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Development and application of bioluminescent *Caenorhabditis elegans* as multicellular eukaryotic biosensors

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Abstract We describe a novel approach to assess toxicity to the free-living nematode *Caenorhabditis elegans* that relies on the ability of firefly luciferase to report on endogenous ATP levels. We have constructed bioluminescent *C. elegans* with the *luc* gene under control of a constitutive promoter. Light reduction was observed in response to increasing temperature, concentrations of copper, lead and 3,5-dichlorophenol. This was due to increased mortality coupled with decreased metabolic activity in the surviving animals. The light emitted by the transgenic nematodes gave a rapid, real-time indication of metabolic status. This forms the basis of rapid and biologically relevant toxicity tests. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Firefly luciferase; Bioluminescence; Eukaryotic biosensor; Toxicology; ATP; *Caenorhabditis elegans*

1. Introduction

Whole cell biosensors offer a powerful new approach to environmental monitoring and toxicity testing, measuring bioavailability of toxins and biological effects rather than total concentrations obtained by traditional analytical techniques [1]. The free-living soil nematode *Caenorhabditis elegans* is an excellent candidate for a biosensor that represents multicellular eukaryotes [2]. Sensitivity of *C. elegans* to many heavy metals is similar to that of mammals [3] indicating potential for evaluating toxicity to humans.

Here we demonstrate the assessment of bioavailability and toxicity of xenobiotics by changes in the metabolic state of *C. elegans*, as measured in vivo by firefly luciferase luminescence. The firefly luciferase catalyses the oxidation of luciferin, a reaction that requires ATP and produces light [4]. When the other substrates are in excess, the amount of light emitted reflects the ATP levels and provides a measure of the metabolic state of the organism [5,6]. The use of firefly luciferase to specifically measure ATP is standard practice and has been validated in vivo by comparison with conventional ATP assays [6]. Any conditions affecting metabolism of the luminescent biosensor can be detected by a change in light levels [1,7]. In this study, we have constructed bioluminescent *C. elegans* and describe their use as biosensors by exposure to elevated temperature, the heavy metals copper and lead and the organoxenobiotic 3,5-dichlorophenol (3,5-DCP), commonly used as a wood preservative. This forms the basis of a robust, rapid and biologically relevant approach to toxicity testing and addresses ethical concerns by providing an alternative to testing on higher eukaryotes.

2. Materials and methods

2.1. Nematode culture

C. elegans was routinely cultured at 25°C on NGM agar plates with *Escherichia coli* OP50 as a food source [8]. Liquid culture was carried out in complete S medium [9] supplemented with 40 g (wet weight) l^{-1} of *E. coli* OP50, at 25°C and 160 rpm. Medium (100 ml) was inoculated with the worms washed from two 6 cm NGM plates and incubated for 5 days.

2.2. Construction of luc marked C. elegans

A *luc* gene with a deleted peroxisome targeting sequence (*luc*Δ) [7] was placed under control of the *C. elegans* constitutive promoter *let-858* [10] present in the vector pPD103.05 [11], to create the plasmid pPECL1. This leads to constitutive expression of cytosolic firefly luciferase in all cells of the nematode [10]. pPECL1 was diluted together with pRF4 (harbouring the dominant collagen mutation, *rol-6*(*su1006*), which confers a roller phenotype [12]) in TE, to a concentration of 200 μg ml^{-1} each and microinjected into the gonads of wild-type *C. elegans* strain N2 [13]. F1 roller progeny were selected to establish stable transgenic lines. A spontaneously integrated line (100% transmission rate), designated strain PE39 (*feIs1*[*rol-6*(*su1006*) *let-858*::*luc*Δ]), was selected for the present work.

2.3. Measurement of luminescence in vivo

Nematodes were centrifuged (2 min, 200×*g*), washed once in citric phosphate buffer at pH 6.5 supplemented with 1% dimethyl sulphoxide (DMSO) and 0.05% Triton X-100 (to increase permeability of luciferin), resuspended in 5 ml of the same buffer and split into four replicate 1 ml samples in luminometer cuvettes (Clinicon). Luminescence was measured after 30 min incubation with D-luciferin (100 μM, Molecular Probes) to obtain maximum levels, except where stated otherwise. A BioOrbit 1251 luminometer and the Multiuse software package (version 1.01) were used; measurements were integrated over a period of 12 s, at 25°C with shaking; and results expressed in relative light units (RLU), where 1 RLU equals 10 mV ml^{-1} s^{-1}. Background readings were subtracted from sample readings. Wild-type *C. elegans* (strain N2) provided with luciferin yielded background luminescence readings. Light levels were expressed as % of the maximum luminescence per nematode (total numbers) or per live nematode, obtained for controls (exposure to 25°C and ddH_2O).

2.4. Measurement of luminescence in vitro

Worms used in bioassays were resuspended in 50 μl ddH_2O, frozen in liquid nitrogen and kept at −70°C until further analysis. The commercial kit 'Luciferase Assay System' (Promega) was then used according to the manufacturer's instructions. Briefly, the frozen samples were ground to fine powder using a small handheld pestle (Konte)

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precooled in liquid nitrogen. Lysis reagent was added to the samples and broken down further (15 strokes). Samples were incubated on ice for 15 min and the mixing process was repeated. Tissue debris was removed by centrifugation (4°C, 12 000 × *g*). The cleared extract and the luciferase assay reagent were equilibrated to 25°C prior to mixing and measurement of luminescence. Settings for the luminometer were as for the in vivo measurements. Addition of a cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin, phenylmethylsulphonyl fluoride and chymostatin) prior to grinding samples did not increase final luminescence readings.

2.5. Bioassay conditions

The nematodes were grown in liquid culture and collected by centrifugation (2 min, 200 × *g*, room temperature) in 50 ml tubes (Nunc). Nematode density in bioassays was approximately 1×10^4 ml^{-1}. Prior to exposure to the various bioassay conditions nematodes were washed in ddH_2O, followed by a wash in the test solution/ddH_2O at the test temperature and resuspension in 10 ml of fresh test solution/ddH_2O at the test temperature. In the temperature assay, incubation was carried out for 1 h at 24, 31 or 36°C in orbital incubators (160 rpm). Exposure to copper, lead or 3,5-DCP solutions was carried out for 2 h, at 25°C and 160 rpm. The concentrations tested were 0, 0.08, 0.16, 0.24, 0.40, 0.8 and 1.6 mM copper ($CuSO_4 \cdot 5H_2O$), 0, 0.025, 0.05, 0.075, 0.125, 0.25 and 0.5 mM lead ($Pb(NO_3)_2$) and 0, 0.09, 0.18, 0.27, 0.36, 0.45 and 0.6 mM 3,5-DCP in ddH_2O and were all adjusted to pH 5.5. Test solutions were prepared fresh from acidified (1% 1 N nitric oxide) 16 mM copper and 5 mM lead stocks or from a 3 mM stock of 3,5-DCP. All glassware was acid washed. Measurement of luminescence was performed at 25°C after resuspension in fresh buffer as described above. Bioassays were performed on separate occasions and the trends found to be identical.

2.6. Enumeration of nematodes and assessment of lethality

The total number of worms in 5 μl aliquots from each luminometer cuvette was determined using a counting cell. Nematode numbers in replicate cuvettes did not differ significantly in any of the experiments. To estimate lethality, 5 μl samples of each suspension were placed on NGM plates. Immobile worms that failed to respond to probing with a needle were scored as dead. Addition of 1% DMSO and 0.05% Triton X-100 prior to luminescence readings had no effect on lethality.

2.7. Statistical analysis

A significance level of 0.05 was chosen. Analysis of variance was carried out on nematode numbers, temperature bioassay and in vitro assays. Least significant differences (5%) were calculated when $P \leq 0.05$. Regression analysis was carried out for the copper, lead or 3,5-DCP bioassays.

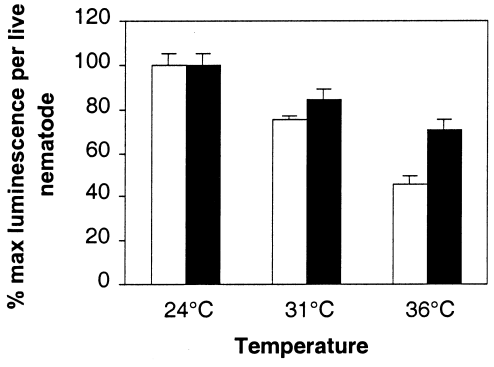

Fig. 1. Effect of elevated temperatures on *C. elegans* bioluminescence. Thermal stress temperatures were 31 and 36°C, whereas 24°C is within the *C. elegans* normal growth range. Luminescence was measured after resuspension of the nematodes in buffer at 25°C, 5 min (□) and 30 min (■) after adding luciferin. Data are expressed as a percentage of the maximum luminescence per live nematode. Error bars represent the S.E.M. of four replicates.

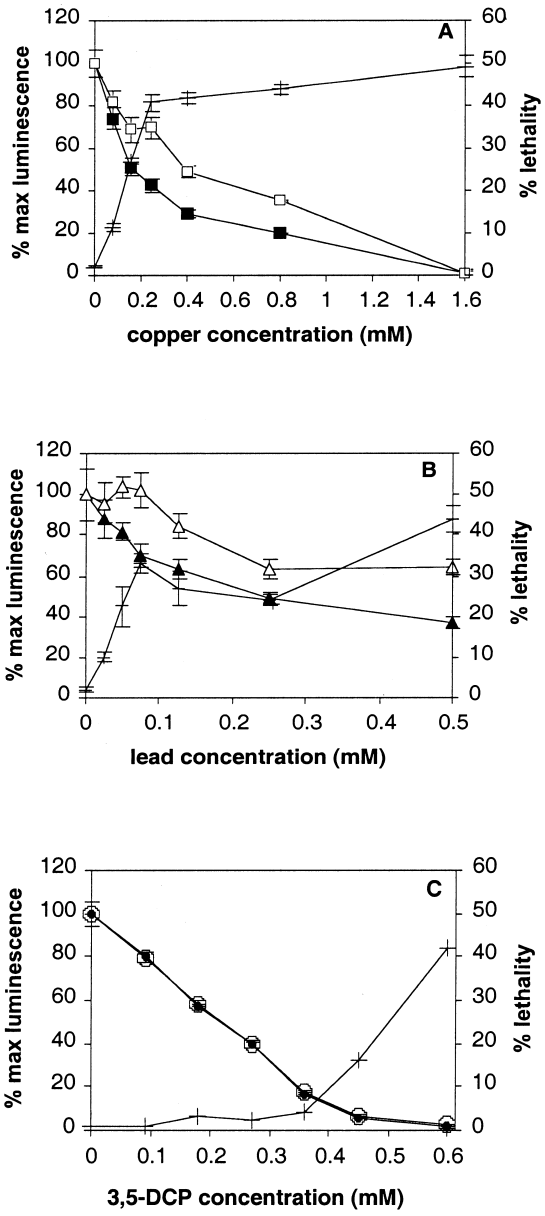

Fig. 2. Effect of copper, lead and 3,5-DCP on bioluminescence and lethality of *C. elegans*. Error bars represent the S.E.M. of four replicates. A: Luminescence per nematode (■) or per live nematode (□) after exposure to copper. B: Luminescence per nematode (▲) or per live nematode (△) after exposure to lead. C: Luminescence per nematode (●) or per live nematode (○) after exposure to 3,5-DCP. +, lethality.

3. Results

3.1. Effect of thermal stress, copper, lead and 3,5-DCP on *C. elegans* bioluminescence (bioassays)

Lethality was very low in the thermal stress experiment (0.5% at 24°C, 1.2% at 31°C and 1.6% at 36°C). Nematodes were resuspended in buffer at 25°C prior to luminescence measurements, so that dependence of luciferase activity on temperature did not interfere with assessment of metabolic status. Exposure to 31 and 36°C led to a significant decrease in light output per live nematode, as measured 5 min after adding luciferin (Fig. 1). Light levels of nematodes exposed to 31 and 36°C recovered partially when they were incubated at

25°C for 30 min prior to measuring. After this period of time, the light output of nematodes exposed to 31°C was no longer significantly lower than that of 24°C samples, but that of 36°C samples was still significantly lower than the others.

Exposure of the nematodes to copper or lead resulted in considerable lethality and decreased light levels (Fig. 2A,B). The regressions of $\log_{10}$(% of maximum luminescence per total or per live nematode) on copper concentration were both highly significant and explained most of the variability in bioluminescence ($r^2 = 94.4$ and 91.2% respectively, $P < 0.001$ for both). Thus, light output per total number of nematodes and per live nematode significantly decreased with increasing copper concentrations. These data indicate that lethality alone did not account for luminescence decline and surviving nematodes emitted less light. The decline in luminescence with increasing concentration of lead was significant ($r^2 = 77.6\%$, $P < 0.001$). In contrast, when data were analysed per live nematode, the regression explained very little of the variability in bioluminescence ($r^2 = 49.8\%$). This suggested that lethality was responsible for most of the decrease in luminescence in this experiment.

Luminescence and lethality in the 3,5-DCP bioassay are shown in Fig. 2C. The regressions of $\log_{10}$(% of maximum luminescence per total or per live nematode) on 3,5-DCP concentration were both highly significant ($r^2 = 93.6$ and 94.8% respectively, $P < 0.001$ for both). Lethality was very low (less than 5%) up to 0.36 mM 3,5-DCP, whereas luminescence decreased dramatically with increasing exposure levels, declining to 20% of its maximum upon exposure to 0.36 mM 3,5-DCP. Hence, the effect of mortality was negligible in this experiment and luminescence decline resulted from sublethal effects.

3.2. *Effect of thermal stress, copper and 3,5-DCP on luciferase expression and activity (in vitro assays)*

Differential expression of the *luc*Δ transgene or alterations in the activity of luciferase, rather than changes in metabolic activity, might account for the observed decrease in bioluminescence upon exposure of the transgenic worms to elevated temperatures (Fig. 1), copper or 3,5-DCP (Fig. 2). In order to investigate this possibility, in vitro luciferase assays were carried out on the nematodes exposed to temperature, copper and 3,5-DCP. In these assays, ATP was provided at a saturating concentration (verified experimentally by increasing the ATP concentration by a factor of two with no change in light levels), so that differences in light output reflected levels of active luciferase in cells. Bioluminescence values were divided by the number of live nematodes in the sample at the point of freezing and expressed as the percentage of maximum luminescence per 'live' nematode.

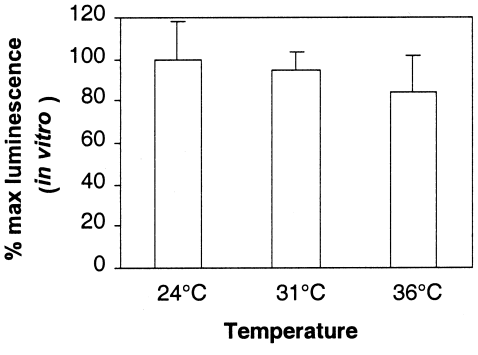

Fig. 3. In vitro luminescence of extracts of nematodes exposed to different temperatures. Data are expressed as a percentage of the maximum luminescence per live nematode (prior to freezing). Error bars represent the S.E.M. of four replicate extracts.

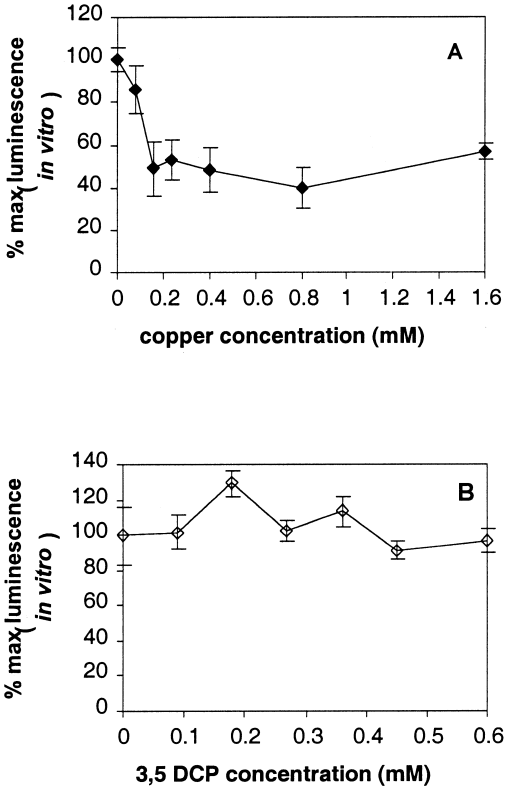

Fig. 4. In vitro luminescence of extracts of nematodes exposed to (A) copper and (B) 3,5-DCP. Data are expressed as a percentage of the maximum luminescence per live nematode (prior to freezing). Error bars represent the S.E.M. of three (copper) or four (3,5-DCP) replicate extracts.

There was no evidence ($P > 0.05$) for altered luciferase levels or activity in samples exposed to 24, 31 and 36°C (Fig. 3) or to the various concentrations of 3,5-DCP (Fig. 4). In vitro luminescence of samples exposed to 0.16–1.6 mM copper was depressed when compared to that of low levels of copper (0 and 0.08 mM) (Fig. 4). Nevertheless, increasing copper levels from 0.16 to 1.6 mM did not decrease in vitro luminescence further. Exposure to copper at concentrations higher than 0.08 mM led to a decrease in expression of the *luc*Δ and/or activity of luciferase. However, further increases in copper levels did not decrease expression of the *luc*Δ and/or activity of luciferase further. This contrasted with the continuous decline in the in vivo assay (Fig. 2), observed for nematodes exposed to low levels of copper or increasing concentrations of the metal.

4. Discussion

Here we report the first example of transgenic *C. elegans* expressing luciferase and the use of in vivo luminescence as a measurement of the toxic effect of compounds on this multicellular eukaryote. Bioluminescent nematodes responded to representative environmental stresses (increasing temperatures and concentrations of copper, lead and 3,5-DCP) by a de-

crease in light. Reduction in light on exposure to lead was caused mainly by an increase in mortality. However, exposure to heat shock, copper and 3,5-DCP resulted in a decline in luminescence due to a lower metabolic state of surviving nematodes. Luciferase is degraded rapidly with a half-life of 3 h in mammalian cells and 45 min in *E. coli* [14]. Differential expression of luciferase and the enzyme's instability could have resulted in lower levels of active luciferase in animals exposed to more adverse conditions. Levels of active luciferase were assessed by in vitro assays and did not correlate with the in vivo luminescence measurements, excluding the above hypothesis. The decrease in luminescence in nematodes exposed to elevated temperatures, copper and 3,5-DCP reflected their ATP levels and thus metabolic state. The strong causal link between in vivo luminescence and cellular ATP levels has been demonstrated previously [6]. The in vivo assay provides a rapid measure of sublethal effects of toxicity. In addition, as illustrated by lead exposure, bioluminescence assays provide a much faster measure of acute toxicity than conventional methods.

Traditional toxicity assays rely mainly on assessment of lethality and results are expressed as concentrations of toxicant causing 50% lethality (LC_{50}) after a certain exposure time. Reported copper and lead 24 h LC_{50} values [3,15–20] were respectively 2–13-fold higher and up to 4.5-fold higher than those depressing luminescence by half in 2 h in the present study (approximately 0.16 mM copper and 0.45 mM lead). Thus, besides being faster and less labour intensive, bioluminescence assays are a more sensitive method for assessing toxicity than conventional lethality assays. Any environmental condition that can impair metabolism, disrupt the electron transport chain and activate protective mechanisms that require energy expenditure will have some effect on luminescence [21].

Transgenic *C. elegans* with the *lacZ* gene under control of heat shock promoters have been developed and show promise as biosensors [2,22–24]. However, when compared with luminescence, such assays are lengthy to perform and present some technical difficulties as not all animals show induction of the transgene. This makes spectrophotometric determination of β-galactosidase less reliable than in situ histochemical staining, which in turn is difficult to quantify [22]. In addition, exposure conditions for minimum staining (0.074 mM copper for 8 h or 0.030 mM lead for 16 h [24]) show that sensitivity is not improved when compared to that of the bioluminescence assays developed here.

Bioluminescent biosensors have the advantage of being a rapid, sensitive and easily quantifiable method for assessing toxicity. Luminescence provides integrated information on the effects of test conditions on the metabolism of the whole organism. The assays lend themselves well to rapid screening and we are currently adapting them to a 96 well microplate format.

C. elegans biosensors represent a more complex level of biological organisation and a higher trophic level than the bacterial and yeast luminescent biosensors already available [1,7]. This is pertinent when predicting toxicity to humans or implications for environmental health. This approach can be used more generally to evaluate *C. elegans* metabolic status and might be especially useful in ageing studies [25] and for the development and screening of new nematocide drugs.

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