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RESEARCH ARTICLE

Construction and comparison of yeast whole-cell biosensors regulated by two RAD54 promoters capable of detecting genotoxic compounds

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ABSTRACT

Two yeast enhanced green fluorescence protein (yEGFP) yeast reporter vectors, pR1558-yEGFP and pR406-yEGFP, which are regulated by two RAD54 promoters containing 406-bp and 1558-bp DNA sequences, respectively, were constructed using molecular biological techniques and transformed into yeast for the screening of genotoxins. The constructed biosensors were named W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP. To quantify biosensor performance, both transformed yeast cells were exposed to multiple doses of genotoxins including methylmethane sulfonate (MMS; a DNA alkylating agent), 4-nitroquinoline-N-oxide (4-NQO; a DNA cleavage agent), 5-fluorouracil (5-Fu; an inhibitor of polymerases and topoisomerases) and colchicine and canavanine (affecting other biochemical activities). The yeast bioassay performance was analyzed using fluorescence-activated cell sorting (FACS) and Multi-Mode Reader in a 96-well black microplate. The observed W303-1A/R1558-yEGFP dose-effect relationship was more obvious and the maximum inductions were 5.96-fold (MMS), 2.19-fold (4-NQO) and 2.71-fold (5-Fu); the corresponding values for W303-1A/R406-yEGFP were 2.53-, 1.50- and 1.91-fold, respectively. It is suggested that it is best to select the entire RAD54 promoter when constructing recombinant yeast cells for screening mutagens.

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KEYWORDS
RAD54 promoter; yeast cell; chemical mutagen; yEGFP; dose-effect relationship

Introduction

The effects of genotoxins on the environment, both from anthropogenic and natural sources, are currently of major concern. In some cases, these toxins pose a potential carcinogenic threat to animals and humans (Barbosa et al., 2010; Goodman & Wilson, 1991; Ross et al., 2000). At present, genotoxicity is commonly assessed using a batch method that was developed by Ames. In this test, specifically developed strains of the bacterium *Salmonella typhimurium* are exposed to chemicals and then plated out on selective growth media. The resulting colony counts are used to measure the frequency of different types of genetic damage (Ames et al., 1973; Bartsch et al., 1980; Irving & Elfarra, 2013; Lah et al., 2008; Maron & Ames, 1983). However, this test is not optimal. Some compounds that yield negative results in the Ames assay lead to cancer in animal models, and some that yield positive results in the Ames assay do not threaten humans (Kirkland et al., 2014). These differences are associated with differences in the repair efficiency and metabolism of compounds between bacteria (prokaryotes) and humans (eukaryotes).

Yeast cells are very common eukaryote cells and represent an attractive alternative to the use of traditional bacterial-based detection systems, such as the Ames test and the SOS chromo test. Yeast cell-based detection systems appear to detect a broader range of genotoxins and can better reflect the DNA-damage response in higher eukaryotes, including humans. For this reason, many investigators have developed whole recombinant yeast cells to screen genotoxins. Some of these systems employ the yeast enhanced green fluorescence protein (yEGFP) gene, which is codon-optimized for transcription in yeast, as a DNA-damage reporter gene that is regulated by the RAD54 promoter (Kwon et al., 2007; Samarajeewa et al., 2014; Schmuckli-Maurer & Heyer, 2000; Siede et al., 1985; Walsh et al., 2002). However, the range of RAD54 promoter is unclear; therefore, a wide range of RAD54 promoter lengths have been selected for use in recombinant yeast cells for the rapid high-throughput screening of chemical mutagenesis agents. Some researchers have selected the entire sequence of approximately −1.8 kb between SUT1 and RAD54 ORF as a promoter (Cole et al., 1987; Mazin et al., 2010; Tan et al., 2003), whereas others have used a sequence of approximately −0.5 kb (Cole & Mortimer, 1989; Zhang et al., 2013). The susceptibility of these two RAD54 promoters in response to chemical mutagens differ, but it is yet unclear which promoter is better for chemical mutagenesis screening.

Materials and methods

Materials

pLZ-yEGFP plasmid, *E. coli* DH5α and *Saccharomyces cerevisiae* W303-1A (genotype MATα, ade2-11, his3-11, leu2-3, trp1-1, ura3-1) were maintained in our laboratory (Hygienic

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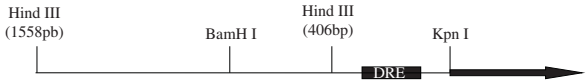

Figure 1. pR1558AD54-yEGFP yEGFP yeast reporter vector regulated by the −1558-bp RAD54 promoter.

Figure 2. pR406-yEGFP yEGFP yeast reporter vector regulated by the −406-bp RAD54 promoter.

Toxicology Laboratory, Yangzhou University Medical College). Restrictive endonuclease and T4 ligase were purchased from the TaKaRa Company (Kyoto, Japan). All plasmid extraction kits and purification kits were purchased from AxyGen (Silicon Valley, CA). Agarose gel DNA purification recovery kits were purchased from Beijing Tiangen Biotech Beijing Co., Ltd (Beijing, China). An epifluorescence microscope (Ti-U, Nikon, Tokyo, Japan), an ABI-2720 thermocycler (Applied Biosystems, Foster City, CA) and a Multi-Mode Reader (Synergy2, BioTek Instruments Inc., VT) were used in these experiments. The primers used were synthesized by Zhongkehuada Boke Biotech Co., Ltd (Beijing, China).

Methods

Two RAD54 promoters regulating yEGFP reporter vectors

All of the DNA upstream of the translational start ATG codon of the RAD54 gene to the next open reading frame (ORF) was amplified by PCR from the isolated yeast W303-1A genome. We divided the promoter into two parts for PCR. First, we used the forward primer 5′-AAAAAGCTTGAAGTTTAG-ATGAGTAAGGAA-3′ containing a restriction site for Hind III (underlined) and the reverse primer 5′-TAGGGATCCT AATACGGTATAACGTAAACC-3′ containing a restriction site for BamH I (underlined) to amplify RAD54-1, resulting a sequence of approximately half of the promoter length. Then, the fragment neighboring RAD54-2, which contains a Hind III restriction site, was amplified using the following PCR primers designed to include both BamH I (forward primer) and Kpn I (reverse primer) restriction sites: 5′-TTAGGATCCCT ATAAGGGTTCCTTCGGGAGGA-3′ and reverse 5′-TCTACCGGT CAGTTATAAGGAAATATA TATG-3′. The aim of this procedure was to avoid the Hind III restriction site on the yeast reporter vector of pLZ-yEGFP. A touchdown PCR program was executed under the following conditions: predenaturation of the template for 5 min at 94 °C; denaturation of the template for 45 s at 94 °C; annealing of the primers for 45 s at 62 °C; elongation for 1 min at 72 °C; and repeat all previous steps for 2 cycles. The annealing temperature was then gradually decreased from 60 °C to 50 °C in 2 °C increments using 2 cycles at each temperature. When the annealing temperature reached 50 °C, 15 cycles of amplification were performed. Finally, the products were extended at 72 °C for 5 min.

The amplified RAD54-1 promoter was purified using a DNA fragment purification kit, digested with Hind III and BamH I and then cloned into a Hind III/BamH I-digested pLZ-yEGFP vector to yield pRAD54-1-yEGFP. RAD54-2 was purified, digested with BamH I and Kpn I and then inserted into pRAD54-1-yEGFP to yield pR1558-yEGFP (Figure 1).

The pR1558-yEGFP vector was digested with Hind III to yield the vector fragment and then ligated with T4 ligase to form the vector pR406-yEGFP (Figure 2), in which the yEGFP reporter is regulated by a −500-bp RAD54 promoter, the basic promoter containing a DRE (DNA damage-responsive element).

Yeast transformation

Yeast transformations were performed using the LiAc transformation procedure. Yeast transformants were selected on SD/-ura selective medium supplemented with 2% glucose. PCR identification was performed using DNA extracted from the transformants as template. To ensure that the positive transformants expressed yEGFP in response to DNA damage, cells that had been exposed to methylmethane sulfonate (MMS) at 200 μg/ml for 12 h were examined using epifluorescence microscopy. The positive clones were named W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP.

Fluorescence assays

Before running the assay, selective SD/-ura plates were inoculated with the yeast W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP cytosensors. The plates were incubated at 30 °C for 3–4 days and then stored at 4 °C. The day before running the assay, 5 ml of SD/-ura selective medium was inoculated with a single colony of the biosensors. The cultures were grown for 24 h at 30 °C with vigorous orbital shaking at 250 rpm. At the late log phase, the yeast biosensor cultures were diluted (1:3) with SD/-ura in a sterile dish.

For exposure in 96-well black plates (Costar), 100 μl of the yeast culture was pipetted into each well. The yeast were exposed to different doses of MMS (a DNA alkylating agent), 4-nitroquinoline N-oxide (4-NQO; a DNA cleavage agent), 5-fluorouracil (5-Fu; an inhibitor of polymerases and topoisomerases) and other cytotoxic substances, such as colchicine, canavanine and tetracycline, by adding 1 μl of stock solutions of the agents (MMS dissolved in ethanol, the others in sterilized saline) to each well such that the final concentration of the correspondent solvents was 1.0%. The solvents were used as blank controls in each experiment. Each sample concentration was assayed in triplicate. The yeast were exposed for 12 h according to the published reports. Fluorescence was measured directly using a Multi-Mode Reader (cytofluorescent multi-well plate reader assay) with excitation at 485 nm and emission at 530 nm. Yeast culture density was determined by measuring the OD600 in 96-well transparent plates (Costar) at the same time to test whether each substance was toxic to yeast cells.

After the samples had been measured using the Multi-Mode Reader, 100 μl of each cell culture was added to 1 ml of PBS for fluorescence-activated cell sorting (FACS) analysis. All surviving cells were gated, and yEGFP expression in the population was measured using flow cytometry (BD FACSCalibur™ flow cytometer). yEGFP fluorescence was excited at 488 nm in the FL1 channel. The typical sampling rate was set in HI mode, and a minimum of 100,000 treated

cells were analyzed in each sample to determine yEGFP fluorescence intensity. To assess the fraction of yEGFP-positive cells, a gate was drawn around a population of fluorescence-compensated blank control cells. Any cell in a subsequent sample that lay outside that gate was considered yEGFP-positive. Cell Quest Pro software was used to analyze the data.

Statistical analysis

To determine biosensor yEGFP fluorescence as a function of DNA-damaging agent concentration in our fluorescence assay, the mean fluorescence intensity was calculated for each of at least three independent trials. This value was then compared with the mean fluorescence intensity of control samples from the same culture. The ratio of the mean fluorescence intensity of three independent exposure trials to that of non-exposure trials (blank controls) was considered the yEGFP induction ratio (IR) for each dose. In our cytofluorescent multi-well plate reader assay, IR was calculated using the equation

$$IR = Di/OD600(i)/Dn$$

where Di is the mean fluorescence intensity at concentration (i), Dn is the mean fluorescence intensity for each blank control, and OD600 (i) is the mean fluorescence intensity of the yeast culture at concentration (i). The OD600 measured in at least three independent trials was averaged. This value should be greater than 0.3.

The IR value of the FACS data was calculated using the equation

$$IR = Di/Dn$$

The data were analyzed using Excel 2007. According to the ISO standards, an IR of greater than 1.5 is considered as genotoxic response (Lu et al., 2015).

Results

As seen in Figure 3, W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP, when exposed to 200 mg/L MMS for 12 h, emitted intense green fluorescence, and the biosensors that were not exposed to the tested genotoxins exhibited much less intense green fluorescence. These results confirm that the cells were induced to express yEGFP in the presence of DNA damage-causing agents. However, it was difficult to distinguish which of the two cytosensors exhibited more intense fluorescence under epifluorescence microscopy.

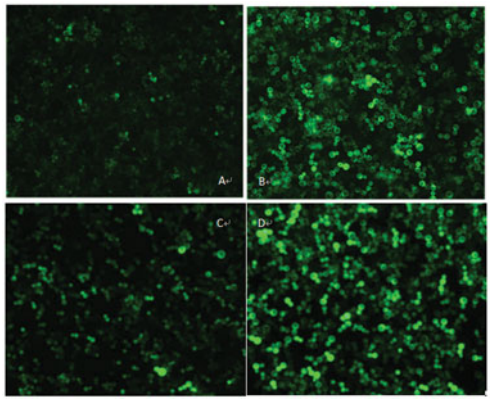

Figure 3. Epifluorescence images of yeast cells exposed to the genotoxin MMS. Fluorescence images of W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP; after exposure to 200 μg/ml of MMS (B, D) for 12 h, W303-1A/R1558 and W303-1A/R406-yEGFP exhibited more intense fluorescence than non-exposed controls (A, C). However, the fluorescence of the W303-1A/R1558-yEGFP strain (B) did not differ markedly from that of the W303-1A/R406-yEGFP strain (D).

As shown in Figure 4, the dose–response relationships of yEGFP expression were analyzed after exposure of the W303-1A/R1558-yEGFP and W303-1A/R406-yEGFP cytosensors to MMS, an alkylating mutagen, using FACS; an IR peak was observed at 200 μg/ml. However, the dose-dependent responses for W303-1A/R1558-yEGFP were stronger than those for W303-1A/R406-yEGFP. The former exhibited a maximum IR of 5.96 (FACS), and the latter exhibited a maximum IR of 2.53. An obvious dose–response relationship was also observed after exposure of W303-1A/R1558-yEGFP to 4-NQO, a DNA-cleavage mutagen, showing a maximum IR of 2.19 at 1.25 μg/ml. Although a dose–response relationship also existed for W303-1A/R406-yEGFP, this response was clearly inferior to the former and yielded a maximum IR of 1.78. In response to exposure to 5-Fu, a mutagen that inhibits DNA polymerase, exhibited similar results as that of 4-NQO for 12 h, but the dose–response curves increased very sharply and did not significantly differ; maximum IR values were 2.53 and 1.91.

Regarding the Multi-Mode Reader (Figure 5) results, when the W303-1A/R1558-yEGFP cytosensor was treated with MMS, 5-Fu and 4-NQO for 12 h, the dose–response curves yielded maximum IR values of 3.03 (MMS), 2.71 (5-Fu) and 2.32 (4-NQO), respectively. However, the W303-1A/R406-yEGFP cytosensor exhibited dose-dependent relationships with MMS

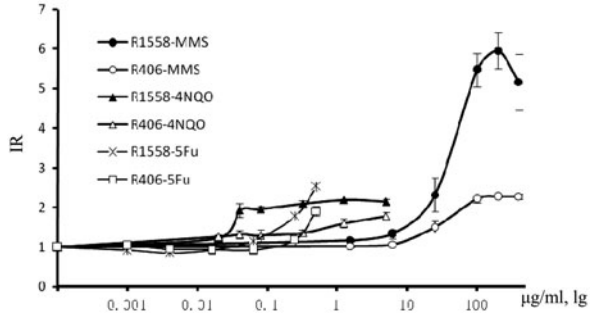

Figure 4. Genotoxin dose–response relationship for both cytosensors, as determined using FACS (R1558: W303-1A/R1558-yEGFP; R406: W303-1A/R406-yEGFP).

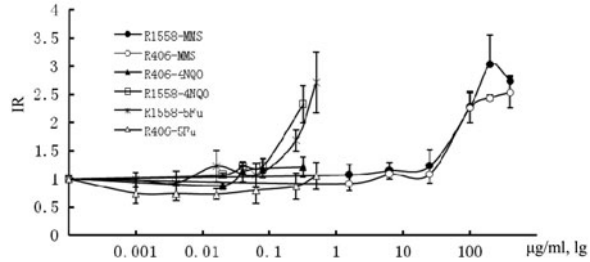

Figure 5. Genotoxin dose–response relationships for both cytosensors as measured using Multi-Mode Reader. (R1558: W303-1A/R1558-yEGFP; R406: W303-1A/R406-yEGFP).

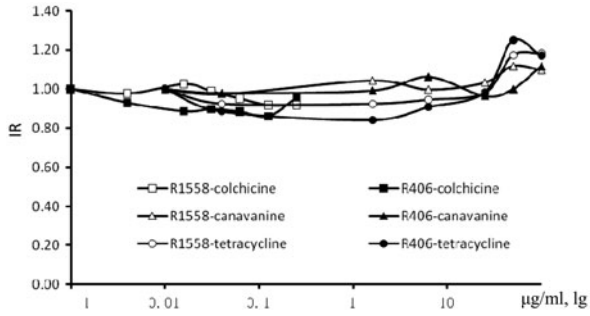

Figure 6. Dose–response relationships for cytotoxic chemicals do not cause DNA damage in the recombinant yeast cells (R1558: W303-1A/R1558-yEGFP; R406: W303-1A/R406-yEGFP).

only; no dose-dependent effects were seen with 4-NQO or 5-Fu.

Additionally, FACS was more sensitive than Multi-Mode Reader because the maximum IRs obtained in the former experiments were higher than those obtained in the latter experiments.

To verify that both cytosensors were selective for genotoxic effects, the cells were exposed to cytotoxic chemicals that do not cause DNA damage, including colchicine, canavanine and tetracycline. When the biosensors were exposed to canavanine or tetracycline at concentrations from 0.1 to 100 μg/ml, the maximum induction of yEGFP fluorescence was below the established positive result threshold of 1.5-fold, and no significant dose relationships were found. Similar results were observed when the cells were exposed to colchicine at concentrations from 0.016 to 0.5 μg/ml; the maximum yEGFP induction was 0.99-fold after 12 h of treatment (Figure 6). Furthermore, the yEGFP fluorescence emitted by both cytosensors does not appear to be activated by cytotoxic chemicals under epifluorescence microscopy (data not shown).

These results were determined using FACS. The results obtained using Multi-Mode Reader were the same as those obtained using FACS; for this reason, the Multi-Mode Reader data are not shown.

Discussion

The use of yeast cells as biosensors is increasingly important for the analysis of a variety of biologically toxic chemical compounds, including genotoxins. Due to these biosensors, genotoxin bioassays have undergone considerable development and are now widely used. Yeast cells present the same advantages as prokaryotic cells in that they are easily cultivated and manipulated, grow rapidly and are cost-effective. While retaining the properties of unicellular organisms, yeasts are true eukaryotes; therefore, their gene regulation and transcription and protein translation are similar to those of mammalian cells. Thus, compared with existing eukaryotic genotoxicity bioassays, yeast biosensors provide higher throughput, strong resistance to the cytotoxicity of the chemical compounds tested, lower consumption of the compound used and are sensitive to a broad spectrum of genotoxins. However, promoter choice is very important and sometimes critical in constructing such biosensors.

RAD54 is one of the key proteins involved in the homologous recombination and repair of DNA. This protein has been shown to play a role in the homologous recombination-related repair of DNA double-strand breaks (Boronat & Pina, 2006). Therefore, the RAD54 promoter can be activated by DNA damage, which is responsive to a wide range of mutagens and carcinogens. Some researchers have engineered various reporter genes downstream of the promoter for the rapid, high-throughput screening of chemical genotoxins. However, the promoter regions used to drive the fluorescence signal differed because the RAD54 promoter has not yet been fully characterized.

Promoters play an important role in gene expression and gene regulation because they contain specific DNA sequences such as response elements, which provide a secure initial binding site for RNA polymerase and for transcription factors that recruit RNA polymerase. These transcription factors have specific activator sequences that attach to specific promoters and regulate gene expression. In eukaryotes, this process is very complicated, and many factors are necessary for RNA polymerase II binding to the promoter Ptashne & Gann, 1997). For the RAD54 promoter, Cole previously demonstrated that −406 bp of the region upstream from the ATG start codon, which contains a 29-bp damage regulatory sequence (DRS), is sufficient to account completely for the response of the wild-type gene regarding both constitutive expression and damage-inducible expression (Cole et al., 1987). For convenience, some researchers selected 500 bp of the region upstream of the RAD54 structural gene, the core promoter, to construct the recombinant yeast cells to screen chemical mutagens (Afanassiev et al., 2000; Cole & Mortimer, 1989; Henriques et al., 1989; Schmuckli-Maurer & Heyer, 2000). However, based on our results, the region from −406 bp to −1800 bp may contain additional regulatory elements that can work in concert with other regulatory regions or contain additional transcription factor-binding sites that direct the level of transcription of the RAD54 gene (Billinton et al., 1998; Carey, 1998; Mazin et al., 2010). A comprehensive understanding of the promoter's role in transcriptional regulation is necessary for accurately reconstructing biosensors. In eukaryotic cells, the transcription of most genes is regulated by specific DNA sequences including enhancers and cis-acting elements. Some DNA sequences that are targeted by multiple transcription factors can achieve a hyperactive state, thus leading to increased transcriptional activity. Although eukaryotic promoters are diverse and can be difficult to characterize, recent studies show that these promoters can be divided into more than 10 classes. A wide variety of algorithms has been developed to facilitate the detection of promoters in genomic sequences, and promoter prediction is a common element of many gene prediction methods. In our study, we used promoter prediction methods to search the DNA sequence from −406 bp to −1800 bp in the RAD54 promoter to obtain possible multiple transcription factor-binding sites, such as ABF1, TAF, GCN4, ADR1 and REB1 (Carey, 1998; Carey et al., 1992; Lieb et al., 2001; Mencia et al., 2002; Ptashne & Gann, 1997; Yu et al., 2003). It was found that there are 12 ABF1, 11 TAF, 4 GCN4, 2 ADR1 and 1 REB1 transcriptional factors in respectively −1588 bp RAD54 promoter region. However, in −406 region

no of ADR1 and REB1 was found and there only have 4 numbers of ABF1, 3 TAF and 1 GCN4. The numbers and the frequencies of these transcription factor binding sites are much greater than those in −406 bp region. These differences mediate stronger synergistic effects on transcription.

To quantify the yeast sensor performance, reporter genes encoding enzymes, such as Lac Z, chloramphenicol acetyltransferase (CAT) and luciferase (LUC), were used (Bullock & Gorman, 2000; Carey, 1998; McNabb et al., 2005). For the determination of enzyme activity, the yeast cell wall has to be disrupted and substrates added; these procedures are time-consuming and costly. GFP protein is derived from the jellyfish *Aequorea victoria* and emits a green fluorescence that can be measured directly without cell disintegration and without the addition of any cofactors. The use of GFP as a reporter renders the bioassay procedure faster and easier. However, in routine applications, established genotoxic tests are performed in a microplate reader; i.e., when using Multi-Mode Reader to generate qualitative measurements, the induced yEGFP signal of the biosensors must be normalized based on OD600 or protein concentration in yeast cells due to the decreased viability of yeast cells when treated with the tested chemicals. OD600 and yeast protein measurements vary depending on the colorimetric instruments used or on the methods used for protein determination (Griffith & Wolf, 2002). This can lead to large errors and render the biosensors insensitive to the genotoxic chemicals. Flow cytometry can be utilized to quantify the fluorescence signal densities of uniform cell populations while removing any debris or dead cells that interfere with the final data and provide the average density as the summary of the overwhelming and unnecessary amount of information. This level of accuracy and sensitivity demonstrates the advantages of using Multi-Mode Reader analysis.

Although flow cytometry sorters are very accurate, even high-speed sorters are often not fast enough to achieve the desired results. A high-speed sorter can sort up to 10^6 cells per hour when dealing with subpopulations that comprise up to 20% of the whole population. This rate is too low for many experiments. However, Multi-Mode Reader analysis is widely used in research in the pharmaceutical and biotechnological industry and in academic organizations because it can be performed in microplates, such as 96- or 384-well microplates. This procedure is very fast and provides high-throughput. With the apparent insensitivity of these techniques to some DNA damage agents, further study is required for potential improvements to enhance biosensor sensitivity, such as utilizing host strains with compromised cell-wall integrity or deficient DNA damage repair enzymes.

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Disclosure statement

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