematodes. For this reason, it is of paramount importance to apply stringent thresholds when defining adducts to eliminate the possibility of false positives. The conservative adduct levels chosen here (defined as a reduction in relative *ced-1* signal in excess of 95%) were deemed sufficiently rigorous and experimentally sound, because no DNA adducts could be detected in any of the 40 *C. elegans* maintained under optimal growth conditions. Likewise, no significant adducts could be measured in any of the 10 nematodes chronically exposed to 20 μg/g copper, a compound not associated with DNA adducts. In contrast, long term exposure to BaP at a concentration of 5 μg/g agar induced an irrefutable response displaying four measurable DNA adducts, of which three resulted in a total inhibition of *ced-1* amplicon formation. Similarly, one adduct was identified in nematodes subjected to 3 μg/g Fla and one adduct in nematodes exposed to a mixture of 0.5 μg/g BaP and 1 μg/g Fla (Fig. 3). Assuming a random distribution of DNA damage, the use of a Poisson equation (Van Houten et al., 2000) estimates a frequency of 2.6 lesions/100 kb or 2524 lesions/genome at 5 μg/g BaP and 0.7 lesions/100 kb or 631 lesions/genome at 3 μg/g Fla, or the mixture 0.5 μg/g BaP and 1 μg/g Fla.

DNA adducts provide a measure of genotoxicity at the time of sampling because the net abundance represents the sum of the dynamic process of adduct formation and DNA repair during the cell cycle. Therefore, analysis of DNA adduct may be used both as a measure of the extent of exposure to genotoxic chemicals and as a predictor of response to exposure. The biological significance and effect of an adduct is dependant on genomic location/target and the efficiency of repair. For the most part, DNA adducts are ephemeral, as DNA-repair mechanisms rapidly excise the adducted structures and replace them with the original moiety. However, a few adducts are durable as the adducted element is mistranslated and a mutant cell is produced. The persistence of DNA adducts formed by this class of compounds suggests an organism is exposed chronically to the stressor. Therefore, the occurrence of DNA adducts provide a means to investigate the qualitative and quantitative relationships between formation of DNA adducts, subsequent DNA processing, and appearance of deleterious lesions in target tissues (Shugart, 2000).

This study demonstrates the feasibility of detecting DNA adducts in single nematodes. Clearly, further detailed (compound-specific) dose response and sensitivity analysis are a prerequisite to allow a translation into meaningful quantitative genotoxic assays. Similarly, it is possible that true environmental/toxicological relevance may only be achievable when this technique is translated to a nematode species found in natural soils, such as *Plectus accuminatus* (Stürzenbaum et al., 2005) or *Acrobeloides* and *Aphelenchus* species (Blakely et al., 2002; Li et al., 2005) rather than a laboratory culture of *C. elegans*. Nevertheless, the assay presented here proved highly sensitive and specific and, thus, clearly is a promising tool for quantitative analysis of DNA damage in single microscopic model and sentinel nematodes.

Acknowledgements

This work was supported by the Royal Society, the EU 6th Framework Network "NoMiracle" and the National Science Foundation — International Opportunities for Science and Engineering–Western Europe Program OISE-0454159.

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