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Construction and evaluation of two biosensors based on yeast transcriptional response to genotoxic chemicals

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ABSTRACT

It has been well established that essentially all microbial mutagens are rodent carcinogens, yet current mutagen detection systems are limited by their detection sensitivity. Here we report the construction of a pair of hypersensitive biosensors by optimizing both reporters and the host strain. The resulting *RNR3-yEGFP* and *HUG1-yEGFP* reporters and the septuple yeast mutant in combination with the automated protocol not only remarkably enhance the detection sensitivity, but also allow a high throughput screen of environmental genotoxins. This system is deemed much more sensitive than similar yeast and bacterium-based tests for all selected chemicals examined in this study.

1. Introduction

Genotoxic agents induce DNA or chromosomal damage and lead to mutation and cell death (Phillips and Arlt, 2009). It has now been well-established that diseases like cancer are due to the accumulation of genetic variations that promote uncontrolled cell proliferation (Vineis and Perera, 2007); hence it is not surprising that essentially all genotoxic agents are rodent carcinogens (Granath et al., 1999; Strauss, 1992), making it extremely critical to detect environmental genotoxins that may exist in low abundance and to assess their cancer risk (Lynch et al., 2011).

Budding yeast *Saccharomyces cerevisiae*, as a unicellular model eukaryotic microorganism, is well characterized and its genome can be readily manipulated, making it highly suitable as a biosensor. Based on the transcriptional response of yeast cells to DNA damage (Fu et al., 2008), various genotoxicity testing systems have been developed (Afanassiev et al., 2000; Ichikawa and Eki, 2006; Jia et al., 2002), one of which has been developed into a commercially available GreenScreen GC assay. Compared with the traditional bacterium-based detection systems such as Salmonella/microsome or Ames test (Ames et al., 1973) and SOS chromotest (Quillardet et al., 1982), the yeast cell-based detection systems appear to be able to detect a broader range of genotoxins, are environmentally friendly, and can better reflect the DNA-damage response in higher eukaryotes including humans (Jia et al., 2002). Indeed, a human-cell based GreenScreen HC assay utilizing a *GADD45a-GFP* (Hastwell et al., 2006) reporter has also been developed.

The yeast-based biosensors consist of two components, the promoter of a DNA damage-responsive gene as the sensor and a reporter. The *RNR3* gene is used as a sensor, since its expression is nearly undetectable during normal cell growth but is strongly induced after DNA damage (Elledge and Davis, 1989; Kolberg et al., 2004). Although a series of genetic manipulations make the *RNR3-lacZ* system highly sensitive (Zhang et al., 2011), it still has room to improve into an applicable biosensor. Firstly, the *lacZ* reporter relies on a colorimetric assay of the β-galactosidase activity, which requires cell disruption. In order to explore highly-efficient and simple-operating genotoxic testing systems, we wish to replace the *lacZ* reporter with a *yEGFP* gene encoding yeast-enhanced green fluorescent protein optimized for expression in *S. cerevisiae* (Cormack et al., 1997; Tsien, 1998). GFP as a reporter of gene expression is considered non-invasive when illuminated in living cells and can be detected directly, hence offering an opportunity to develop highly automated live-cell

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testing systems. Secondly, a yeast-cell based *HUG*1-*GFP* biosensor was reported recently as a sensitive genotoxic testing system to detect multiple genotoxins (Benton et al., 2007). *HUG*1 (hydroxyurea and UV and gamma radiation induced), which is regulated by the Mec1 checkpoint pathway (Basrai et al., 1999), and is inducible by a DNA-alkylating agent and γ radiation, appears to be more sensitive than known genes previously studied. However, it has not been directly compared with *RNR*3, and has only been examined in select gene inactivation backgrounds (Benton et al., 2008). Thirdly, although several reports (Benton et al., 2008; Jia and Xiao, 2003; Walsh et al., 2005; Zhang et al., 2010, 2008, 2011) examined the effects of various genetic manipulations on the enhancement of yeast genotoxicity testing systems, they used different reporter systems and a comparative study is lacking. Finally, there has not been an attempt to combine all the advances into a single system to make it a useful biosensor.

The major challenge facing yeast-based (as well as other biomarker) testing systems is to detect the extremely low doses of genotoxins in the environment. The overall objective of the current study was to construct a hypersensitive biosensor suitable for the automated detection of a broad range of genotoxic pollutants. We report the construction of such a biosensor and its comparison with other currently used biosensors.

2. Materials and methods

2.1. Yeast strains, plasmids and biosensor system construction

The haploid *S. cerevisiae* strain BY4741 (*MAT***a** *his*3Δ0 *leu*2Δ0 *met*15Δ0 *ura*3Δ0), its quintuple (*snq*2Δ::*KanMX*, *pdr*5Δ::*LEU*2, *cwp*1Δ::*hisG*, *cwp*2Δ::*HIS*3, *yap*1Δ::*hisG*) mutant WXY2908 and septuple (quintuple plus *rad*1Δ::*hisG mag*1Δ::*hisG*) mutant WXY3009 were cultured at 30 °C in YPD medium. Plasmid YCplac33 (YCp, *URA*3) (Gietz and Sugino, 1988) was used as the cloning vector and plasmid pUG36 (a gift from Dr. J.H. Hegemann, Heinrich-Heine University, Germany) serves as a donor for *yEGFP* and T_{CYC1}. In addition, plasmid M4366 (*HO-hisG-URA*3-*hisG-HO*) (Voth et al., 2001) and pBluescript (Stratagene) were used for integrating the complete biosensor cassette into the yeast genome, followed by selection of spontaneous *URA*3-*hisG* excision through homologous recombination.

A PCR-driven gene splicing by the overlap extension method (gene SOEing) (Heckman and Pease, 2007) was employed for the construction of reporters. Firstly, fragments containing *RNR*3 or *HUG*1 promoters were amplified, while *yEGFP* open reading frame (ORF) and T_{CYC1} extensions were also amplified. Secondly, the three individual fragments were used as templates in one reaction to amplify the splicing fragment containing *RNR*3-*yEGFP-CYC*1 or *HUG*1-*yEGFP-CYC*1; the overlapping primers were designed for the PCR-mediated reactions (Supplementary Table S1). The 1.6-kb *RNR*3-*yEGFP-CYC*1 or the 1.5-kb *HUG*1-*yEGFP-CYC*1 cassette was released by *Pst*I-*Eco*RI digestion and cloned into YCp33, with the two resulting recombinants designated *RNR*3-*yEGFP* biosensor and *HUG*1-*yEGFP* biosensor, respectively.

To create stable testing systems, we integrated the biosensor cassettes P_{RNR3}-*yEGFP*-T_{CYC1} or P_{HUG1}-*yEGFP*-T_{CYC1} into the yeast genome. Briefly, the above cassettes were inserted into the *Pst*I-*Eco*RI sites of pBluescript, which were then released by the flanking *Eco*RI–*Bam*HI sites and reinserted into plasmid pM4366. Finally, the biosensor cassette and a *hisG-URA*3-*hisG* selectable marker were isolated as a single *Not*I fragment and integrated into the host genome at the dysfunctional *HO* locus (Jia et al., 2002; Voth et al., 2001). Positive clones were confirmed by PCR identification and the flow cytometry assay.

To create the septuple deletion strain, the *RAD*1 and *MAG*1 genes were sequentially deleted from the quintuple mutant strain (Zhang et al., 2011). The *rad*1Δ::*hisG-URA*3-*hisG* disruption cassette was released by *Sal*I digestion of pΔrad1::Blast, a gift from Dr. E. Perkins (NIESH, USA). The *rad*1Δ::*hisG* derivative was obtained by selection of 5-fluoro-orotic acid (5-FOA)-resistant colonies (Boeke et al., 1984). A *mag*1Δ:: *hisG-URA*3-*hisG* cassette was released by *Eco*RI–*Bgl*II digestion of a *mag*1Δ::*hisG-URA*3-*hisG* disruption plasmid (Chen et al., 1990) prior to transforming the above sextuple deletion strain. Once the septuple deletion strain was confirmed, the 5-FOA-resistant colonies were obtained in a similar manner.

2.2. Test chemicals and DNA-damage exposure

DNA-damaging chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and include methyl methanesulfonate (MMS), 4-nitroquinoline-N-oxide (4-NQO), phleomycin and three typical oxidative chemicals hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (*t*-BHP) and methyl viologen (MV, also known as paraquat) as well as two chemotherapeutic drugs chlorambucil and cisplatin. In addition, L-(+)-(*S*)-canavanine, chloramphenicol, ClonNAT and formaldehyde were used as a non-genotoxic agent. 4-NQO, chlorambucil and cisplatin were dissolved in DMSO at and stored at −20 °C, chloramphenicol was dissolved in anhydrous ethanol and stored at −20°C, chloramphenicol was dissolved in anhydrous ethanol and stored at −20°C, while other chemicals were dissolved in sterile distilled H_2O and stored at 4 °C. DMSO alone in the experimental dose range had no apparent effect on the sensors or cell survival (data not shown). All the above chemical solutions were added directly to the yeast cultures before incubation.

Yeast cells carrying the biosensors were incubated in fresh YPD medium at 30 °C overnight with shaking until mid log-phase and then diluted to an $OD_{600\ nm}$ of 0.1. 1-ml aliquots were dispensed into a 24-well plate for the genotoxin exposure, with one well remaining untreated to serve as a control.

After drug exposure, cells were collected and washed in fresh PBS buffer, resuspended in fresh PBS, and put on ice in the dark until the flow cytometry analysis.

2.3. Flow cytometry (FCM) analysis

Becton Dickinson FACS Aria TM III flow cytometer was used to measure fluorescence intensity. At least 10,000 treated cells were analyzed for each sample to determine the *yEGFP* fluorescence intensity. Treated cells were labeled by propidium iodide (PI) to separate live from dead cells, and the *yEGFP* fluorescence in the viable cell population was measured. To assess the fluorescence-positive population, cells without drug exposure served as a negative control gate.

To determine the performance of *yEGFP*-inducible expression and investigate the relationships among the *yEGFP*-fluorescent intensity, exposure time and drug dose, the test data exported from FCM analysis were processed by using the Flow-Jo 7.6 software, and the mean *yEGFP* fluorescence intensity was calculated as a reference to quantify the *yEGFP* inducible expression. Results are expressed as multiple changes over the same untreated cells.

2.4. Confocal microscopy

Confocal microscopy images were acquired by using Zeiss NOL-LSM 710 with EC Plan-Neofluar 40 × /0.75 objective. Two signals were acquired sequentially. Track 1 includes GFP and DIC brightness channels with a 488 nm excitation (4.5%, master gain 828 for GFP, 278 for DIC brightness) and a 508–601 nm filter, while rack 2 was a PI channel with a 561 nm excitation (2.0%, master gain 680) and a 566–719 nm filter. ImageJ software was used to gather semi-quantitative data regarding relative intensities of both biosensors,