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Application of yeast cells transformed with GFP expression constructs containing the *RAD54* or *RNR2* promoter as a test for the genotoxic potential of chemical substances

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Abstract

Yeast strains transformed with high copy number plasmids carrying the gene encoding a green fluorescent protein optimised for yeast (yEGFP3) under the control of the *RAD54* or *RNR2* promoter were used to investigate the activity of potentially DNA-damaging substances. The assays were performed on 96-well microtitre plates in the presence of different concentrations of the test substances. The synthesis of GFP protein was measured through the fluorescence signal and cell growth was monitored by absorption. Here, we demonstrate that this system can be used as a biosensor to assess the genotoxic potential of drugs and other chemical substances. The use of microtitre plates will enable full automation of the system and allows the inclusion of internal reference standards in each assay. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: GFP reporter; Genotoxicity; MMS; DNA damage; Chemical substances; Yeast

1. Introduction

The Science Research Development Programme on Environment and Climate of the European Community (1994–1998) emphasised the need for the development of novel biosensors capable of measuring new parameters of relevance to the environment. It also stressed the importance of improving assessment methods. In this regard, the development of a genotoxicity test which can detect critical levels of different DNA-damaging substances in environmental samples, e.g., water, is of great importance. At present, genotoxicity is usually assessed using the Ames Test [1,2]. In the Ames Test, specifically developed strains of the bacterium *Salmonella typhimurium* are exposed to chemicals and then plated out on selective growth media. Colony counts of the different strains growing on the selective media are used as a measure for the frequency of different

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types of genetic damage. The Ames Test does not lend itself to automation as a continuous monitoring system. Moreover, the positive results are interpreted as an indicator of potential carcinogenic activity which could be misleading since bacteria (prokaryotes) are sufficiently different from humans (eukaryotes). To which extent biosensor data are a reliable source for risk assessment is an ongoing debate [3,4]. These differences are associated with the efficiency of repair and with variations in metabolism of compounds in different cell types [5,6]. The most recent advance in genotoxicity measurement has been the 'SOS' chromotest [7]. This is also a bacterial assay, but it gives attention to the SOS-repair response using an enzyme linked reporter system. It has been used in a semiautomatic microtitre plate batch assay [8,9].

Although lacking many metabolic pathways found in animals and humans, basic repair mechanisms as a response to genetic damage are more similar between yeast and mammals [10,11]. There are many different yeast genes whose transcription is induced upon exposure to DNA-damaging agents [12,13]. The promoters of two such damage-inducible genes, *RAD54* and *RNR2*, were used in the construction of ''reporter plasmids'', such that they control the expression of the green fluorescent protein (GFP) gene. Thus, cells containing the ''reporter plasmid'', when exposed to DNA-damaging chemicals, induce GFP expression and consequently emit green fluorescent light [14,15]. This system was further optimised by substituting the original wild type GFP gene, with the yEGFP mutant derivative, which is both brighter and faster forming [16]. *RAD54* is a DNA-damage-induced gene in *Saccharomyces cerevisiae* [17,18]. The *RAD54*-yEGFP reporter's activity correlates with increasing concentrations of the alkylating agent methyl methane sulphonate (MMS) [16]. The reporter does not respond to chemicals that delay mitosis, e.g., nocodazol, and responds appropriately to the genetic regulation of DNA repair. As an extension of this system, the *RAD54* promoter was replaced with the promoter of the *RNR2* gene, which is also up-regulated in response to DNA-damage [19–21]. In the present report, we used these two reporter systems to assess their sensitivity towards a range of substances to get a broader view of the general applicability of the system. Thus, a basis for further development of a highly sensitive assay that can be used in toxicology and environmental monitoring is provided.

2. Materials and methods

2.1. Strains and growth conditions

The yeast strains used in this study are listed in Table 1. Yeast cells (FF 18984 with and without pWDH444 or pUMGP5 plasmids) were grown on F1 medium: per l of final solution: 2 g $(NH_4)_2SO_4$; 3 g KH_2PO_4; 0.55 g $MgSO_47H_2O$; 0.06 g $CaCl_2$; 0.1 g NaCl; 1 mg H_3BO_3; 1 mg $CaSO_4$ $5H_2O$; 1 mg KI; 5 mg $FeCl_3$ $6H_2O$; 7 mg $ZnSO_4$ $7H_2O$; 20 mg leucine; 20 mg histidine; 30 mg lysine; 62 μg inositol; 14 μg thiamine HCl; 4 μg pyridoxine; 4 μg pantothanate; 0.3 μg biotin; 20 g glucose [22]. All components of the medium had the quality 'cell culture tested' or 'insect cell culture tested' and were received from Sigma.

2.2. Fluorescence assays

In all tests we used two types of 96-well microtitre plates. For the measurement of fluorescence: black plates (Nunc 'immuno-module F16'; Cat.#: 475515); for the measurement of cell density by absorption: transparent plates (Greiner; Cat.#: 655 101). In some experiments we used plates with black walls and transparent bottoms (Dunn; Cat.#: 215003) to measure fluorescence and absorption in the same plate. MMS, camptothecin, aphidicolin, *cis*-platinum(II)diamine-dichloride, *N*-nitroso-*N*-methylurea and ethidium bromide were obtained from Sigma

Table 1
Yeast strain used

Strain	Plasmid	Relevant genotype
Host strain		
FF 18984		*MATa leu2-3,112 ura3-52, lys2-1, his7-1*
Transformed reporter strains		
FF18984	pWDH444	as FF 18984 and *RAD54-GFP, KanMX3, URA3*
FF18984	PUMGP5	as FF 18984 and *RNR2-GFP, KanMX3, URA3*

(Cat.#: M-4016, C-9911, A-0781, P-4394, N-1517, E-8751), Psoralen from Molecular Probes (Cat.# P-96045), Mitomycin C, Chlorambucil, Streptonigrin, Phleomycin, Actinomycin D and Chromomycin A_3 from Fluka (Cat.#: 69824, 23125, 85885, 79305, 01817, 27100). All other substances were obtained from the natural product chemistry department of the Hans Knöll Institute (Dr. Dornberger, HKI).

Single colonies of yeast strains, grown on selective medium, were used to inoculate F1 medium (5 ml) and grown to mid log phase (0.3–0.4 AU at 600 nm). Cells were collected by centrifugation and resuspended in fresh medium to a final concentration of 0.1 AU. Solutions of MMS or test substances (20 μl per well) were distributed in parallel in two plates (black and transparent) to measure fluorescence and growth. We used eight wells (one-microtitre plate column) for each concentration of MMS or test substance. In the first column we added only 20 μl of water or diluent as zero concentration reference. In each well, 80 μl of cell suspension were added and fluorescence (on black plate; ext. = 485/10 nm and em. = 520/10 nm) and absorption (on transparent plate 670 nm) values were measured (biolumin™ 960, Molecular Dynamics). This measurement was taken as time 0. For incubation, plates were sealed with adhesive film (Nunc, Cat.#: 236366), and incubated at 30°C with shaking. The measurements were repeated at the time points indicated. When black plates with transparent bottom were used all measurements were made on a single plate.

2.3. Calculation and graphical representation

For each column of eight wells, that is for each set of replicate concentrations, the mean value was calculated and the fluorescence values were normalised by the absorption values for each well. The normalised fluorescence values were plotted against the substance concentration and incubation time. Error bars show the standard deviation of the normalised fluorescence signals (fluorescence/absorption) for each column (eight wells). All calculations and plots were made with KaleidaGraph™ software and MS Excel™ 4.0.

2.4. Construction of a multicopy plasmid, containing GFP under the transcriptional control of the RNR2 promoter

The *RNR2* promoter has not been fully characterised, so all the DNA upstream of the transcriptional start ATG codon to the next open reading frame (ORF) was amplified by PCR. The primers used to generate the 1568 bp fragment incorporated flanking restriction sites, allowing direct replacement of the *RAD54* promoter (*Pac*I and *Bam*HI). The PCR product was ligated into the *Sma*I site of pUC19 to produce pUC-RNR2. This plasmid was then digested with *Pac*I and *Bam*HI to remove the *RNR2* promoter, and the fragment ligated into the corresponding sites of pWD444 to produce pUMGP5. Primer sequences are shown in Table 2.

2.5. Western blot analysis of yEGFP expression

Cells were grown in F1 medium (2 ml) and treated with different concentrations of MMS as indicated. Cell density at start of incubation was (0.1 AU_{600}). After 16 h of incubation under continuous agitation at 30°C cells were collected and washed twice in PBS and resuspended in 1 ml PBS. Optical density was measured at 600 nm for relative quantitation of cells in each sample. Cell extracts were prepared as described [23]. Extracts were loaded on SDS-PAGE and transferred to a nitrocellulose mem-

Table 2
Primers used in the construction of the *RNR2*-yEGFP promoter–reporter plasmid

RNR2BAM	5′-CCT CC**G GAT** CCC GTA CCT TCC AGC ATT GTC C-3′
Amplifies the *RNR2* promoter, incorporating a *Bam*HI restriction site (**bold**) into its 5′ end	
RNR2PAC	5′-CCT CCG **TTA ATT AAC** ATG GTA ATT GGA CAA ATA AAT ACG-3′
Amplifies the *RNR2* promoter, incorporating a *Pac*I restriction site (bold) into its 3′ end	

brane after electrophoretic separation. yEGFP was visualised using a GFP specific antibody (Molecular Probes A-6455) and horse radish peroxidase conjugated secondary antibody with the ECL system (Amersham).

3. Results

3.1. Sensitivity of the RAD54-yEGFP reporter system to different MMS concentrations and incubation time in the microtitre plate assay format

It was shown previously that the brightness of the fluorescence signal obtained from yeast strains carrying the RAD54-yEGFP promoter–reporter construct on the pWDH444 plasmid correlates with exposure to increasing concentrations of MMS [16]. MMS is an alkylating agent that can act on DNA leading to damage and mutagenesis. MMS is regularly used as a reference substance for assessing the mutagenic and DNA-damaging potential of uncharacterised chemicals [24]. Using an online sampling device, it could be clearly demonstrated that the MMS-induced strength of the fluorescence signal increased with incubation time in a yeast culture transformed with the RAD54-yEGFP construct [25]. To study if a comparable sensitivity could be obtained in smaller sample volumes, such as the microtitre plate format routinely used in pharmaceutical companies, we distributed equal volumes of a culture of yeast cells containing the RAD54-yEGFP construct in microtitre plates and treated the cells with various concentrations of MMS (1.6×10^{-5}% to 1.7×10^{-2}% f.c.; 1% of MMS = 0.12 mol/l or 13 mg/ml). Fluorescence was then measured at different times during the incubation. In Fig. 1(A and B), the fluorescence at 520 nm normalised by absorption at 670 nm is displayed for four time points at 0, 4, 16 and 20 h in response to MMS concentration. All values are the mean value of 8-microtitre plate wells (independent samples) and the standard deviations are indicated. The minimal concentration of MMS to which cells start to respond was 2×10^{-3}% after 4 h, and 1×10^{-3}% after 16 h. A maximum of the fluorescence signal is reached after approximately 14–16 h incubation. After 4 h only a weak increase in fluorescence could be observed with the RAD54-GFP reporter construct (Fig. 1B).

As an alternate sensor system, the *RAD54* promoter was replaced with the promoter of the *RNR2* gene. *RNR2*, a small subunit of the ribonucleoside diphosphate reductase, is essential for the synthesis of nucleotide precursors and was shown to be induced by MMS treatment [21,26]. As described above, we performed fluorescence measurements in response to MMS treatment with yeast cell cultures carrying the RNR2-yEGFP construct. The strength of the fluorescence signal obtained with the *RNR2* promoter is much stronger than with the *RAD54* promoter (Fig. 1B and C).

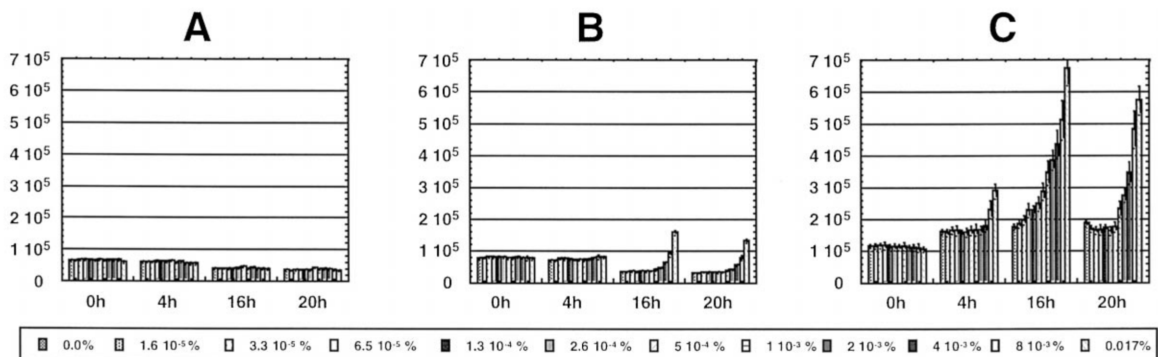

Fig. 1. Comparison of MMS-dependent fluorescence in untransformed yeast (A), and in yeast transformed with RAD54-GFP (B) or in yeast with RNR2-GFP (C) promoter–reporter constructs. Intensity of the fluorescence signal is dependent on incubation time (0, 4, 16, 20 h) and MMS concentration (0.0–0.017%) as indicated. The fluorescence signal displayed is normalized for cell density. The values presented were obtained dividing the value of the fluorescence measurement through the value of the absorption as described in Section 2.

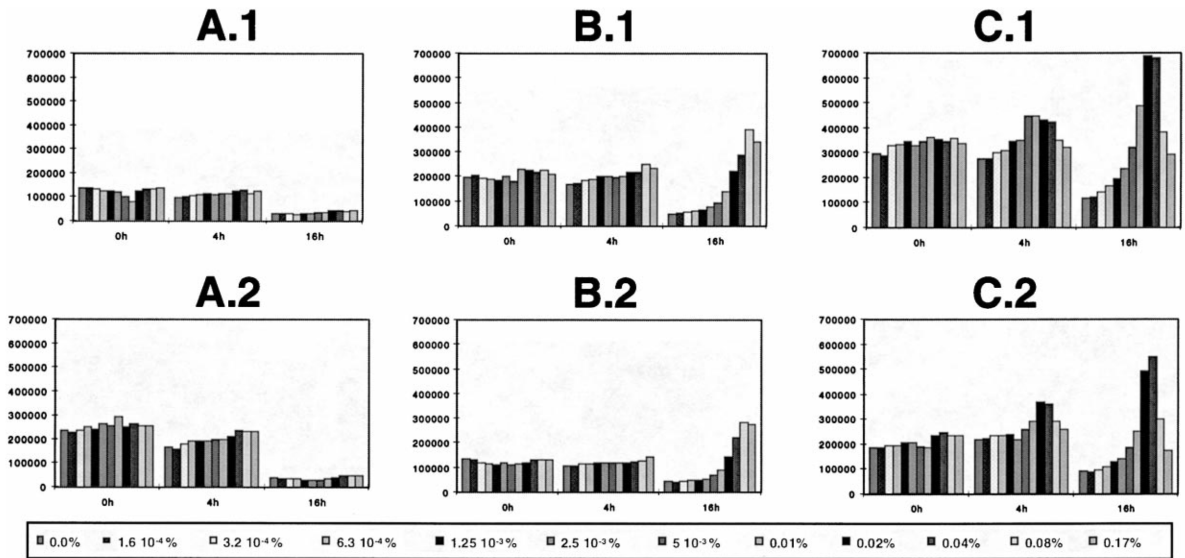

Fig. 2. Dependence of fluorescence signal on cell density in untransformed yeast (A), and in yeast transformed with RNR2-GFP (B) and with RAD54-GFP (C) promoter–reporter constructs. In samples A1, B.1, C.1 a starting cell density of 0.21 AU_{670} and in samples A.2, B.2, C.2 of 0.42 AU_{670} was used. The fluorescence values displayed are normalised for cell density (fluor. 520 nm/abs. 670 nm). Fluorescence signals were measured at the time indicated. Increasing concentrations of MMS are indicated.

For both reporter systems, the cell density used in each well directly affected the strength of the fluorescence signal obtained (Fig. 2). This significant change in induced fluorescence is critical for the sensitivity of the assay. It can be seen in Fig. 2C with RNR2-GFP that after 16 h in the presence of 5×10^{-3}%, MMS fluorescence was induced by 3-fold at a cell density of 0.21 AU_{600} but only 2-fold when the cell density was doubled (0.42 AU_{600}). Similar in Fig. 2B with RAD54-GFP signals were higher when the lower cell density was used. Throughout our experiments, an even lower initial density of 3×10^{6} cells/ml (0.1 AU_{600}) was used that gave detectable fluorescence signals already after 4 h of incubation (Fig. 1).

To ensure that the fluorescence readout observed is indeed the result of an induction of yEGFP synthesis, we performed experiments with the RAD54-GFP and the RNR2-GFP systems and measured the amount of yEGFP at the protein level. After 16 h of incubation, cells were lysed and protein extracts were prepared. Aliquots of the extracts, based on the cell number after incubation, were analysed by Western blot with a GFP specific antibody. The results are displayed in Fig. 3. In both yeast strains, a significant amount of GFP is made without MMS induction, and as expected from the fluorescence data the amount of GFP is much lower with the *RAD54* promoter construct (Fig. 3A,B, lane 1). Treatment with MMS resulted in a significant in-

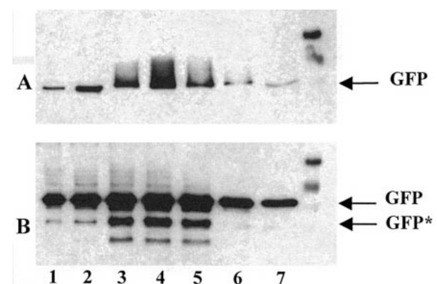

Fig. 3. Western blot analysis of GFP induction in yeast strains transformed with RAD54-GFP (A) or RNR2-GFP (B). Cells were grown for 16 h in the presence of MMS: 0.0% (1), 0.00313% (2), 0.00625% (3), 0.0125% (4), 0.025% (5), 0.05% (6), 0.1% (7). The position of the band representing the green fluorescent protein is marked with GFP. GFP* indicates degradation products of GFP that are visible at very high expression levels.

crease in GFP protein per cell in both cases. The faster migrating bands in Fig. 3B, from yeast cells with the RNR2-GFP construct, are degradation products only visible at higher protein concentrations or perhaps the result of increased degradation due to the high protein concentration. These results correlate well with the fluorescence signal obtained after MMS treatment, demonstrating that the change in fluorescence observed with MMS is the result of increased yEGFP synthesis. Here in Fig. 3 as well as in Fig. 2 at higher concentrations of MMS a lack of induction of GFP and consequently fluorescence is observed. An explanation for this observation is that at high concentrations of MMS protein synthesis is immediately inhibited upon treatment preventing GFP synthesis in response to DNA damage as well.

3.2. Substances with different properties induce the fluorescence signal

We selected a set of substances that are known to have a potential for DNA or RNA interaction. We also included substances that have other known sites of action. All substances used are listed and categorised in Table 3. The substances include DNA-binding, -modifying and -cleaving molecules as well as topoisomerase and polymerase inhibitors. We also used substances that are primarily active on other macromolecules within the cell. If more than one mode of activity is known, only one well-established activity is listed. In general, we anticipate that most substances will be active on more than one molecular target. Substances known to react with nucleic

Table 3
Substances used and short description of known and assumed functional mechanisms

DNA intercalation	
Actinomycin D	Inhibition of RNA and DNA synthesis [Pincus and Rekosh, BBRC 121 (1984) 1031–1041]
Ethidium bromide	Selective effect on mitochondria described [Hayakawa et al., J. Biol. Chem. 273 (1998) 20,300–20,307]
Non-intercalative DNA binding	
Chromomycin	[Gao and Patel, Biochemistry 28 (1989) 751–762; Simon et al., FEBS Lett. 353 (1994) 79–83]
Netropsin	Minor groove binding (cleavage) [Portugal and Waring, Eur. J. Biochem. 167 (1987) 281–289]
Psoralen	[Ussery et al., Methods Enzymol. 212 (1992) 242–253]
Alkylation of DNA	
MMS	[G.S.-F. Lee et al., J. Mol. Biol. 223 (1992) 617–626]
Mitomycin C	[Galloway et al., Mutat. Res. 400 (1998) 169–186]
N-nitroso-*N*-methylurea	[A.I. Gorin et al., Biull. Eksp. Biol. 81(8) (1976) 674–677]
Chlorambucil	[Kundu et al., Pharmacol. Biochem. Behav. 49 (1994) 621–624]
Cleavage of DNA	
Bleomycin	[Gavin et al., Anal. Biochem. 263 (1998) 26–30]
Phleomycin	[Bennett, Mol. Cell Biol. 19 (1999) 1800–1809]
cis-Platinum(II) diamine-dichloride	(G.L. Cohen et al., Science 203 (1979) 1014–1019]
Daunomycin	(Daunorubycin) [Gerwitz, Biochem. Pharmacol. 57 (1999) 727–741]
Streptonigrin	Topoisomerase II dependent cleavage (Yamashita et al., Cancer Res. 50 (1990) 5841–5844]
Inhibitors of polymerases or topoisomerases	
Aphidicolin	Inhibitor of DNA polymerase a and d [Ikegami et al., Nature 275 (1978) 458–459]
Camptothecin	Inhibitor of topoisomerase I [Kjeldsen et al., J. Mol. Biol. 228 (1992) 1025–1030]
Other biochemical activities	
Antimycin A	Inhibitor of mitochondrial energy production [Guidarelli et al., Biochem. J. 328 (1997) 801–806]
Colchicine	Interferes with microtubule organisation in higher eukaryotes, but not in yeast [Parry, Mutat. Res. 287 (1993) 23–28]
Tetracycline	Inhibitor of protein synthesis in prokaryotes (interaction with 30 S subunit) [Hlavka and Boothe (Eds.), The Tetracyclines, Springer, Berlin, 1985]

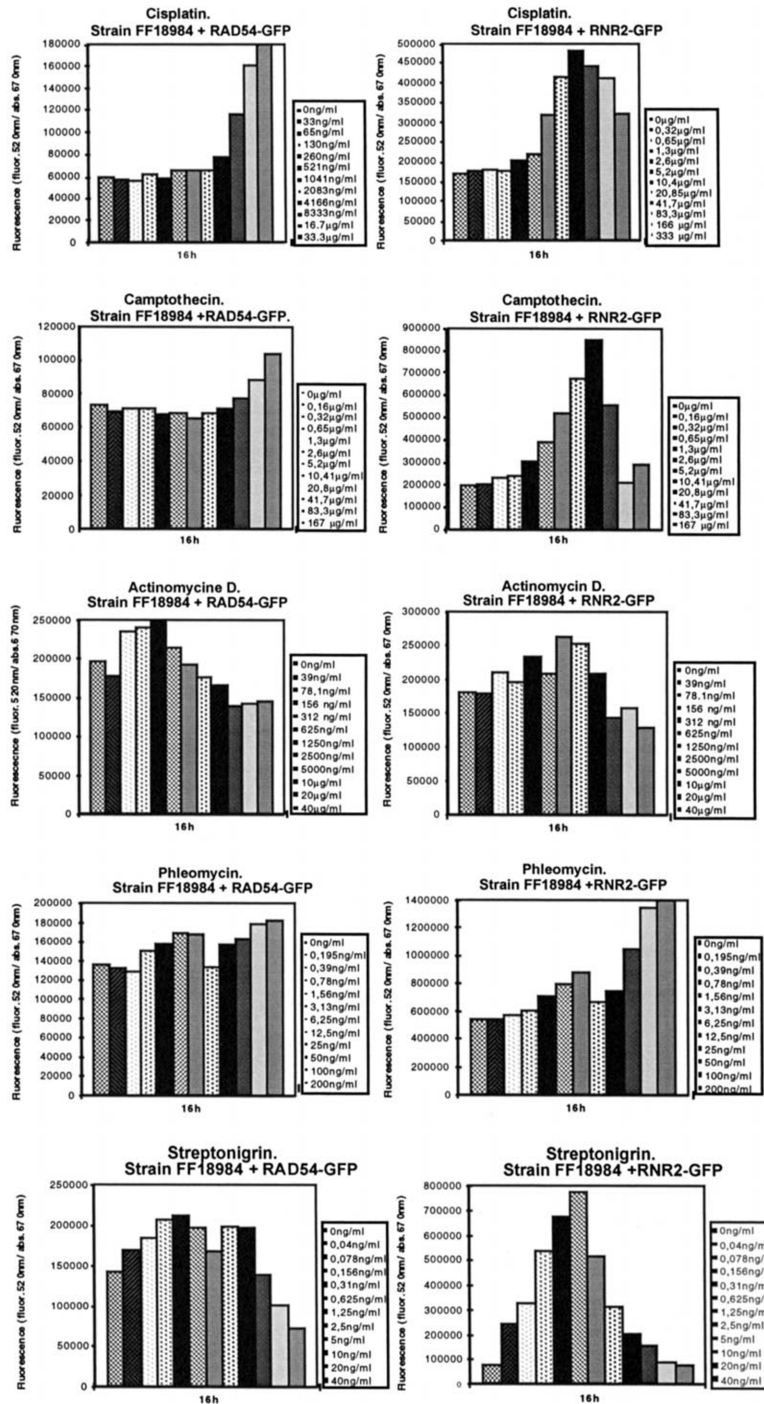

Fig. 4. Selection of fluorescence response profiles after treatment with increasing concentration of selected chemicals. Representative results obtained with the chemical substances indicated. Diagrams show the dose response of fluorescence after incubation with cisplatin, camptothecin, actinomycin D, phleomycin and streptonigrin for 16 h. Results with the yeast strain containing the prRAD54-yEGFP are shown on the left panels and results with the yeast strain containing prRNR2-yEGFP on the right panels.

acids will also react with proteins, and substances known to be active at a protein target will also interact with nucleic acids. For most substances tested, we observed comparable positive or negative results with both the RAD54-GFP reporter and the RNR2-GFP reporter. As with MMS, a wide concentration range was used for each test substance. Examples of concentration dependent fluorescence signals obtained with both reporter systems and control experiments with the untransformed parent yeast strain are presented in Fig. 4. It can be seen that both reporter systems responded to cisplatin, a strong DNA-cleaving agent, to camptothecin, an inhibitor of topoisomerase I, to aphidicolin, an inhibitor of DNA polymerases α and δ, and to actinomycin D, an intercalator that inhibits mainly transcription but to some extent also replication. It is interesting to note, that in the case of actinomycin D, after a slight increase at a medium concentration, no further increase is visible — most likely due to the strong inhibitory effect on transcription, a prerequisite for GFP synthesis. At higher concentration of several test substances a lack of GFP induction is observed (Fig. 4). As mentioned before this is most likely inhibition of protein synthesis. However, the range of concentration, in which actinomycin D induces fluorescence, as well as the signal (value) were much smaller compared with other substances. Some substances, known to have mutagenic or DNA-damaging potential, like psoralen or benz-pyrene, did not induce activity in our system under the conditions used. All results obtained are summarised in Table 4. For comparison, published results of the Ames Test or the SOS Test are included (from the Genotoxicity Database at http://www.pasteur.fr/units/pmtg/sommaireuk.html [27]).

It can be seen that the results obtained in our study correlate well in the case of the strong alkylating and DNA-cleavage inducing agents: MMS, cisplatin, *N*-nitroso-*N*-methylurea. In the case of actinomycin D, the positive result seen in our assay may be the result of replication inhibition at lower concentra-

Table 4
Summary of results obtained with various chemical substances

Substance	Concentration	RAD54pr.	RNR2pr.	Ames test	SOS test
Actinomycin D	37.4 ng/ml–38.3 μg/ml	+	+	−	−
Bleomycin	36 ng/ml–36,6 μg/ml	−	not tested	+	+
Daunomycin	15.6 ng/ml–16 μg/ml	−/?	−	not found	not found
Ethidium bromide	81 ng/ml–83.3 μg/ml	?	?	+	−
Mitomycin C	33 ng/ml–33 μg/ml	−	−	not found	+
Antimycine	46 ng/ml–46.6 μg/ml	+	not tested	not found	not found
MMS	0.2 μg/ml–1.3 mg/ml	++++	++++	+	+
Cisplatin	0.22 μg/ml–230 μg/ml	++++	++++	+	+
Streptonigrin	1.6 ng/ml–1667 ng/ml	++	++++	not found	+
Tetracycline	54.7 ng/ml–56 μg/ml	−	−	not found	−
Colchicine	15 ng/ml–15.4 ng/ml	−	−	not found	not found
Camptothecin	33 ng/ml–35 μg/ml	++	++++	not found	not found
Netropsin	36 ng/ml–36.6 ng/ml	−	not tested	not found	not found
Phleomycin	0.18 μg/ml–182 μg/ml	+	+	not found	+
Aphidicolin	17.76 ng/ml–18.2 μg/ml	++++	+++	not found	not found
Chlorambucil	0.10 μg/ml–109 μg/ml	+	−	not found	not found
Psoralen	40 ng/ml–40 μg/ml	−	−	not found	+
Chromomycin A3	18 ng/ml–18.2 μg/ml	−	−/?	not found	−
N-nitroso-*N*-methylurea	1.5 μg/ml–1650 μg/ml	++++	++++	+	+

Name of substances and range of concentrations used are indicated.
+ = Indicates induction of the yEGFP reporter and cumulation indicates increased signal strength. '**?**' = Indicates unclear interpretation. − = No effect observed. −/? = Unclear interpretation due to fluorescence of substance that most likely can be interpreted as negative. The Ames and SOS test results are taken form the Genotoxicity Database: http://www.pasteur.fr/units/pmtg/sommaireuk.html [27], and no indication of significance is made.

tions, leading to fluorescence induction, before inhibition of transcription at higher concentrations prevents GFP synthesis. As such it may represent an important class of genotoxins that to date have escaped current tests. The negative result seen with bleomycin, mitomycin C and psoralen, all active in either the Ames or SOS tests, could be due to enzymatic inactivation, lack of cell penetration, detoxification or other properties specific to yeast.

4. Discussion

The monitoring of the DNA-damage potential of chemical substances, is a central task in understanding the risk that can be derived from an enrichment of these substances in the environment. Moreover, understanding the mechanisms that are responsible for the detrimental effects of these substances can help to find countermeasures if severe contamination occurs. To assess the activity of a large number of substances, measurements of toxicity are required that can be fully automated and reliable on large scale.

The induction of a fluorescence signal driven by promoters that respond to DNA-damage in the yeast *S. cerevisiae* provides a simple system to monitor the activity of substances that could posses mutagenic potential at low concentrations. The microtitre plate format used for incubation and measurement allows simple automation with well established microtitre plate handling systems used in high throughput screening.

We have shown that the small reaction volumes available in the wells of a standard microtitre plate are fully sufficient to obtain and measure the induction of GFP fluorescence driven by the *RAD54* promoter upon treatment with low levels of MMS. We found that the sensitivity to low concentrations of MMS is not only proportional to MMS concentration, but also to the ratio of MMS to cell mass. To obtain reproducible sensitivity, the cell concentration used at the start of incubation must be equal in each assay. For good sensitivity a starting cell density at 0.1 AU_{600} was used. Using the promoter of the *RNR2* gene (whose product is required in the biosynthesis of nucleotides), an even greater sensitivity to MMS was found. Independent of the promoter used, only small variations were found between the data points of individual wells, as indicated by the standard deviation given in the figures. This clearly shows that under standard conditions a high reproducibility can be obtained for each analysis.

The higher activity of the *RNR2* promoter is consistent with the recent data presented by Jelinsky and Samson [12]. In this work the change in transcription of all yeast genes was measured in response to 0.1% MMS. Although the concentration of MMS was relatively high in comparison to our study, they describe a 3.9-fold increase in mRNA level for *RNR2* and only a 2.8-fold increase for *RAD54*. However, Jelinsky and Samson also report several genes whose transcription is up regulated to an even greater extent than *RNR2* in response to DNA damaging agents. The promoters of these genes may allow the construction of even more sensitive reporter systems for potential genotoxins.

As expected from the results with MMS, other substances that modify bases in DNA, such as *N*-nitroso-*N*-methylurea and cisplatin, show comparable induction of the fluorescence reporter system. Chlorambucil, that is also described as an alkylating substance is significantly less active. The larger molecule mitomycin C, which also possesses alkylating and cytotoxic activity [28], was not found to activate *RAD54* promoter driven GFP expression. One explanation could be that the intracellular concentration obtained in the yeast was not sufficient for activity, or that reduction, a step most likely required for activation, is not efficient in yeast.

From the two related antitumor substances bleomycin and phleomycin, a clear effect was only found with phleomycin. Although, the somewhat lower concentrations used in the assay with bleomycin could have been below the active concentration, it is also possible that bleomycin was inactivated by the yeast cysteine proteinase (Ycp1, Gal6). Overexpression of this proteinase increases the resistance to bleomycin [29]. Recently it was shown that yeast carrying the metabolising system of cytochrome *P*450 showed enhanced gene conversion by bleomycin [30] suggesting that metabolic bleomycin modification could also lead to an activity in our system.

The lack of effect seen with the DNA cleaving drug daunomycin could also be due to a lack of

accumulation of molecules in the yeast cell, because of permeability barriers or detoxification mechanisms.

Of all the other substances known to have a potential for direct interaction with DNA, psoralen (with UV light and without), chromomycin, netropsin, ethidium bromide and actinomycin D, only ethidium bromide and actinomycin D were found to lead to fluorescence. The unclear result indicated for ethidium bromide (indicated '?'), is due to the high fluorescence induction by this chemical even in the wt yeast cells without the reporter system after incubation. It is unclear whether this is the result of intercalation into mitochondrial DNA, or the formation of highly fluorescence adducts. Western blot analysis of GFP after ethidium bromide treatment only resulted in a slight increase of GFP production (data not shown). The lack of induction by psoralen well known to damage DNA by forming UV-induced photoadducts [31,32] was at first quite surprising. It was shown previously that a wide range of psoralen derivatives could activate β-galactosidase under the control of the RAD54 or RNR2 in yeast [26]. The absence of fluorescence in response to unsubstituted psoralen, a good crosslinker in vitro, used in our study therefore must be attributed to a lack of penetration into the yeast cell. It is planed to include other derivatives that show better cell penetration, e.g., trimethylpsoralen, in future studies.

In the case of these well-known DNA-damaging substances, a limitation of our present system becomes clear. Although our reporters clearly respond to DNA damage, detoxifying mechanisms of the yeast cell prevent access to the site of action, the DNA, and thus no activation of damage response can be observed. This limitation can be overcome by following predictable routes that make yeast more sensitive to chemicals. These include enhanced permeability of the yeast cells [33,34] and inhibition of efficient detoxification by drug resistance mechanisms [35].

Treatment with drugs that do not interfere with RNA polymerase but block topoisomerase I or DNA polymerases α and δ gave a strong induction of fluorescence, comparable to the signal obtained with the alkylating/cleaving reagents MMS, cisplatin and nitrosourea. Further investigation should reveal whether this response is mediated by secondary strand breaks, changes in negative supercoiling or stabilisation of single stranded DNA.

Drugs acting on particular targets that are not present in yeast, colchicine and tetracycline, did not show any activity. Interestingly, the inhibitor of the electron transfer in the respiratory chain, antimycin, clearly activated the fluorescence of the *RAD54* promoter–reporter system. A possible mechanism could be DNA damage by free radicals [36]. In this context it is noteworthy that UV-irradiation and treatment with small alkylating substances induces constitutive expression of alcohol dehydrogenase genes (*ADH2*, *ADH4*) that also provide resistance to antimycin [37].

5. Perspective

Induction of GFP expression by the promoters of two DNA-damage repair genes occurred in response to various substances that can lead to significant levels of DNA damage. The presence of biologically active amounts of genotoxic substances in the environment poses a significant risk to public health and this yeast based system, that analysis the toxic potential of isolated substances, could provide a very robust system for large scale analysis of environmental samples. Using yeast instead of mammalian cell culture and animal tests helps to reduce the need for experiments on animals and avoids the time consuming and expensive set up for mammalian tissue culture.

In contrast to the established Ames and the SOS tests [1,2,7], which are bacterially based, our yeast system provides all the typical features of eukaryotic cell architecture and metabolism. For example, yeast possess mitochondria and a cell nucleus, into which the chromosomal DNA is packaged in a highly structured form, analogous to that found in mammalian cells. Bacteria share none of these important features. Furthermore, the DNA repair mechanisms of yeasts and humans are strikingly similar. A lack of sensitivity, due to differences in metabolism and drug uptake could be overcome by expressing the respective mammalian enzyme in the yeast reporter strain. For example the expression of mammalian cytochrome *P*450 enzymes with their corresponding

oxido-reductase recycling enzymes, has been shown to promote DNA damage by aflotoxins in yeast [38]. Complemented by knock out of yeast genes that provide efficient detoxification [35], we should be able to develop a reliable assay system for induced DNA damage.

A number of mutant yeast strains now exist with altered phenotypes, including more permeable cell walls, or impaired export-pumps (that otherwise confer drug resistance on the cells). A screen of such mutants is now underway, and it is hoped that strains with enhanced genotoxin sensitivity will be identified, thus improving upon the present ''limits of detection'' (LOD) for those chemicals known to activate the reporter. Similarly, ''export-pump'' mutants may be found that show responses to a broader class of mutagens than the strain currently employed in our tests.

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References

[1] B.N. Ames, W.E. Durston, E. Yamasaki, F.D. Lee, Carcinogens are mutagens: a single test system combining liver homogenates for activation and bacteria for detection, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 2281–2285.

[2] D.M. Maron, B.N. Ames, Revised methods for the Salmonella mutagenicity test, Mutat. Res. 113 (1983) 173–215.

[3] K. Yoshikawa, Anomalous nonidentity between Salmonella genotoxicants and rodent carcinogens: nongenotoxic carcinogens and genotoxic noncarcinogens, Environ. Health Perspect. 104 (1996) 40–46.

[4] B.A. Fetterman, B.S. Kim, B.H. Margolin, J.S. Schildcrout, M.G. Smith, S.M. Wagner, E. Zeiger, Predicting rodent carcinogenicity from mutagenic potency measured in the Ames Salmonella assay, Environ. Mol. Mutagen. 29 (1997) 312–322.

[5] P. Hubner, P.M. Groux, B. Weibel, C. Sengstag, J. Horlbeck, P.M. Leong-Morgenthaler, J. Luthy, Genotoxicity of ethyl carbamate (urethane) in Salmonella, yeast and human lymphoblastoid cells, Mutat. Res. 390 (1997) 11–19.

[6] P. Poli, A. Buschini, A. Candi, C. Rossi, Bleomycin genotoxicity alteration by glutathione and cytochrome *P*-450 cellular content in respiratory proficient deficient strains of *Saccharomyces cerevisiae*, Mutagenesis 14 (1999) 233–238.

[7] P. Quillardet, O. Huisman, R. Dari, M. Hofnung, SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 5971–5975.

[8] L. Nylund, C. Rosenberg, P. Jäppinen, H. Vainio, Genotoxicity of kraft pulp spent liquors from different types of chlorination procedures, Mutat. Res. 320 (1994) 165–174.

[9] F. Raabe, S. Janz, G. Wolff, H. Merten, A. Landrock, T. Birkenfeld, R. Herzschuh, Genotoxicity assessment of waste product of aluminum plasma etching with the SOS chromotest, Mutat. Res. 300 (1993) 99–109.

[10] T. Weinert, Yeast checkpoint controls and relevance to cancer, Cancer Surv. 29 (1997) 109–132.

[11] S.E. Crichlow, S.P. Jackson, DNA end-joining from yeast to man, Trends Biochem. Sci. 23 (1998) 394–398.

[12] S.A. Jelinsky, L.D. Samson, Global response of *Saccharomyces cerevisiae* to an alkylating agent, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 1486–1491.

[13] E.C. Friedberg, G.C. Walker, W. Siede, DNA Repair and Mutagenesis, ASM Press, Washington, DC, 1995, p. 596.

[14] R.M. Walmsley, N. Billinton, W.-D. Heyer, Green fluorescent protein as a reporter for the DNA damage-induced gene *RAD54* in *Saccharomyces cerevisiae*, Yeast 13 (1988) 1535–1545.

[15] R.M. Walmsley, W.-D. Heyer, Patent application, 1997, (PCT) 9706414.1.

[16] N. Billinton, M.G. Barker, C.E. Michel, A.W. Knight, W.-D. Heyer, N.J. Goddard, P.R. Fielden, R.M. Walmsley, Development of a green fluorescent protein reporter for a yeast genotoxicity biosensor, Biosens. Bioelectron. 13 (1998) 831–838.

[17] G.M. Cole, D. Schild, S.T. Lovett, R.K. Mortimer, Regulation of *RAD54*- and *RAD52-lacZ* gene fusions in *Saccharomyces cerevisiae* in response to DNA damage, Mol. Cell. Biol. 7 (1987) 1078–1084.

[18] G.M. Cole, R.K. Mortimer, Failure to induce a DNA repair gene, *RAD54*, in *Saccharomyces cerevisiae* does not affect DNA repair or recombination pathways, Mol. Cell. Biol. 9 (1989) 3314–3322.

[19] S.J. Elledge, R.W. Dawis, Identification and isolation of the gene encoding the small subunit of ribonucleotide reductase from *Saccharomyces cerevisiae*: DNA damage inducible gene required for mitotic viability, Mol. Cell. Biol. 7 (1987) 2783–2793.

[20] S.J. Elledge, R.W. Davis, DNA damage induction of ribonucleotide reductase, Mol. Cell. Biol. 9 (1989) 4932–4940.

[21] S.J. Elledge, Z. Zhou, J.B. Allen, T.A. Navas, DNA damage and cell cycle regulation of ribonucleotide reductase, Bioessays 15 (1993) 333–339.

[22] R.M. Walmsley, D.C.J. Gardner, S.G. Oliver, Stability of a cloned gene in yeast grown in chemostat culture, Mol. Gen. Genet. 192 (1983) 361–365.

[23] F.M. Asubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Short Protocols in Molecular Biology, Wiley, New York, 1997, pp. 13–51.

[24] G.S.-F. Lee, K.S. Blonsky, D.V. Lee, E.A. Savage, A.R. Morgan, R.C. von Borstel, Base alterations in yeast induced by alkylating agent with different Swain–Scott substrate constants, J. Mol. Biol. 223 (1992) 617–626.

[25] A.W. Knight, N.J. Goddard, P.R. Fielden, M.G. Barker, N. Billinton, R.M. Walmsley, Development of a flow-through detector for monitoring genotoxic compounds by quantifying the expression of green fluorescent protein in genetically modified yeast cells, Meas. Sci. Technol. 10 (1999) 211–217.

[26] D. Averbeck, S. Averbeck, Induction of the genes *RAD54* and *RNR2* by various DNA damaging agents in *Saccharomyces cerevisiae*, Mutat. Res. 315 (2) (1994) 123–138.

[27] P. Quillardet, M. Hofnung, The SOS chromotest: a review, Mutat. Res. 297 (1993) 235–279.

[28] D. Siegel, N.W. Gibson, P.C. Preusch, D. Ross, Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells, Cancer Res. 50 (23) (1990) 7483–7489.

[29] N.G. Kambouris, D.J. Burke, C.E. Creutz, Cloning and characterization of a cysteine proteinase from *Saccharomyces cerevisiae*, J. Biol. Chem. 267 (1992) 21570–21576.

[30] P. Poli, A. Buschini, A. Candi, C. Rossi, Bleomycin genotoxicity alteration by glutathione and cytochrome *P*-450 cellular content in respiratory proficient and deficient strains of Saccharomyces cerevisiae, Mutagenesis 14 (1999) 233–238.

[31] S.T. Isaacs, C.K. Shen, J.E. Hearst, H. Rapoport, Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA, Biochemistry 16 (1977) 1058–1065.

[32] B.H. Johnston, M.A. Johnson, C.B. Moore, J.E. Hearst, Psoralen-DNA photoreaction: Controlled production of mono- and diadducts with nanosecond ultraviolet laser pulses, Science 197 (1977) 906–911.

[33] L. Staleva, L. Waltscheva, E. Golovinsky, P. Venkov, Enhanced cell permeability increases the sensitivity of yeast test for mutagens, Mutat. Res. 370 (1996) 81–89.

[34] T. Morita, Y. Iwamoto, T. Shimizu, T. Masuzawa, Y. Yanagira, Mutagenicity tests with a permeable mutant of yeast on carcinogens showing false-negative in Salmonella assay, Chem. Pharm. Bull. 37 (1989) 407–409.

[35] E. Balzi, A. Goffeau, Yeast multidrug resistance: the PDR network, J. Bioenerg. Biomembr. 27 (1) (1995) 71–76.

[36] A. Guidarelli, E. Clementi, L. Brambilla, O. Cantoni, Mechanism of the antimycin A-mediated enhancement of *t*-butylhydroperoxide-induced single-strand breakage in DNA, Biochem. J. 328 (1997) 801–806.

[37] C. Morawetz, U. Hagen, Effect of irradiation and mutagenic chemicals on the generation of *ADH2*- and *ADH4*-constitutive mutants in yeast: the inducibility of Ty transposition by UV and ethylmethanesulfonate, Mutat. Res. 229 (1990) 69–77.

[38] C. Kaplanski, C.P. Wild, C. Sengstag, Rearrangements in minisatellite sequences induced by aflatoxin B1 in a metabolically competent strain of *Saccharomyces cerevisiae*, Carcinogenesis 9 (1998) 1673–1678.