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A Novel Yeast-Based Reporter Assay System for the Sensitive Detection of Genotoxic Agents Mediated by a DNA Damage–Inducible LexA-GAL4 Protein

Kohei Ichikawa and Toshihiko Eki*
Division of Bioscience and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, Toyohashi, Aichi 441-8580

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Yeast-based genotoxicity testing systems can sensitively detect DNA damaging agents in the environment. We have developed a novel "indirect" reporter assay system based on a recombinant yeast containing both a sensor and a reporter plasmid. The sensor plasmid contains a gene encoding the artificial transcription factor of the *Escherichia coli* LexA DNA binding domain fused to the transcriptional activation domain of yeast Gal4p, which is regulated by the DNA damage–inducible *RNR2* promoter. The reporter plasmid contains the *E. coli lacZ* gene with the LexA binding site in the 5′-upstream region, allowing transcriptional activation by the induced LexA-GAL4 protein. The activity of DNA damage–dependent β-galactosidase (β-gal) in the "indirect" reporter assay system was compared with that of a current yeast-based "direct" reporter system. The "indirect" system exhibited 1.5- to 5-fold greater β-gal activity upon induction by alkylating agents or camptothecin. To increase the sensitivity of the new reporter system further, several deletion yeast strains were tested, and enhanced induction of reporter activity was observed in DNA repair-deficient *mag1*Δ cells. The "indirect" 96-well microtiter plate assay system is a potentially inexpensive and sensitive method for detecting genotoxic activities in a wide range of compounds, and in polluted environmental samples.

Key words: DNA damage, genotoxicity test, LexA-GAL4 fusion protein, sensitive reporter assay, yeast mutant.

Abbreviations: β-gal, β-galactosidase; DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; GFP, green fluorescent protein; MMS, methyl methanesulfonate; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; SD, synthetic dextrose minimal; SDMH, 1,2-dimethyl hydrazine dihydrochloride; YPD, yeast extract-peptone-dextrose.

Both natural and man-made genotoxic chemicals can damage DNA, resulting in cell death or cancers due to accumulated genetic mutations (*1*). Sensitive, simple methods are therefore required for screening and monitoring a wide range of environmental pollutants and synthetic compounds for potential genotoxic activity. The Ames test, based on the sensitivity of *Salmonella* strains to carcinogenic chemicals (*2*), has been used extensively, but compounds producing Ames-negative responses can, in fact, be carcinogenic to animals, and *vice versa*. Recently, improved genotoxicity tests have been established by harnessing cellular responses to DNA damage. In bacteria, the "SOS chromotest" (*3*) and "*umu*" test (*4*) use enzyme-linked reporter systems to monitor the induction of cellular transcription caused by DNA damage. In eukaryotes, several yeast-based genotoxicity tests have been developed using reporter assays linked to DNA damage-inducible promoters (*5*–*7*). Yeast-based systems have certain advantages compared to bacterial systems. Although, like bacteria, *Saccharomyces cerevisiae* is unicellular, easily manipulated and readily genetically modified, yeast are eukaryotic organisms and thus respond to various DNA-damaging agents in a manner more similar to mammalian cells than bacteria do. These systems contain an *E. coli lacZ* or green fluorescent protein (GFP) reporter gene linked to the DNA damage-inducible promoter of the *RAD54, RNR2*, or *RNR3* gene, and can detect a wider range of genotoxicants compared to bacterial genotoxicity tests (*5*–*7*). To be practical and widely applicable, a genotoxicity test must be sufficiently sensitive to both identify weakly-mutagenic chemicals and monitor low concentrations of genotoxic agents in environmental samples. To date, two major approaches to increasing the sensitivity of the reporter assay have been investigated. One approach has been to select gene promoters that are sensitively and specifically induced by DNA damage. Promoters of the double-stranded break-repair gene *RAD54*, and the ribonucleotide reductase subunit genes *RNR2* and *RNR3* (which respond to a wide range of DNA damage) are currently used in reporter systems (*6*, *7*). However, recent yeast DNA microarray data suggest that other promoter genes could be more specifically and easily induced (*8*, *9*). The second approach investigated to date has been to use DNA repair–defective yeast mutants as hosts. For example, the introduction of a *dam-3* mutation into an *E. coli* PQ37 strain enhances the sensitivity of the SOS chromotest (*10*). Similar attempts have been reported with the

*To whom correspondence should be addressed. Tel: +81-532-44-6907, Fax: +81-532-44-6929, E-mail: eki@eco.tut.ac.jp

RNR3-lacZ system in which enhanced activity of the *lacZ* gene product, β-galactosidase (β-gal), was observed in deletion mutants with several inactivated DNA repair pathways (*11*).

The present paper describes a novel 96-well assay system based on a recombinant yeast containing both a sensor and a reporter plasmid. Increased induction of β-gal activity by genotoxic agents was observed, and evidence is provided that this reporter system can monitor genotoxic agents with greater sensitivity than current reporter systems. In addition, three mutants defective in DNA repair and cell wall integrity have been tested as hosts in order to increase further the sensitivity of the yeast-based genotoxicity assay system.

MATERIALS AND METHODS

Chemicals—Methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and 1,2-dimethyl hydrazine dihydrochloride (SDMH) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Hydrogen peroxide (H_2O_2, 30% solution) was purchased from Santoku Chemical Industries Co. Ltd. (Tokyo, Japan), camptothecin from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), zinc sulfate ($ZnSO_4 \cdot 7H_2O$) from Nacalai Tesque (Kyoto, Japan), and actinomycin D from SERVA Feinbiochemica (Heidelberg, Germany). A stock solution of camptothecin (1 mg/ml) was prepared in dimethylsulfoxide (DMSO) and serially diluted with distilled water immediately prior to use.

Yeast Strains—Yeast strain DF5 (*MAT***a**, *his3-*Δ*200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52*) (kindly provided by Dr. Helle Ulrich, Max Plank Institute for Terrestrial Microbiology, Marburg, Germany) was used as the host for the reporter assay. Experiments using gene disruptants utilized wild-type BY4741 (*MAT***a**, *his3-*Δ*1, leu2-*Δ*0, met15-*Δ*0, ura3-*Δ*0*), and *mag1*Δ, *slg1*Δ, and *erg6*Δ BY4741 strains in which the pertinent gene was replaced with the *kanMX* gene (*12*). These strains were purchased from Invitrogen Corp. (Carlsbad, CA). Yeast cells were grown at 30°C in YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose (*13*). The gene-disrupted BY4741 strains were maintained in YPD medium containing 200 μg/ml genecitin (Invitrogen Corp.). Yeast cells were transformed with plasmid DNA using a modified lithium acetate protocol (*14*). Transformants were selected on synthetic dextrose minimal (SD) medium (*13*), and independent colonies were streaked on fresh selection agar plates before use.

Construction of Plasmids—Reporter plasmids for the "direct" reporter system were prepared using *E. coli*-yeast shuttle vector YEp365 (*15*) containing a 2 μm origin, *E. coli lacZ* and yeast *LEU2* genes (kindly donated by Dr. Hiroshi Uemura, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). A 1,613-bp upstream sequence of the *RNR2* gene, including 46-bp of the open reading frame, was amplified from *Saccharomyces cerevisiae* genomic DNA (S288C strain) by PCR using KOD polymerase (Toyobo, Osaka, Japan) and the following primer set: RNR2PSmaF1 (5′-TTCCCGGGACCGTACCTTCCAGCATTGTCC-3′) and RNR2PSalR1 (5′-TTGTCGACGGACAATGCATCGGCAGCAGCT-3′). A 724-bp fragment of the *RNR3* gene with 46-bp of the open reading frame was also amplified using the primer set: RNR3PSmaF1 (5′-TTCCCGGGTGTTGTCGTCGCTGGAGGCG-3′) and RNR3PSalR1 (5′-TTGTCGACGAATTGAACGGGCTCTTTGCGG-3′). The amplified DNAs were successively digested with *Sma*I (Takara, Kyoto, Japan) and *Sal*I (Toyobo, Osaka, Japan), and purified by gel electrophoresis. The DNAs were cloned into the S*ma*I–*Sal*I–digested YEp365 DNA, resulting in the reporter plasmids, YEp365-^{p}*RNR2-lacZ* and YEp365-^{p}*RNR3-lacZ*. The YEp365-^{p}*RNR3-lacZ* was used as a positive control for the current "direct" reporter system (*7*). In the "indirect" reporter system, two kinds of plasmids, a sensor plasmid and a reporter plasmid, were prepared. The sensor plasmid contained the *lexA-GAL4* gene encoding the *E. coli* LexA DNA binding domain fused to the transcriptional activation domain from yeast Gal4p. Transcription is driven by the *RNR2* promoter, and the LexA-GAL4 protein activates the reporter gene on the second plasmid. The sensor plasmid was constructed using the shuttle vector pGMT20 (*16*) containing a 2 μm origin, *GAL1* promoter, and an auxotrophic marker *TRP1* gene (provided by the DNA Bank, RIKEN BioResource Center, Ibaraki, Japan). Briefly, pGMT20 DNA was digested with *Sph*I and *Bam*HI to remove a *GAL1* promoter fragment, and then self-ligated after blunting the DNA ends using a TaKaRa BKL kit. The *lexA-GAL4* fusion gene fragment (2.8 kb) was amplified from the template plasmid pLexA-Pos (identical to pSH17-4 [*17*]) with the *lexA-GAL4* and *HIS3* genes (Clontech, Palo Alto, CA) by PCR using the Expand High Fidelity PCR system (Roche Diagnostics, Basel, Switzerland) and a primer set (lexA-GAL4-SmaF: 5′-GGGATGAAAGCGTTAACGGCCAGGCAACA-3′, and lexA-GAL4-R: 5′-GGCCAAGATTGAAACTTAGAGGAGTATAG-3′). The DNA was successively treated with the TaKaRa BKL kit and cloned into the *Sma*I site of the resultant plasmid to make the promoterless *lexA-GAL4* plasmid pGMT20-*lexA-GAL4*. The *RNR2* promoter fragment was amplified from yeast genomic DNA using the Expand High Fidelity PCR system and a primer set (RNR2PSmaF1 and RNR2blunt-R: 5′-GGTAATTGGACAAATAAATACG-3′), and then cloned into the *Sma*I-digested pGMT20-*lexA-GAL4* DNA to generate the sensor plasmid pGMT20-^{p}*RNR2-lexA-GAL4*. The reporter plasmid p8op-lacZ (identical to pSH18-34 [*18*]) was obtained from Clontech and contains a *lacZ* gene that is regulated by eight copies of the *lexA* operator sequence at the 5′ upstream region and the *HIS3* gene. DNA primers were purchased from Sigma Genosys (St. Louis, MO) and Proligo LLC (Boulder, CO). Nucleotide sequences were determined using a dye-terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and an automated DNA sequencer (Applied Biosystems model 377XL, Foster City, CA); data were assembled and analyzed using AutoAssembler (version 1.4.0, Applied Biosystems) and Genetyx MAC (version 9, Software Development, Co., Tokyo), respectively.

β-*Galactosidase Assay*—β-Gal activity was measured using a 96-well microtiter plate assay (*19*, *20*) modified as follows. An overnight culture of yeast was diluted to an optical density of 0.15 at 600 nm (OD_{600}) with YPD medium, and then further diluted 5-fold with selective medium. After shaking at 30°C for 2 h, 90 μl of cell suspension was mixed with 10 μl of 10-fold concentrated stock solutions of test chemicals in a 96-well flat-bottom

microtiter plate (No. 3585, Corning Inc., Corning, NY) using a 12-channel pipettor. Cells in the microtiter plate were incubated for 6 h with the chemicals under vigorous shaking (220 rpm), then the OD_{590} of each well was determined using a microtiter plate reader (ImmunoMini NJ-2300, Nalge Nunc International, Rochester, NY). Cell culture conditions affected the expression levels of genes involved in DNA repair and in stress responses (*21*). For example, MMS-inducible genes, including DNA repair genes, were highly expressed in stationary phase culture (*22*). Therefore, these culture conditions (*i.e.*, dilution of the inoculant, incubation time for β-gal induction) were optimized using the direct reporter assay system prior to conducting the genotoxicity experiments. Induction at OD_{600} = 0.15 was higher than that obtained from inoculants at OD_{600} of 0.3 or 0.6. In addition, the highest induction was observed after 6 h treatment (time course data not shown).

The induced culture was directly subjected to the 96-well format β-gal assay. 20 μl of cell suspension was transferred into a new flat-bottom 96-well plate and stirred with a mixture of 35 μl of Y-PER® reagent (Pierce, Rockford, IL) and 85 μl of 1.1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma-Aldrich Inc.) solution in 1.65× Z buffer (36.6 mg/ml $Na_2HPO_4{\cdot}12H_2O$, 9.74 mg/ml $NaH_2PO_4{\cdot}2H_2O$, 1.24 mg/ml KCl, 0.406 mg/ml $MgSO_4{\cdot}7H_2O$, 0.27% β-mercaptoethanol). After 10 min incubation at 25°C, 56 μl of 1 M $Na_2CO_3$was added to stop the reaction, and the absorbance of each well at 405 nm (A_{405}) and 590 nm (A_{590}) was determined using the microtiter plate reader. Underestimation of β-gal activity caused by cell debris light scattering and absorption was corrected as follows: β-gal activity = $(A_{405} - 1.18 \cdot A_{590})/OD_{590}$.

For each data point, the mean value of at least three independent reactions was calculated. Fold induction for each experiment was calculated as the ratio of β-gal activity in the presence and absence of each chemical.

RESULTS

Development of the "Indirect" Reporter Assay System—Several yeast-based reporter systems for detecting genotoxicity have previously been developed using a reporter plasmid regulated by a DNA damage-inducible promoter (*5*–*7*). In the present study, we designed a novel yeast-based reporter system consisting of two plasmids: a sensor plasmid and a reporter plasmid. In previously-described reporter systems, a reporter gene is "directly" regulated by the promoter in the same plasmid. In contrast, a reporter gene in our system is "indirectly" regulated by an artificial transcription factor whose expression is controlled by the promoter. Therefore, in this paper, we call previously-described yeast-based systems "direct" reporter assay systems, and our system an "indirect" reporter assay system. In our system, the sensor plasmid encodes the artificial LexA-GAL4 fusion protein driven by a DNA damage-inducible promoter. When DNA damage occurs, the induced and accumulated LexA-GAL4 protein binds to the operator sequences at the 5′ region upstream of the *lacZ* gene on the reporter plasmid p8op-lacZ, and activates the gene. A similar vector system has previously been used in a LexA-mediated yeast two-hybrid system in order to enhance reporter activity mediated by protein-protein interactions (*17*, *18*). In this two-hybrid system, p8op-lacZ is the most sensitive reporter plasmid constructed. This plasmid contains eight *lexA* operator sequences to which the bait protein (fused with the LexA DNA binding domain) can bind, and exhibits up to 100-fold higher activities than that by other plasmid with one or two copies of *lexA* operators (*18*). Thus, our "indirect" reporter system was assumed to express enhanced reporter activities by using the p8op-lacZ reporter plasmid and its activator LexA-GAL4 protein.

For our DNA damage-inducible promoter, we used the regulatory sequence of the *RNR2* gene, since *RNR2* transcripts can be strongly activated in a DNA damage-specific manner by various genotoxicants (*8*, *23*, *24*). We have constructed a "direct" reporter system compatible for use in 96-well microtiter plates using an *RNR2* promoter containing modifications to the *RNR3-lacZ* system, previously reported by Jia *et al*. (*7*). To this end, the reporter activities of two DNA damage-inducible promoters (*i.e.*, *RNR2* and *RNR3*) were investigated. The yeast strains DF5[YEp365-P*RNR2-lacZ*] and DF5[YEp365-P*RNR3-lacZ*] containing the *RNR2-lacZ* and *RNR3-lacZ* reporter plasmid, were prepared and their reporter activities in response to several cytotoxic and genotoxic chemicals were examined (Fig. 1). The chemicals included DNA alkylating agents (MMS, SDMH), an oxidizing agent (H_2O_2), a topoisomerase I inhibitor (camptothecin), a transcriptional inhibitor (actinomycin D), and a heavy metal (zinc). The mechanistic activities of these agents are well documented (*1*). Enhanced induction by MMS in our *RNR3-lacZ* system was detected as previously reported (*7*): for example, induction in the *RNR3-lacZ* system at 0.01% MMS is 1.7-fold higher than that in the *RNR2-lacZ* system (Fig. 1A). However, β-gal induction by SDMH or $H_2O_2$was similar in both systems (Fig. 1, B and C), and camptothecin weakly induced β-gal activity in the *RNR2-lacZ* system but not in our *RNR3-lacZ* system (Fig. 1D). These data suggest that both "direct" reporter systems with the *RNR2* and *RNR3* promoter exhibit essentially similar properties.

The yeast strain DF5[YEp365-P*RNR2-lacZ*] with the *RNR2-lacZ* reporter plasmid was used as the "direct" reporter system. As the "indirect" reporter system, the sensor plasmid pGMT20-P*RNR2-lexA-GAL4* was constructed and transformed into DF5 cells together with the reporter plasmid p8op-lacZ, generating strain DF5[pGMT20-P*RNR2-lexA-GAL4*, p8op-lacZ]. β-gal activities induced by various concentrations of the alkylating agent MMS were examined using DF5[YEp365-P*RNR2-lacZ*] and DF5[pGMT20-P*RNR2-lexA-GAL4*, p8op-lacZ] (Fig. 2). Although the induction of β-gal activity in each strain was dependent on the concentration of MMS and peaked at 0.02% MMS, the level of induction in DF5[pGMT20-P*RNR2-lexA-GAL4*, p8op-lacZ] is approximately 1.5-fold higher than in DF5-[YEp365-P*RNR2-lacZ*]. At lower concentrations of MMS, β-gal induction in the two strains differs significantly: for example, induction in the "direct" and "indirect" system at 0.005% MMS is 4.6-fold and 16.8-fold, respectively. At higher concentrations, β-gal activity generally decreases in both systems. Similar phenomena have been consistently observed in other "direct" reporter systems, probably due to increased cytotoxicity (*6*, *7*).

Induction by Various Genotoxic Chemicals of the "Direct" and "Indirect" Systems—Several cytotoxic and genotoxic

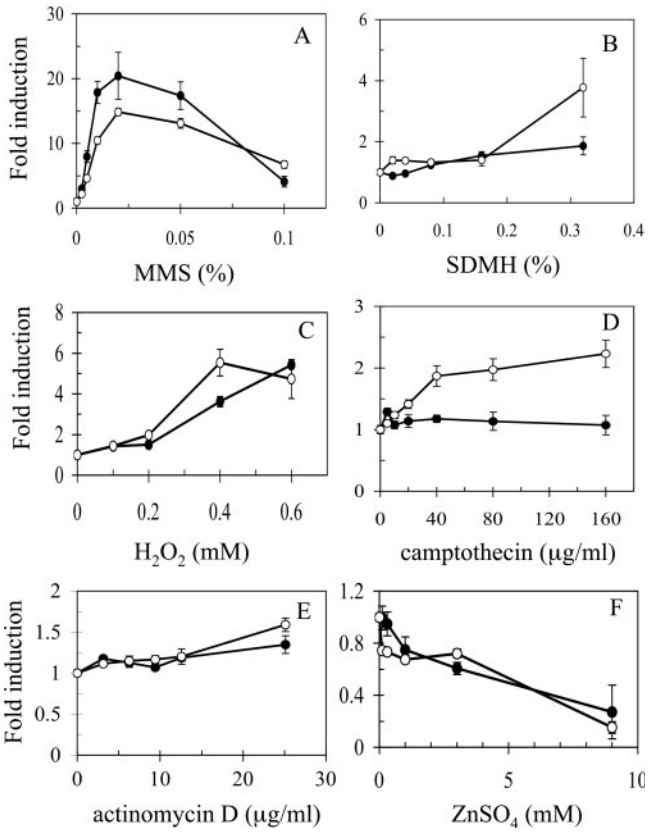

Fig. 1. **Induction of β-gal activity by various toxic chemicals in the "direct" reporter assay system.** (A) MMS; (B) SDMH; (C) H_2O_2; (D) camptothecin; (E) actinomycin D; and (F) $ZnSO_4$. The fold induction was calculated as the ratio of β-gal activity in treated cells to that in untreated cells. Data points with standard deviations are the averages of at least three independent reactions in yeast DF5 cells with YEp365-^{p}*RNR2-lacZ* (open circles) and with YEp365-^{p}*RNR3-lacZ* (solid circles).

chemicals including EMS, SDMH, H_2O_2, camptothecin, actinomycin D, and zinc were tested in both reporter assay systems. The higher sensitivity of the "indirect" reporter system was clearly observed with both EMS and camptothecin (Fig. 3, A and D), and was comparable to that observed with MMS (Fig. 2). The highest induction observed in the "indirect" *vs.* "direct" reporter systems was 4.3-fold at 0.25% EMS, and 4.9-fold at 40 μg/ml camptothecin. As demonstrated by the marked enhancement of induction at low concentrations of these chemicals, the "indirect" reporter system described here could detect these genotoxic agents more sensitively than current "direct" reporter systems. Strong induction by H_2O_2 (Fig. 3C), and weak or no induction by SDMH (Fig. 3B) and actinomycin D (Fig. E) were observed in both the "direct" and "indirect" systems. Zinc inhibited β-gal activity in the *RNR2-lacZ* reporter system but not in the "indirect" system (Fig. 3F). Although the mode of action of zinc is unknown, similar inhibition by zinc has been observed in our *RNR3-lacZ* system (Fig. 1F). Table 1 summarizes and compares our experimental results to genotoxicity database results obtained using the Ames test and SOS chromotest (see legend, Table 1). The sensitivities of the yeast-based systems are comparable to or higher than those published for the Ames test and SOS chromotest. For

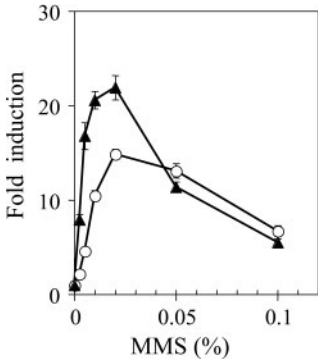

Fig. 2. **Enhanced induction of β-gal activity by MMS in the "indirect" reporter assay system.** The fold induction was calculated as the ratio of β-gal activity in treated cells with MMS to that in untreated cells. Data points with standard deviations are the averages of at least three independent reactions in yeast DF5 cells with YEp365-^{p}*RNR2-lacZ* (open circles) and with pGMT20-^{p}*RNR2-lexA-GAL4* and p8op-lacZ (solid triangles).

example, threshold doses at which β-gal activity increased 2-fold are approximately 0.10 mM MMS, 10 mM EMS and 1 mM H_2O_2 in both the Ames test and SOS chromotest, whereas the corresponding doses for the "direct" and "indirect" reporter systems are 0.20 and 0.05 (MMS), 1.45 and 0.48 (EMS), and 0.20 and 0.18 mM (H_2O_2).

Different Responses of the Reporter Systems in Knockout Strains—It has previously been reported (*11*) that the induction of reporter activity in some DNA repair-deficient mutants can be greatly enhanced compared to wild-type cells. Specifically, the *mag1*Δ mutant, defective in base excision repair, was shown to enhance β-gal induction by alkylating agents (*11*). In the present study, the effects of *MAG1, ERG6*, and *SLG1* deletions on β-gal induction by various toxic chemicals were studied using our *RNR2-lacZ* reporter system, and the sensitivities of our new reporter systems towards genotoxicants were assessed. Since Erg6p is involved in the biosynthesis of ergosterol (*25*), and Slg1p is a sensor protein for cell wall integrity (*26*, *27*), the deletion of these genes could enhance the induction of β-gal activity by causing cell wall defects and thus increasing cellular permeability. This hypothesis was tested using BY4741 *erg6*Δ and *slg1*Δ strains; BY4741 was chosen because it is a host for the *Saccharomyces* Genome Deletion Project (*12*). Levels of β-gal activity induced by MMS (Fig. 4A), EMS (Fig. 4B), SDMH (Fig. 4C), H_2O_2(Fig. 4D), camptothecin (Fig. 4E), and actinomycin D (Fig. 4F) were determined in the wild-type, *erg6*Δ, *slg1*Δ, and *mag1*Δ BY4741 strains containing the YEp365-^{p}*RNR2-lacZ* plasmid. In *mag1*Δ cells, induction of reporter activity was markedly enhanced in the presence of the alkylating agents MMS and EMS, whereas these agents had little effect on either the wild-type or the other two deletion strains (Fig. 4, A and B). Significantly, β-gal activity was particularly enhanced by lower concentrations of these chemicals, indicating increased sensitivity of the *mag1*Δ cells. For example, 0.01% MMS or 0.5% EMS maximally induced β-gal activity in *mag1*Δ cells, compared to 0.02 % MMS or 1.0% EMS for wild-type cells. Enhanced induction was observed in *mag1*Δ and *erg6*Δ cells treated with 0.35% SDMH (Fig. 4C), and in *mag1*Δ cells treated with camptothecin

(Fig. 4E). Interestingly, the induction of *mag1*Δ cells by hydrogen peroxide resulted in a pattern clearly different from that observed with the other strains (Fig. 4D). In *slg1*Δ cells, high doses of H_2O_2 slightly enhanced induction; both *erg6*Δ and *slg1*Δ cells showed little induction of β-gal activity by any of the other agents tested.

DISCUSSION

We report here a novel "indirect" reporter assay system using recombinant yeast containing both a sensor and a reporter plasmid, and show that these engineered strains exhibit increased sensitivity to certain toxic chemicals. As

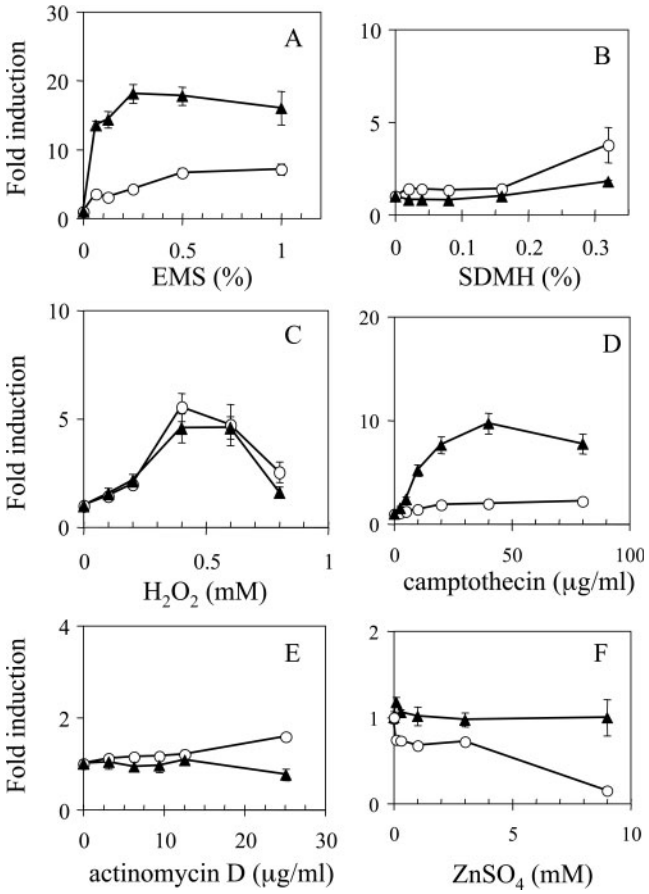

Fig. 3. **Induction of β-gal activity by various toxic chemicals.** (A) EMS; (B) SDMH; (C) H_2O_2; (D) camptothecin; (E) actinomycin D; and (F) $ZnSO_4$. Data points with standard deviations are the averages of at least three independent reactions in yeast DF5 cells with YEp365-^{p}*RNR2-lacZ* (open circles) and with pGMT20-^{p}*RNR2-lexA-GAL4* and p8op-lacZ (solid triangles).

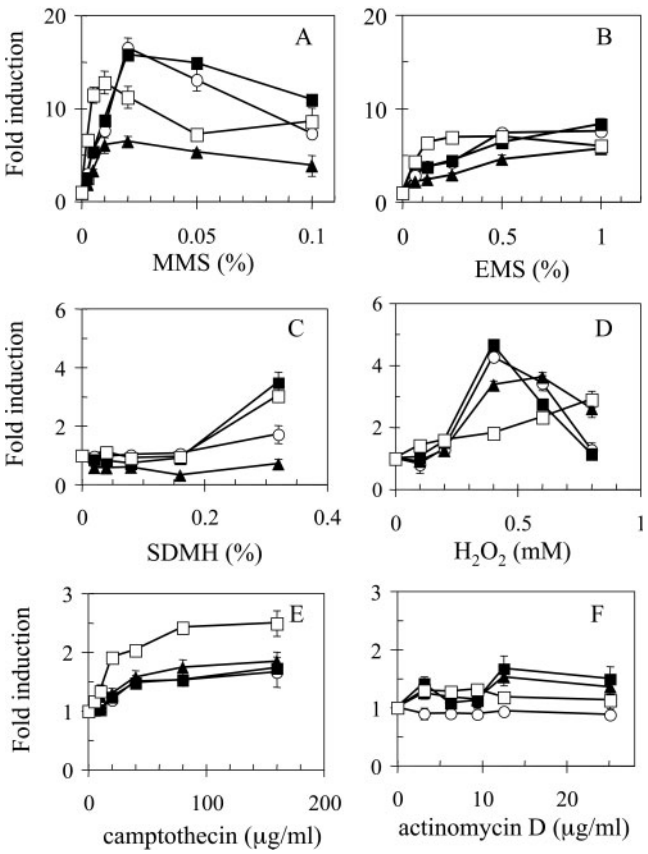

Fig. 4. **Induction of β-gal by various toxicants in mutants defective in cell wall integrity and DNA repair.** Data points with standard deviations are the averages of at least three independent reactions in yeast cells with YEp365-^{p}*RNR2-lacZ*. Yeast strains used: BY4741 (wild-type) (open circles); BY4741 *erg6*Δ (solid squares); BY4741 *slg1*Δ (solid triangles); and BY4741 *mag1*Δ (open squares).

Table 1. **Summary of the reporter assays and comparison with other genotoxicity test results.**

Agents	Maximum fold induction in reporter assay[a] (optimal dose[b])		Ames test[c]	SOS chromotest[c]
	Indirect method	Direct method		
MMS	21.9 (0.02%)	6.0 (0.02%)	+	+
EMS	18.1 (0.25%)	2.9 (1.0%)	+	+
SDMH	1.5 (0.32%)	1.8 (0.32%)	+[d]	–
H_2O_2	5.5 (0.4 mM)	4.6 (0.4–0.6 mM)	–	+
camptothecin	9.7 (40 μg/ml)	2.2 (80 μg/ml)	Not found[e]	Not found
actinomycin D	1.2 (25.1 μg/ml)	1.1 (12.6 μg/ml)	–	–
$ZnSO_4$	<0.7 (0.1 mM)	1.2 (0.1 mM)	Not found	–[f]

[a]Maximum fold of induction observed in the experiment. [b]Concentration inducing maximal β-gal activity, or the highest concentration tested, within experimental limits. [c]Results for the Ames test and SOS chromotest were obtained from the Genotoxity Database: www.pasteur.fr/recherche/unites/pmtg/toxic/index.html (*36*). [d]Positive results were obtained only in alkyltransferase deficient strains (*37*). [e]Data unavailable from references and databases. [f]Results were obtained with zinc acetate (*38*).

we intended, β-gal activity in the "indirect" reporter system using the *RNR2* promoter was induced to significantly higher levels by incubation with either DNA alkylating agents (MMS and EMS) or a topoisomerase I inhibitor (camptothecin) that causes strand breaks, compared to a reporter system containing a single plasmid (Figs. 2 and 3). Compared to the "direct" system, induction was increased 1.5-to 5-fold in the "indirect" system by these agents. Previous experiments by Jia *et al*. (*7*) showed that the copy number of the reporter plasmid (*i.e.*, multiple copies versus a single copy) in a cell did not affect the level of β-gal induction. Since both "direct" and "indirect" reporter systems contain multiple copies of the plasmids, enhancement of β-gal activity in the "indirect" reporter system is likely due to the introduction of the reporter plasmid p8op-lacZ regulated by the artificial transcriptional activator LexA-GAL4 protein. Estojak *et al*. (*18*) showed that a *lacZ* reporter plasmid containing increased numbers of *lexA* operators exhibits significantly strong β-gal activity in a LexA-mediated yeast two-hybrid system, and p8op-lacZ with eight LexA binding sites was the most sensitive reporter plasmid in the system (*18*). Thus, the enhanced induction of β-gal activity in our "indirect" reporter system is likely due to significantly increased transcription of the p8op-lacZ reporter gene via DNA damage–induced LexA-GAL4 protein.

Compared to MMS, EMS and camptothecin, significantly less β-gal enhancement was observed with SDMH, hydrogen peroxide, and actinomycin D (Fig. 3, B, C, and E). Although the reasons for this are unclear, genetic variations in drug metabolism and signal transduction of DNA damage in the strain used might cause different transcriptional responses.

In order to increase the sensitivity of the genotoxic agent assay system further, deletion mutants defective in DNA repair or cell wall integrity were investigated. Since mutants defective in each repair pathway exhibit relatively specific sensitivities to DNA damaging agents, a panel of yeast DNA repair-deficient mutants has been used to screen anticancer drugs (*28*, *29*). A similar attempt using yeast DNA repair mutants has been applied in the *RNR3-lacZ* reporter system (*11*). In our *RNR2-lacZ* reporter system, only *mag1*Δ cells exhibited enhanced induction of β-gal activity when treated with alkylating agents (Fig. 4A and B). The observed enhancement is presumably due to the accumulation of damaged DNA caused by defects in base excision repair mediated by Mag1 DNA glycosylase. These results are consistent with previous findings (*11*) that MMS and EMS induce the *RNR3-lacZ* reporter system. In addition, since increased cell-wall permeability makes yeast more sensitive to chemicals (*30*, *31*), we predicted that cell-wall defective mutants would exhibit enhanced reporter activities towards various chemicals. Erg6p is involved in membrane biogenesis, and Slg1p in cell wall integrity: deletion of *ERG6* increases the sensitivity of yeast to many small lipophilic drugs (*32*) and deletion of *SLG1* increases sensitivity to bleomycin (*27*). We, therefore, tested the effect of deleting both these genes in our reporter assay. In contrast with *mag1*Δ cells, deletion of these two genes only marginally affected β-gal induction when the cells were exposed to genotoxicants (Fig. 4); it is possible that the observed limited effect arises from the existence of redundant genes or pathways. The present results, however, do not exclude the possibility that deleting genes involved in cell wall integrity or stress response can enhance the cellular response to genotoxic chemicals. It would be interesting to test strains further with genes whose mutation is known to cause altered sensitivity to oxidative stress (*33*) or to cell-wall damaging agents (*34*).

To date, yeast-based reporter assay systems for genotoxicity testing have utilized the *RAD54*(*5*), *RNR2*(*6*), and *RNR3*(*7*) promoters. The *RAD54-GFP* system developed by Walmsley *et al*. (*5*) has been improved, converted into a 96-well format assay, and is commercially available as the GreenScreen® assay (*35*). This system directly monitors fluorescence caused by chemical induction of the reporter gene, and an alternative version using the *RNR2* promoter exhibits increased sensitivity (*6*). However, both systems require overnight incubation of the culture with the test chemicals. A genotoxicity reporter assay based on the *RNR3* promoter has been described. Although this "direct" reporter system has not been applied to a 96-well format assay, it is more sensitive than other reporters currently used (*7*). Jia *et al*.(*7*) reported that their *RNR3-lacZ* reporter system showed approximately 3-fold higher induction by MMS than that in the *RNR2-lacZ* system. Our *RNR3-lacZ* reporter system also exhibited approximately 1.7-fold higher β-gal activity by MMS than that in our *RNR2-lacZ* system (Fig. 1A). However, induction by SDMH or hydrogen peroxide was similar in both our "direct" reporter systems (Fig. 1, B and C), and our *RNR3-lacZ* system did not induce β-gal activity by campthothecin (Fig. 1D). Although it is not clear why the induction of β-gal activity in our *RNR3-lacZ* system was lower than that reported by Jia *et al*.(*7*), the difference in either the yeast strain used or the cloned promoter region (*i.e.*, 0.88-kb *vs.* 0.72-kb) might cause the variable response.

Compared with our "direct" and "indirect" reporter systems, the "indirect" reporter system exhibits significantly higher induction of β-gal activity than does the "direct" system (Figs. 2 and 3). Regarding the future of the "indirect" reporter system, introduction of other DNA-damage responsible gene promoters and mutants such as *mag1*Δ into the "indirect" reporter assay system may improve both sensitivity and specificity to various genotoxic chemicals. In addition to increased sensitivity, the "indirect" reporter system is compatible with 96-well microtiter plates, allowing high-throughput assays. Furthermore, the "indirect" reporter gene can be easily changed from the current *lacZ* gene to, for example, the GFP gene, by replacing the reporter plasmid. Once a set of sensor plasmids with various promoters are made, they can be used in multiple assay formats by combining them with the appropriate reporter plasmid regulated by *lexA* operator. Finally, by virtue of its high sensitivity and high throughput potential, the "indirect" reporter assay system described here could conveniently, sensitively and economically monitor low concentrations of genotoxic agents in polluted environmental samples, detect weakly-carcinogenic chemicals, or screen for potential anti-tumor compounds in the biome.

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