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DEVELOPMENT OF A YEAST BIOSENSOR STRAIN FOR THE IDENTIFICATION OF GENOTOXIC COMPOUNDS

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Abstract:

Advances in combinatorial chemistry have provided the pharmaceutical industry with innumerable lead compounds that could potentially serve as therapeutic agents. One of the challenges in the further development of such compounds is to rapidly, yet inexpensively, distinguish those that have undesirable effects such as genotoxicity. Thus, a simple biological assay that would permit the identification of potential DNA mutagens, and be adaptable to high-throughput technologies would be cost-effective in screening such lead compounds. The current methods use the Ames and SOS tests involving prokaryotic organisms, while systems that utilize mammalian cell culture and/or animal testing are time-consuming and expensive. Our research has focused on developing the yeast, Saccharomyces cerevisiae, as a convenient and inexpensive eukaryotic biosensor for the identification of genotoxic compounds. The yeast biosensor uses two distinct bioluminescent reporters within the same cell. The first reporter is the Renilla (sea pansy) luciferase gene, which is expressed at a continuous rate to allow standardization. The second reporter is the firefly luciferase gene fused to gene promoters that are induced when cells are exposed to DNA mutagens. By monitoring changes in the ratio of firefly to Renilla luciferase activity upon exposure to potential mutagens, one can rapidly assess genotoxicity. We have demonstrated the sensitivity, reliability and convenience of the dual luciferase assay itself, and are continuing to optimize the sensitivity of the biosensor system.

Introduction:

Since the identification of DNA as the molecule of inheritance, the quest for uncovering its maintenance and reproductive mechanisms has been ongoing. Explanations and proposed schemes for these mechanisms have since sparked questions as to how these processes are manipulated in response to environmental stimuli. Much of the work in these areas has been done with rapidly growing prokaryotic organisms such as *Escherichia coli*. These organisms contain several fundamental differences from eukaryotes, such as DNA packaging and repair mechanisms, that restrict inferences into the human population (1). To overcome this problem, the yeast *Saccharomyces cerevisiae* has become an important model organism for eukaryotic research (2). Because it possesses the typical features of eukaryotic cellular architecture and metabolism, and its DNA repair mechanisms are strikingly similar to humans, it presents an ideal model system for investigation (1).

One question that has arisen from this research is how the genetic response to DNA mutagenic agents can be monitored. For example, in pursuit of new therapeutic agents, the pharmaceutical industry seeks a fast, cheap, and reliable means of quickly identifying compounds that may be genotoxic (3). The current standard uses the well-established Ames and the SOS tests, which involve prokaryotic organisms (1). Alternative methods that utilize mammalian cell culture and/or animal testing are both time consuming and expensive (1). The work described here demonstrates that a simple eukaryote, the Baker's yeast *Saccharomyces cerevisiae*, may provide a useful tool for identifying genotoxic compounds.

The *RAD54* gene encodes one of the proteins in *S. cerevisiae* involved in DNA damage repair (4). Importantly for the studies described here, the transcription of *RAD54* is tightly regulated (5), being induced in yeast cells only when the cells are exposed to a variety of DNA mutagens (6). This transcriptional induction results from the binding of transcription factors to specific DNA sequence elements in the *RAD54* promoter region in response to DNA damage (5). In the studies described herein, we take advantage of the transcriptional induction of the *RAD54* promoter in response to DNA mutagens to develop a biological sensor (biosensor) for genotoxins.

This work is an expansion of previous research in which a bioluminescent gene reporter system was developed for *S. cerevisiae* to monitor changes in gene expression in response to environmental stimuli (7). In this system, the luminescent reporter is firefly luciferase that, when exposed to the substrate luciferin, emits light and the rate of light emission is a direct indicator of the amount of firefly luciferase (8). When the firefly luciferase

is coupled to a regulated gene promoter such as that of *RAD54*, the relative changes in luminescence can be directly correlated to changes in transcriptional activity (9). In many different organisms, firefly luciferase has served as one of the best non-toxic and most sensitive methods to measure changes in gene expression (10).

The yeast system developed by McNabb *et al.* (7) is a dual-luciferase assay in which two different bioluminescent reporters are contained within the same yeast cell (Figure 1). The second reporter is the *Renilla* (sea pansy) luciferase, which uses a different substrate for bioluminescence and can be assayed sequentially with the firefly luciferase (9) (Figure 2). The *Renilla* luciferase is fused to a promoter (*SPT15*) that is expressed at a constant rate under all environmental conditions. This reporter serves as an internal control, allowing multiple samples to be compared directly providing enhanced accuracy to the assay. By monitoring the ratio of firefly:*Renilla* luciferase activity, one can rapidly and accurately determine the induction of a test reporter when exposed to changes in environmental conditions (i.e. exposure to DNA mutagens). In this paper, we describe our initial studies directed toward the development of a yeast biosensor strain to monitor genotoxicity.

Materials and Methods:

Yeast strains and media.

For generation of the yeast biosensor, *the Saccharomyces cerevisiae* strain DMY229 (*Mata ura3-52, his3Δ200, leu2Δ1, lys2Δ202, can1Δ::SPT15-Rluc*) was used. Rich (YPD) medium and synthetic complete (SC) medium lacking appropriate auxotrophic selections were prepared as previously described (11).

Plasmid construction.

The promoter region of the *RAD54* gene was obtained by polymerase chain reaction (PCR) using genomic DNA isolated from *S. cerevisiae* (12). The PCR reactions contained 100ng of yeast genomic DNA as template, 100 pmols of the primers oDM0351 (5'-GGCCGGATCCATGATATAGAGCCCACG-, CATATAC-3') and oDM0352 (5'-GGCCGAATTCA GTTATAAGGAAATATATATGGTACC-3'), 2.5 mM $MgCl_2$, 0.2 mM of each nucleotide and 1X PCR buffer (Promega) and 2 units of Taq DNA polymerase (Promega). The PCR product was purified, subjected to restriction enzyme digestion with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites in the polylinker region upstream of the firefly luciferase gene in the plasmid pDM553 (7). The ligation was introduced into *E. coli* DH5[*alpha*](*F-[phi]80lacZΔM15Δ(lacZYA-argF)U169, endA1, recA1, hsdR17(rk- mk+), deoR, thi1, supE44, [lambda]-, gyrA96, relA1*) by transformation using standard procedures as previously described (13). To verify the correct plasmid construction, the plasmid DNA was isolated from bacteria and subjected to restriction digestion. The *RAD54*-firefly luciferase (*RAD54-Fluc*) fusion plasmid was designated pDM573.

Construction of the yeast biosensor strain

For construction of the yeast biosensor strain, DMY229 was grown overnight in YPD and subsequently inoculated into fresh YPD and allowed to grow for four hours at 30°C. The plasmid pDM573 was linearized by restriction enzyme digestion with *Nco*I within the *URA3* gene of the plasmid. The linearized plasmid was introduced into DMY229 by the lithium acetate transformation (14) and the cells were plated on SC medium lacking uracil (SC-Ura). Linearizing the plasmid within the *URA3* gene targets integration of the plasmid to the mutated *ura3-52* locus by homologous recombination (15) resulting in cells that can grow on medium lacking uracil (SC-Ura). Three independently isolated Ura$^+$ colonies were tested for firefly luciferase activity in response to mutagenic treatment.

Luciferase assays.

Assays for firefly and *Renilla* luciferase activities were measured using the dual-luciferase kit according to the manufacturers instructions (Promega). Briefly, 10 to 20 µl of yeast cells were taken directly from a growing culture, diluted with 100 µl of passive lysis buffer (9), and 10 µl of the lysate was immediately transferred to the luminometer. Firefly and Renilla luciferase activities were determined by the sequential addition of 100µl of each substrate as described (9). Bioluminescence was quantified using a Turner Designs TD-20/20 single tube luminometer with an integration time of 10 seconds for each substrate. Each individual dual assay required approximately 30-45 seconds to complete.

Results:

Identification of the biosensor strain.

The integration of the linearized *RAD54-Fluc* plasmid (pDM573) into the *ura3-52* locus of the yeast genome by homologous DNA recombination could occur by recombinational events that result in Ura$^+$ colonies lacking a functional *RAD54-Fluc* gene. To verify that the individual Ura$^+$ colonies contained a functional *RAD54-Fluc*, three individual clones were screened for transcriptional induction in response to exposure to the DNA mutagen ethyl methanesulfonate (EMS), a DNA alkylating agent. Previous studies have demonstrated that the *RAD54* promoter is induced in response to EMS treatment of cells (1); therefore, growth of the putative biosensor strains in the presence of EMS should result in the transcriptional induction of firefly luciferase activity. The three strains were grown for 19 hours in YPD medium in the absence or presence of 0.1% (vol/vol) EMS. The cells were subsequently assayed for firefly and Renilla luciferase activities (Table 1). Strain 10 and strain 12 clearly demonstrated Fluc activity that was induced approximately two-to three-fold in response to EMS exposure at this concentration, while strain 11 had negligible activity. The rest of the studies were conducted using strain 10 since it demonstrated the highest overall luciferase activity suggesting that it might provide the greatest sensitivity and accuracy.

Growth conditions of the biosensor strain during exposure to DNA mutagens.

To determine whether differences in the growth medium for the yeast biosensor strain altered the sensitivity of the bioluminescence assay or altered the mutagenic capacity of the EMS being tested, strain 10 was grown under two different conditions: rich medium (YPD) or synthetic complete medium lacking uracil (SC-Ura). Thus, strain 10 was inoculated into YPD or SC-Ura in the absence or presence of 0.1% (vol/vol) EMS for 20 hours at 30°C. Cells were subsequently assayed for firefly and Renilla luciferase activities (Table 2). One, the basis of these data, the growth conditions, do not appear to alter the transcriptional induction of the *RAD54* promoter in response to mutagen exposure. Moreover, the level of the response is essentially identical under both conditions. Thus, our subsequent studies were performed using YPD medium since yeast cells grow more rapidly in rich medium.

Dosage sensitivity of the biosensor strain.

To examine the dosage sensitivity of the luciferase assay, studies were performed using various concentrations of both EMS and, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Strain 10 was inoculated to YPD medium in the presence of various concentrations of the mutagens. After growth in the presence of EMS (22 hours) or MNNG (5 hours), yeast cells were assayed for firefly and *Renilla* luciferase activity. As shown in Figure 3, growth in the presence of EMS demonstrated luciferase activity that was proportional to the amount of mutagen in the medium. A reproducibly detectable transcriptional response was observed at 0.01% EMS, and increased proportionally with the EMS concentration. At concentrations of EMS higher than 0.25%, the strain failed to grow due to genotoxicity. The mutagenesis with MNNG showed a threshold of 5 μg/ml before a detectable change in firefly luciferase activity was observed, afterwhich, the response increased in proportion to the amount of mutagen. Between 50 and 100 μg/ml of MNNG the transcriptional response appeared to be approaching saturation, suggesting that the maximum inducibility of the *RAD54* promoter had been achieved. Thus, for the two mutagens tested, the yeast biosensor strain responded in a manner that was predictable based on previously published data (1). At present, the sensitivity of the biosensor system is not yet sufficient to be used for the detection of trace levels of mutagen in a given setting. Further improvements in the biosensor strain (as described in the discussion) may allow us to use *S. cerevisiae* as a simple eukaryotic alternative for the identification of genotoxic chemicals.

Discussion:

In an era of combinatorial drug development and high-throughput drug screening, pharmaceutical companies can readily identify numerous drug candidates acting as effectors of given therapeutic targets. One of the problems associated with the identification of numerous agents is ruling out the toxic compounds that will not prove fruitful as curative agents. For the sake of cost efficiency, drug companies must rapidly and inexpensively discard these toxic candidates, such as DNA mutagens, to focus resources on the most promising compounds. Thus, an assay that can rapidly identify potential mutagens and be adaptable to a high-throughput screening platform is a valuable tool for the pharmaceutical industry.

The long-term goal of this research is to generate a system whereby pharmaceutical companies can conveniently screen potential drug candidates for mutagenic activity. Moreover, such a system could also be exploited for the detection of environmental mutagens or genotoxins sometimes found in food products (i.e. alfotoxins). For example, water samples from pools or lakes suspected of being contaminated with a mutagenic agent could be assayed with the biosensor strain. The inducible expression of *RAD54-Fluc* would provide a rapid indication of mutagen contamination and further studies could then be initiated and public health measures employed more rapidly. The simplicity and speed of the assay makes it adaptable for use in the field rather than having to wait for laboratory results, which may require several days, thereby allowing preventive health measures to be instituted rapidly.

While the experiments described in this paper show our initial stages in the development of the yeast biosensor strain, further improvements to the system may add to both the accuracy and sensitivity of the system. For example, the permeability of yeast cells to different chemicals will clearly impact whether or not the biosensor strain responds to a given genotoxin. We chose MNNG and EMS for our initial studies because these chemicals are known to enter yeast cells and to alter *RAD54* expression; however, when screening unknown chemicals for mutagenic activity, cell permeability is an important issue to consider. Fortunately a number of mutant yeast strains exist that have alterations in the cell wall that cause increased permeability. Such strains may prove useful in the continued development of the biosensor system. Alternatively, a genetic screen for mutants with increased sensitivity to a variety of genotoxic agents may allow us to identify strains with increased permeability, thereby broadening the number of chemicals that can enter the yeast cells. Such permeability mutants may also increase the lower level of detection for those genotoxins known to activate the reporter. An additional improvement in the system would be to use other promoters, such as *RNR2*, which are upregulated in response to DNA damage (6). The speed and convenience of the luciferase assay makes it plausible to use multiple independent biosensor strains to screen for genotoxins, thus enhancing the range of potential compounds that can be identified.

The studies described here provide the first application of the yeast dual-luciferase assay to the development of a yeast biosensor useful for identify genotoxic chemicals. However, it should be emphasized that the yeast dual-luciferase system is not restricted to just screening for genotoxins. One could conceivably

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screen for any small molecules that alter a cellular process that could ultimately be assayed by changes in transcription.. For example, high-throughput screens for small molecules that disrupt the interaction between two proteins known to be involved in causing disease could be identified using the dual-luciferase assay coupled with the yeast two-hybrid system (15). We have already constructed a two-hybrid yeast reporter strain based on dual-luciferase technology and have shown that protein-protein interactions can be accurately monitored via the induction of firefly luciferase activity (7). Thus, the number of potential applications for such a simple eukaryotic biosensor system are endless and should prove applicable to the pharmaceutical and biotechnology industry in the future.

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Table 1: Identification of the biosensor strain.

Strain name	Fluc/Rluc (0% EMS)*	Fluc/Rluc (0.1% EMS)*
strain 10	29 +/- 0	69 +/- 0
strain 11	none detected	none detected
strain 12	8 +/- 0.8	16 +/- 1.0

* values shown are the ratio of firefly to *Renilla* luciferase. All values were multiplied by a factor of 1000 to obtain whole numbers.

Table 2: Effect of growth medium on the genotoxicity assay.

Medium	Fluc/Rluc (0% EMS)*	Fluc/Rluc (0.25% EMS)*
YPD	29 +/- 0	77 +/- 7
SC-Ura	22 +/- 0	60 +/- 2

* values shown are the ratio of firefly to *Renilla* luciferase. All values were multiplied by a factor of 1000 to obtain whole numbers.

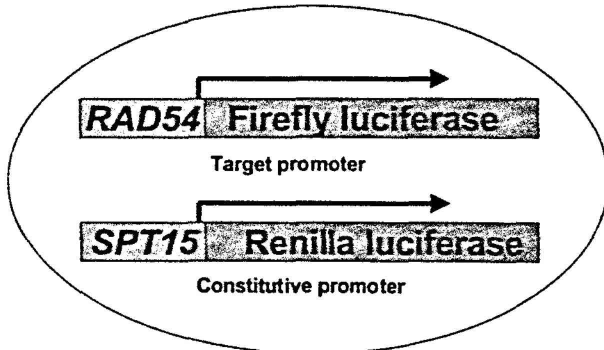

Figure 1. Schematic depiction of the dual-luciferase assay system within the nucleus of a yeast cell. The RAD54-Fluc *(firefly luciferase)is integrated at the* ura3-52 *locus and the* SPT15-Rluc (Renilla *luciferase) is integrated at the* can1Δ *locus (7).*

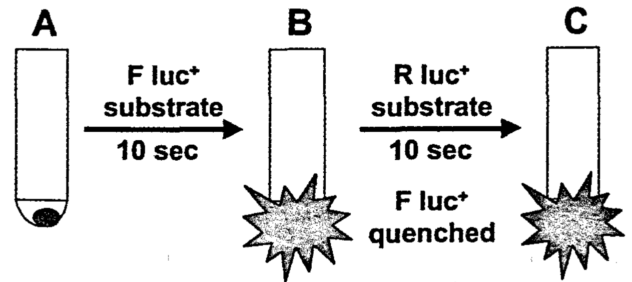

Figure 2. Schematic depiction of the sequential luciferase assay technique. A) sample of lysate is added to a tube in the luminometer; B) the firefly luciferase (Fluc) substrate (luciferin) is added and the luminescence of the sample is measured for 10 seconds; and C) the Renilla luciferase (Rluc) substrate (coelenterazine) is added along with a quenching agent (Promega) that specifically inhibits further Fluc luminescence and the luminescence arising solely from the Rluc is read for 10 seconds.

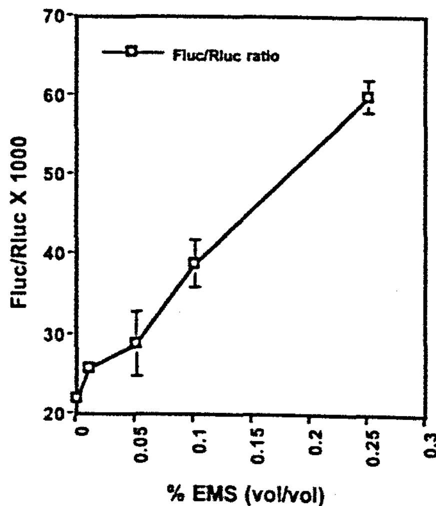

Figure 3. Dose response of the yeast biosensor strain to EMS. The yeast strain was grown in YPD medium containing increasing concentrations of EMS and assayed for firefly (Fluc) and Renilla *(Rluc) luciferase activity. The data shown represent the Fluc/Rluc ratios for each sample. Three independent measurements were done at each concentration of EMS.*

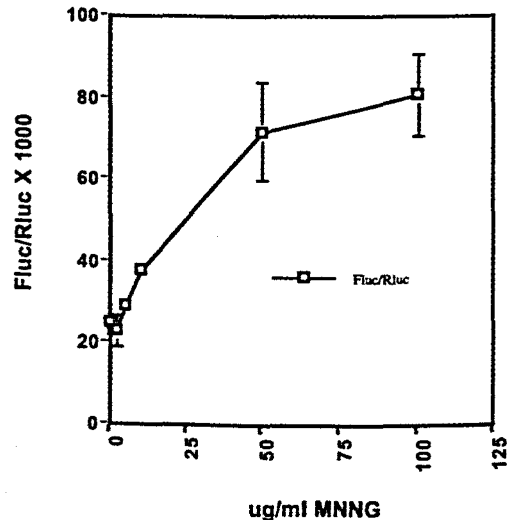

Figure 4. Dose response of the yeast biosensor to MNNG. The yeast strain was grown in YPD medium containing increasing concentrations of MNNG and assayed for firefly (Fluc) and Renilla *(Rluc) activity. The data shown represent the Fluc/Rluc ratios for each sample. Three independent measurements were done at each MNNG concentration.*

Faculty Comments:

Ms. Reed's faculty mentor, David McNabb, had glowing things to say about Ms. Reed's research. He made the following comments:

> I first met Robin when she joined my research lab in the fall of 2000. Robin quickly established herself as invaluable asset to the lab. Since I had just arrived at the University of Arkansas campus and was just setting up my research laboratory, there was an enormous amount of work just to get the lab functional. Robin was in the lab every day helping me unpack, set up equipment, and get organized. Once the lab was functional, she quickly moved on to her research project in which she has made great progress.
>
> Robin's research continued work that I initiated as a postdoctoral fellow on the development of the yeast dual-luciferase assay system as a new technology for monitoring changes in gene expression in the yeast Saccharomyces Cerevisiae. Robin performed many of the experiments that demonstrated the utility and accuracy of the assay system as well as established several of the parameters for its use. Robin also demonstrated the usefulness of the dual-luciferase reporter system in two-hybrid screening, a technique employed commonly for studying protein-protein interactions using yeast. This component of Robin's research is part of a manuscript that is currently being prepared for publication and Robin is one of the co-authors. Robin has since taken the dual luciferase assay system to the level of industrial application. She has demonstrated the utility of the yeast dual-luciferase assay as a potentially powerful screening technique for identifying genotoxic chemicals, the subject of this article. Such a screening technology would be applicable to pharmaceutical companies that are engaged in the development of new therapeutics through combinatorial chemistry. I consider myself lucky to have had Robin as my first undergraduate researcher in the laboratory, and wish that all students displayed her level of motivation for hard work and success.
>
> In addition to her research in my laboratory, Robin has spent the past summer at the University of Arkansas Medical School doing research on tuberculosis with Dr. Kathleen Eisenach. Robin seized the opportunity to work with Dr. Eisenach to broaden her scientific knowledge and technical expertise. During her undergraduate years at the U of A, Robin has also volunteered her time at the public health clinics in Fayetteville, as well as volunteered as a member of a medical mission to Nicaragua to provide free medical care to members of a small community. Thus, her enthusiasm for learning, helping others, and her dedication to hard work is truly inspiring.
>
> Robin's overall academic record at University of Arkansas has been consistently excellent. She has received several awards from the university including: University Scholar, the Chancellor's List, and Fulbright College Student Ambassador. Robin was also the recipient of a SILO/SURF award this year that partially funds her research efforts in my laboratory.
>
> On a personal level, Robin is a very friendly, mature, responsible, and caring individual. I am continually amazed by her academic abilities, work ethic, social responsibility and maturity. Robin will be attending medical school this coming Fall and I have no doubt that she will continue her excellent academic performance to become a well-rounded physician capable of interacting with patients and providing them with the medical information and care they need.

Ms. Reed's faculty advisor, Mack Ivey, had very complimentary things to say about her. He wrote:

> Robin is an exceptional student. I have known her for approximately 3 years. I serve as her academic advisor, and I have had her in several classes. She is currently enrolled in my Cell Physiology course, having taken my Microbial Genetics class last fall. Robin never misses class or review sessions, where her questions are always pertinent and thoughtful. Her performance has been spectacular. In Microbial Genetics, she placed in a tie for the top position in the class of 62 students. She has achieved this while maintaining an extremely active calendar outside of the classroom. She stays busy with her honors thesis research under the capable guidance of Dr. David McNabb. Her extracurricular interests are many, and include service as a Student Ambassador.
>
> Robin is a natural leader. She is highly respected by her peers, and by members of the faculty with whom she has made acquaintance. She is pleasant, outgoing, and friendly. She does not do anything half-heartedly. I am certain that her research will be productive, and that the work she presents will have been carried out carefully and with great diligence and determination.

Jeannine Durdik also had opportunities to observe Ms. Reed's work. She says:

> I had Robin in Immunology lecture and lab and in Mechanisms of Pathology classes. Robin distinguished herself in all three courses. Robin Reed ranks in the upper 1-5% of the pre-medical track seniors and the graduate students at this institution. Robin is the sort of student who will do additional work —in this case added computer problems in which the task was diagnosing virtual patients in the Mechanisms of Pathology course out of shear interest. She really enjoyed the problem solving and asked if something similar couldn't be added to the other course, Immunology. My point is that she likes to think. I assert that thinking is what being a scientist and a scholar is all about.