

# Development of a green fluorescent protein reporter for a yeast genotoxicity biosensor

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## Abstract

A reporter system, constructed for a laboratory screen for new genes involved in DNA repair in the brewer's yeast *Saccharomyces cerevisiae*, has been developed for use in a genotoxicity biosensor. The strain produces green fluorescent protein (yEGFP) when DNA damage has occurred. yEGFP is codon optimised for yeasts. The reporter does not respond to chemicals which delay mitosis, and responds appropriately to the genetic regulation of DNA repair. Data is presented which demonstrate strain improvements appropriate to biosensor technology: improved signal to noise ratio, ease of data collection and uncomplicated material handling. © 1998 Elsevier Science S.A. All rights reserved.

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## 1. Introduction

Genotoxicity is commonly assessed using a batch method developed by Ames (Ames et al., 1973; Maron and Ames, 1983). The methodology of the test does not lend itself to automation into a continuous monitoring system. In the test, specially developed strains of the bacterium *Salmonella typhimurium* are exposed to chemicals, then plated out onto selective growth media. Colony counts of the different strains on selective media indicate the frequency of different types of genetic change. The simplicity and effectiveness of the test has led to its widespread adoption. The test is not perfect. Positive results are interpreted as an indication of the potential for carcinogenic activity, though bacteria (prokaryotes) are sufficiently different to humans (eukaryotes) to allow some misleading results. There are compounds that are Ames-negative yet lead to cancers in animal models, as well as those that are Ames-positive and not a threat to humans. These differences are associated with differences in the metabolism of compounds in the different cell types, and the efficiency with which

repair can be affected. In fact, the majority of DNA damage is seamlessly repaired. Consequently the Ames test, which assays wrongly repaired damage, underestimates the amount of DNA damage happening. The most recent advance in genotoxicity measurement has been the "SOS" chromotest (Quillardet et al., 1982). This is also a bacterial assay but looks at the repair (SOS) response using an enzyme linked reporter system. It has been used in a semiautomatic, microtitre plate batch assay (Nylund et al., 1994; Raabe et al., 1993).

We have previously reported the construction of a GFP reporter for the DNA damage-induced gene *RAD54* in *Saccharomyces cerevisiae* (Fig. 1, reproduced from Walmsley et al., 1998; Walmsley and Heyer, 1997). It was demonstrated that the reporter's activity could be unambiguously correlated with induction of the native *RAD54* protein and showed a predicted dose responsiveness to methyl methane sulphonate (MMS) treatment. A positive result was detectable with MMS within 3 h. At the core of the reporter is the promoter region of *RAD54* fused to the GFP gene from the jellyfish *Aequoria victoria* (reviewed by Cubitt et al., 1995). Measurement of GFP is simple and reagent free: the protein is non-toxic and can be estimated by measuring fluorescence. The *RAD54* gene is not highly expressed so extracts had to be made from treated and untreated cells before relative

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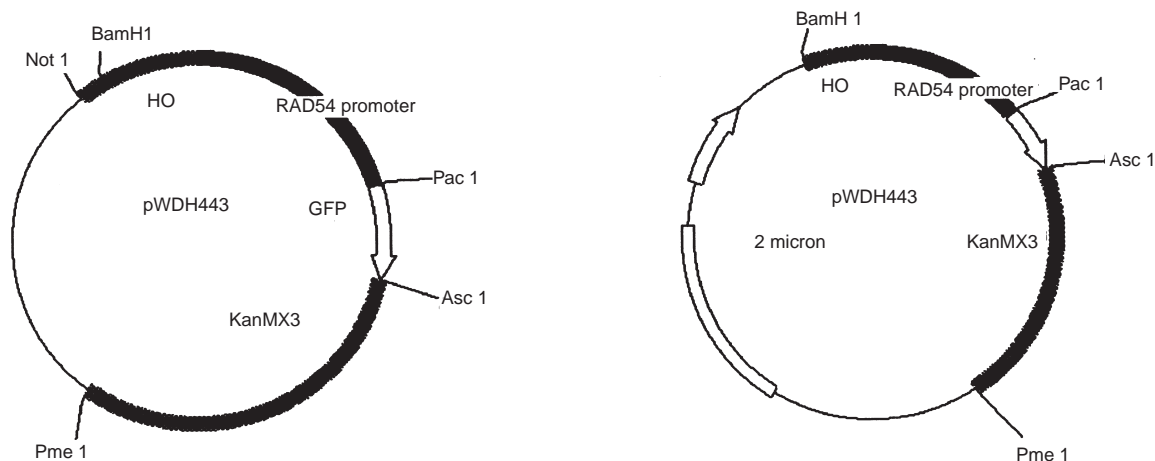


Fig. 1. RAD54-GFP reporter plasmids. Key to annotations: *HO*, a fragment of the yeast *HO* gene; RAD54 promoter, the 1.8-Kb region immediately upstream of the *RAD54* gene; GFP, the green fluorescent protein gene; KanMX3, resistance to kanamycin/G418; *URA3*, the yeast *URA3* gene; 2 micron, a fragment of the natural yeast 2 micron circular plasmid which confers autonomous replication to plasmids. Sequences without annotation are for replication and selection in *E. coli*. All the restriction enzyme cleavage sites shown are unique within the plasmids. pWDH443 can be cleaved at a unique *Bam*HI site to target integration to the yeast *HO* locus.

fluorescence could be assessed. The *RAD54* gene product has a most important function in recombinational repair in *S. cerevisiae* as *rad54* mutations confer the highest degree of sensitivity to ionizing radiation (reviewed in Game, 1993). Rad54p functionally interacts with other recombinational repair proteins (Clever et al., 1997). The RAD54-GFP reporter is currently being used in the EUROFAN programme (the analysis of genes of unknown function; Oliver, 1996, 1997). One thousand yeast strains, each with a different open reading frame deleted, are being transformed with the reporter and the efficiency of MMS induction assessed by fluorescence.

The EC Science Research Development Programme on Environment and Climate (1994–98) emphasized 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 paper, a potential successor to the Ames and SOS chromotest assays is described. It uses eukaryotic cells (the bakers yeast, *Saccharomyces cerevisiae*) instead of bacteria and exploits the DNA-damage-inducible GFP reporter described above. The existing laboratory method for the production of cell extracts is not suited to automation. It would require a complex series of chemical treatment and centrifugation steps to break open the cells, and the separation of cell extracts from cell debris. Because of this we embarked on a programme of reporter and strain development in order to allow a more simple system for use in a biosensor. This has involved the use of a brighter GFP and the identification of a strain with improved DNA damage induced repair activity. This has allowed the reporter to be assayed in whole cells without the need for extraction. The combination of inducible promoter and GFP reporter represents a generic approach to

environmental stress monitoring and lends itself to simple and continuous, high throughput biosensor technology.

## 2. Materials and methods

### 2.1. Strains and growth conditions

Yeast strains used in this study are listed in Table 1. The media YP plus 2% glucose (YPD) and synthetic media plus 2% glucose (SD) were prepared as previously described (Kaiser et al., 1994). For the selection of plasmids, either G418 (Geneticin) was added to a final concentration of 200  $\mu\text{g/ml}$  (w/v) in YPD or uracil was omitted from SD medium.

Table 1  
*Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotypes
YLR030w $\alpha$ FF18984	<i>MAT<math>\alpha</math> his3<math>\Delta</math>200 ura3-52</i> <i>MAT<math>\alpha</math> leu2-3,112 ura3-52 lys2-1 his7-1</i>
Isogenic strains used in Figure 5	
Y300 (WT)	<i>MAT<math>\alpha</math> can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
Y438 (RAD9)	<i>MAT<math>\alpha</math> can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1; rad9::HIS3</i>
Y439 (POL2)	<i>MAT<math>\alpha</math> can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1; pol2::TRP1</i>
Y286 (DUN1)	<i>MAT<math>\alpha</math> can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1; dun1-<math>\Delta</math>100::HIS3</i>

## 2.2. Fluorescence assays

The assessment of GFP fluorescence in cell extracts was performed essentially as described in Walmsley et al. (1998). A stationary phase culture of cells grown in SD medium was used as the inoculum source. Aliquots (30  $\mu$ l) of cells were inoculated into 3 ml of SD in 15-ml test tubes. Half of the tubes were then supplemented with 0.01% MMS (the remaining tubes left as controls). The tubes were incubated at 25°C for 16 h on an orbital shaker at 120 rpm, then adjusted to 0.02% NaN<sub>3</sub> and transferred to an icebox for 75 min, maintaining agitation. The cells were then transferred to 1.5-ml eppendorf tubes, harvested by centrifugation (10 s), washed twice in sterile distilled water, then washed and resuspended in 250  $\mu$ l extraction buffer (20 mM Tris-HCL, pH 7.5, 0.1 M NaCl). For whole cell fluorescent measurements, the washed cells were transferred directly to cuvettes containing 2.75 ml sterile distilled water. For cell extract studies, 100  $\mu$ l of 400–600 nm diameter glass beads were added to the tubes of cells which were then placed in a BIO 101 Fastprep FP120 to mechanically disrupt the cells. Following centrifugation for 30 s, the supernatant was transferred to a clean tube. The pellet of beads was washed in a further 250  $\mu$ l of extraction buffer and this was added to the supernatant from the previous extraction. The extract was adjusted to 0.1 M Tris, pH 11 by the addition of 1 M Tris base, 200  $\mu$ l of the pooled extract plus 2.8 ml of water was then transferred to a cuvette.

Fluorescence measurements were performed with either a Perkin Elmer LS50, or LS5 Fluorescence Spectrometer. The excitation and emission wavelengths were set to 488 and 511 nm, respectively, with a slit width of 10 nm. To correct for variations in cell number/protein extraction efficiency, light absorption was recorded for each cuvette at either 600 nm (for whole cells) or 280 nm (for extracts). The fluorescence values obtained from the fluorimeter were then divided by the absorption readings to give the “brightness value”, an arbitrary unit which is independent of sample concentration, though varies with different fluorimeters. The y-axis on fluorescence scans gives raw data in the form of machine defined (“INT”) units. Other data handling details are dealt with either in the text or in the figure legends.

## 3. Results

### 3.1. Re-engineering of the reporter increases GFP fluorescence

Several modified forms of the GFP have been produced in which absorption and emission characteristics are altered. Commonly, the “S65T” derivative is used because it produces a good quantum yield of fluor-

escence and matches the output of argon ion lasers used in fluorescence activated cell sorters. Recently a new Yeast Enhanced GFP (yEGFP) has been synthesised in which the amino acid coding is biased to the preferred usage of yeasts (Cormack et al., 1997). It has a slightly higher emission peak (~ 515 nm). The formation of the GFP fluorophore from the nascent protein is also less heat sensitive than existing GFPs. The modular design of the reporter construct allowed simple replacement of the S65T derivative with the new yEGFP gene. yEGFP was PCR-amplified using primers that added flanking *Pac1* and *Asc1* restriction enzyme sites and the PCR product was cut with the two enzymes. The S65T GFP gene was released from pWDH444 (Fig. 1) by cutting with the same two enzymes, and gel electrophoresis was used to purify the remaining vector fragment. The PCR fragment was then ligated into the vector. Light output from the new plasmid was more than doubled compared to the S65T derivative (Table 2).

### 3.2. yEGFP allows detection of GFP in whole cells

With the first constructs it was necessary to make cell extracts in order to get a good signal to noise ratio. The limited success with whole cell measurements was partly due to the weak output signal but also a consequence of light scattering. There is only a narrow difference between the excitation (488 nm) and emission (511 nm) wavelengths used in GFP assessment. Using the new construct, together with a more sensitive fluorimeter, it became possible to see the GFP signal as a correctly positioned shoulder on the fluorescence scan. To further reduce the impact of light scattering, a 495-nm glass, sharp cut-off filter was introduced between the sample chamber and the emission-detector. This greatly enhanced the sensitivity of the instrument such that the

Table 2

Comparison of fluorescence output from yEGFP and S65T-GFP reporters. YLR030w $\alpha$  cells were transformed with plasmids containing either the original S65T-GFP plasmid (pWDH444) or the new version containing yEGFP. The cells were grown with or without 0.01% MMS as described in methods and fluorescence measured. Both the constitutive and induced brightness are increased in the yEGFP plasmid containing strain

Reporter	Brightness (BU)–MMS	Brightness (BU) + MMS
yEGFP	100	45
	97	41
	93	47
	Average = 96.7	Average = 44.3
S65T-GFP	45	27
	54	29
	48	26
	Average = 49	Average = 27.3

difference between uninduced and induced cells was the same as that obtained with cell extracts (Fig. 2).

### 3.3. Strain background greatly influences the DNA damage response

In almost any biochemical assay, it is common to find that results vary with strain background. For this reason, pWDH444 was put into several strains from the UMIST laboratory collection. MMS-induced response was found to vary greatly. In the best strain, FF18984, induction was two and a half fold greater than in the strain being used in our genetic screen (Fig. 3(a, b)). There would seem to be a good prospect of improving this by a wider survey of strains.

### 3.4. Following the onset of the stationary phase of growth, uncharacterised fluorescent components of the yeast cell influence GFP estimation

There are many fluorescent chemicals in living cells. These include amino acids such as tyrosine (excitation 275 nm, emission 303 nm), tryptophan (excitation 287 nm, emission 348 nm) and phenylalanine (excitation 260 nm, emission 282 nm), reduced nicotinamides (excitation 360–365 nm, emission 460–470 nm) and oxidised flavins (excitation 395–440 nm, emission 520 nm). In addition, there is the poorly characterised group of chemicals called lipofuscins (Tsuchida et al., 1987; excitation 330–390 nm, emission 430–490 nm). In a number of experiments it was noticed that the background fluorescence detected at the GFP emission wavelength was variable in experiments repeated with the same strain

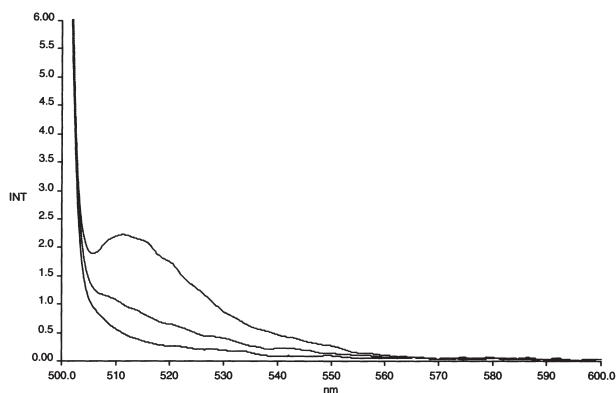


Fig. 2. yEGFP allows detection of GFP in whole cells. Emission spectra (excitation at 488 nm) from 0.2 OD600 units of untransformed and transformed cells (YLR030w $\alpha$ ), with or without 0.01% MMS. Cells were twice washed in sterile water before resuspending in  $1 \times$  TE. The lower spectrum represents the fluorescence measured from untransformed cells with an MMS challenge. The middle spectrum shows YLR030w cells transformed with the RAD54-yEGFP construct without an MMS challenge, whilst the upper spectrum shows the result of an MMS insult on the same transformed cells. This represents a 2.2-fold induction of fluorescence from uninduced to induced transformants. The yEGFP emission maximum is at 512 nm.

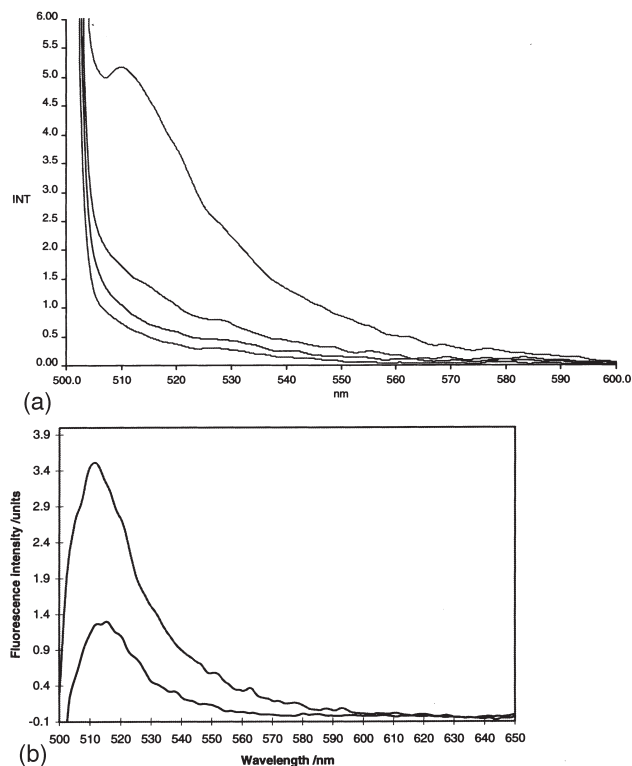


Fig. 3. (a) Strain background greatly influences the DNA damage response. Emission spectra (excitation at 488 nm) from 0.2 OD600 units of transformed and untransformed whole cells (FF18984), with or without MMS. Cells were twice washed in sterile water to remove any autofluorescent media before resuspension in  $1 \times$  TE and further dilution. The lowest spectrum represents untransformed cells with MMS and the next shows the same cells without MMS. The middle spectrum represents cells transformed with the reporter but not challenged with MMS, whilst the peaked spectrum shows the fluorescence from transformed cells challenged with MMS. (b) Simple data handling enhances the GFP emission peak. Data files containing fluorescence readings at 0.5 nm intervals were transferred into Microsoft Excel. Each set of data was normalised by dividing by the OD600 reading of cells loaded into the cuvette, then the background data set for each strain was subtracted from the MMS-induced data set. The resulting data was re-plotted using Excel. In this figure, YLR030w $\alpha$  with yEGFP (lower) and FF18984 with S65T (upper) are compared. Because of this data handling, the fluorescence scale of this plot should not be compared with those in other figures.

(see for example Table 2): this in turn led to a problem in calculating induction ratios. It was concluded that this must reflect some aspect of the cell's physiology. For this reason the fluorescence output spectrum of untransformed cells was followed from inoculation through to stationary phase. It is clear that stationary phase cells have a broad emission peak centred around 520 nm when illuminated at 488 nm. This material appears when cells begin to enter stationary phase (Fig. 4(a)). At its peak, the amount of fluorescence due to this material is similar to the level of constitutive GFP fluorescence. The position of the band suggests that this fluorescence might be due to oxidised flavins and appearance of the peak late in the growth phase is consistent with cells switch-

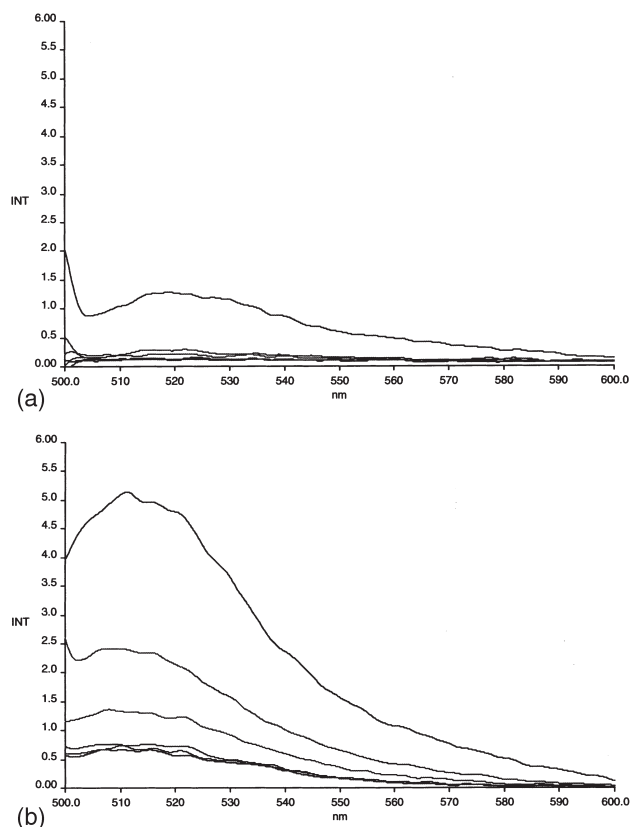


Fig. 4. (a) Appearance of background fluorescence in untransformed cells. Emission spectra for cell extracts (from FF18984) made from aliquots of cells taken every 2 h from growing cultures. The zero hour point occurs immediately after inoculation of the culture. Extracts were illuminated at 488 nm. The five basal spectra correspond to extracts taken after 0, 2, 4, 6 and 8 h (no significant difference). The upper spectrum represents the fluorescence from extracts made after 20 h in culture. Significant fluorescence emission is seen in extracts from 20-h old cells as a broad peak between 505 and 540 nm. (b) Fluorescence spectrum from cell extracts illuminated within the flavin absorbance range. Emission spectra of cell extracts made from FF18984 cells taken every 2 h from a growing culture, beginning immediately after inoculation. The extracts were excited at 395 nm. The lower three spectra correspond to the fluorescence emitted from cell extracts made after 0, 2 and 4 h. The three ever-increasing peaks correspond to samples taken at 6, 8 and 20 h, respectively.

ing from fermentative to oxidative growth as the glucose becomes exhausted. Additional evidence for the putative assignment of the background was gained by scanning fluorescence with excitation at 395 nm to get a more accurate spectrum of oxidised flavins. There was a much greater peak at the position of flavin fluorescence, consistent with the use of an excitation within the main absorbance range of the flavins (Fig. 4(b)). This peak was also absent during the early stages of growth. The variability in data is removed when cells are sampled in identical physiological conditions.

### 3.5. The reporter does not respond to agents that delay mitosis

The *RAD54* promoter region has been extensively studied and elements responsible for both constitutive and inducible transcription have been identified. One of these, the *mlu* box, is found in a number of cell cycle regulated promoters (Johnston and Johnson, 1995). Because DNA damage causes yeasts to delay cell cycle progression prior to mitosis, it was conceivable that part of the induction seen in MMS-treated cells was due to a prolonged period of *mlu* box regulation. To demonstrate that the biosensor's response was specific to DNA damage and not a cell cycle phenotype, cells were exposed to two different chemical agents which can delay mitosis in rather different ways. Hydroxyurea interferes with DNA synthesis at two levels: it inhibits ribonucleotide reductase, so limiting the supply of precursors for DNA synthesis, and it inhibits DNA ligase, an enzyme responsible for repairing nicks (single strand breaks) in the DNA backbone (Johnston, 1980). In contrast, nocodazole inhibits microtubule formation and thus primarily arrests cell growth by preventing the normal segregation of chromosomes during mitosis (Sentin, 1979; Kunkel, 1980). At concentrations of these two chemicals that inhibited cell growth to a similar extent to our MMS treatment regime, there was no detectable induction of the *RAD54*-GFP reporter (Table 3).

### 3.6. *RAD54*-GFP is regulated by *RAD9*, but is not regulated by *DUN1* or *POL2*

The repair pathways of eukaryotes are complex and incompletely understood. In characterising the first reporter construct, it was demonstrated that DNA damage-induction response was curtailed predictably by mutations in the genes *RAD53* (*MEC2/SAD1/SPK1*) and *MEC1*. The ease with which the reporter can be used allowed a further genetic characterisation. A number of different mutants were assessed in an otherwise isogenic

Table 3

The effect of mitotic inhibitors on GFP fluorescence. The induction levels of GFP fluorescence, relative to untreated cells, in transformed cells treated with hydroxyurea or nocodazole at the concentrations shown. The highest concentration in each case caused arrest of cell growth, whilst the two lower concentrations permitted growth at a rate comparable to that of cells treated with 0.01% MMS

% Hydroxyurea	Induction (relative to control)	% Nocodazole	Induction (relative to control)
Control, 0	1	Control, 0	1
0.001	0.95	0.0001	0.88
0.005	1	0.00045	0.99
0.01	1	0.0015	0.79

background (kindly provided by the Elledge laboratory: Zhou and Elledge, 1993; Allen et al., 1994).

The *RAD9* gene product has two roles in DNA repair and is known to be required for *RAD54* induction. It acts in the transient cell cycle checkpoint and as an inducer of genes involved in DNA metabolism and repair pathways (Aboussekhra et al., 1996). Induction of the *RAD54*-GFP reporter was reduced in a *rad9* deletion background compared with an isogenic wild type (Fig. 5). This confirms the previous work.

The *POL2* gene encodes DNA polymerase  $\epsilon$  and mutants are specifically defective in the response to replication blocks. The *POL2* protein has been implicated in DNA damage signalling in the S phase of the cell cycle (Navas et al., 1996), though it is unlikely to function outside of the S phase when the replication complexes are not assembled. Induction of the *RAD54*-GFP reporter was unaffected by the *pol2-12* mutation (Fig. 5).

The *DUN1* gene product is a protein kinase required for the DNA damage induced expression of two compo-

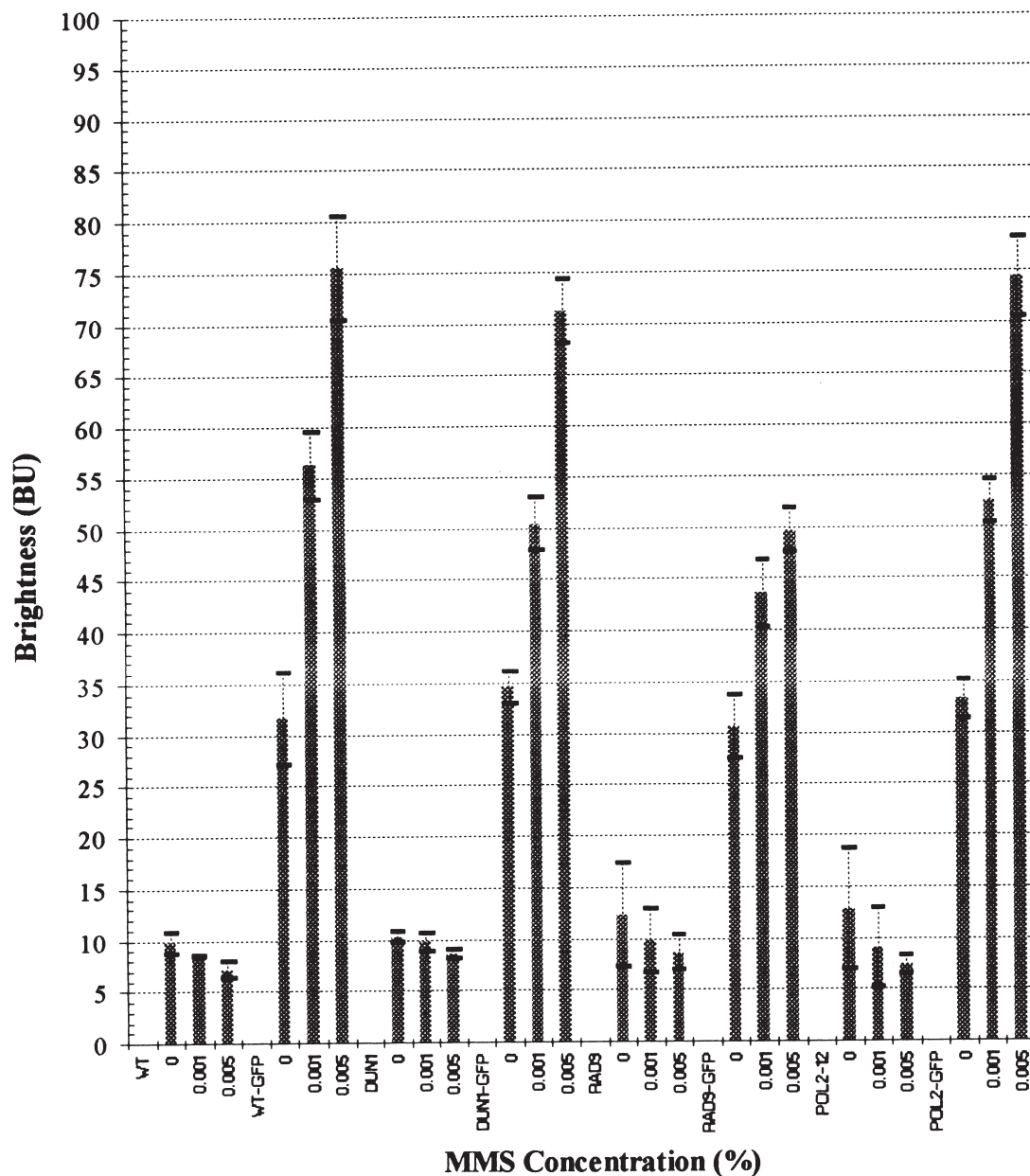


Fig. 5. *RAD54*-GFP induction in repair mutants. Each of the mutant strains (Table 1) was transformed with *WDH444*, then grown in the presence or absence of *MMS* at the concentrations indicated. Each experiment was repeated six times, and the data combined. The brightness in the absence of *MMS* reflects constitutive *RAD54* expression. These data were collected using the *LS5* spectrophotometer so brightness values cannot be compared with those from other figures. *WT*, *DUN1*, *RAD9*, and *POL2-12* are the untransformed mutant strains, *WT-GFP*, *DUN1-GFP*, *RAD9-GFP* and *POL2-12-GFP* are the transformed strains.

nents of ribonucleotide reductase, the products of *RNR2* and *RNR3* (Zhou and Elledge, 1993; Allen et al., 1994). Induction of the RAD54-GFP reporter was unaffected in a *dun1* deletion background compared with an isogenic wild type. Aboussekhra et al. (1996) observed normal DNA damage induction of *RAD51* (like *RAD54*, a member of the *RAD52* group of repair genes) in *dun1* deletion strains. They concluded *DUN1* might be specific only to a subset of *RAD9* dependent genes. Our result supports this view and demonstrates that both members of the *RAD52* epistasis group, *RAD51* and *RAD54*, are in one regulatory network (Fig. 5.)

#### 4. Discussion

A biological assay system for DNA damage has been developed for applications in biosensor technology. The combination of an improved GFP gene, a better host strain and the limitation of light scattering has allowed a shift away from the time consuming and complex assay of cell extracts to measurements of whole cells. Some simple data handling allows the GFP fluorescence peak to be isolated.

It has been possible to demonstrate that the reporter does not falsely respond to cell cycle delays induced either by interference with DNA replication or by interference with the mitotic spindle apparatus. The appearance of strong additional fluorescence late in the growth phase has been tentatively associated with the predicted appearance of oxidised flavins. Further work is being undertaken to confirm this. Relatively simple data handling might allow fluorescence output to be compensated mathematically if simultaneous measurements are taken of the peak height at 520 nm. A preferred solution would be to introduce a step to chemically reduce the oxidised flavins following the exposure regime.

Genetic analysis has demonstrated proper regulation in three different backgrounds: it is dependent on the checkpoint gene *RAD9*; it is insensitive to *DUN1* along with other genes with which it is known to interact; it is insensitive to the putative S-phase signalling pathway mediated by *POL2*. The latter result is consistent with the failure of hydroxyurea to illicit a response and underlines the specificity of the reporter to DNA damage which is separate from the accumulation of DNA replication intermediates.

The modular design of the reporter plasmid gives it great flexibility. The S65T-GFP gene was easily replaced with yEGFP. Similarly, by replacing the *RAD54* promoter with alternative, well-characterised inducible promoter elements, alternative biosensors may be made. If these were constructed using other GFP genes, such as the blue-shifted derivative, more than one output could be obtained from the same cell. Other elements can be replaced to change the selection that is placed on cells

to monitor successful transformation. Much of our development work has been done with replicative plasmid vectors. Integrating reporters have the advantage of stability compared to replicative plasmids and one such version of the RAD54-GFP reporter has already been constructed and demonstrated to work using cell extracts (Fig. 1: Walmsley et al., 1998). Multiple copies can be targeted to chromosome XII using sequences from the ribosomal DNA array (Lopes et al., 1989) and this should increase the signal output further.

An effective biosensor for DNA damage would have several key components. The first of these is a signal transduction system. The living yeast cell responds to DNA damage by activating DNA repair genes and slowing (or stopping) growth to allow time for repair to occur. In our system, it is one of the DNA-damage induced genes, *RAD54*, that has been adapted to provide an output signal in the form of a green fluorescent protein. The second component is a method of measuring the output signal and, in this respect, measurement of fluorescence is simple and reagent free compared to the assay of commonly used enzyme reporters. The only other widely used system which exploits microbes in environmental monitoring, relies upon detecting the decrease in light output when luminous bacteria, *Vibrio fischeri*, are poisoned by a sample (Fielden and Snook, 1996; Fielden et al., 1996). In this paper we have demonstrated that even using a relatively poorly expressed promoter, the signal from GFP is sufficient to allow estimation in whole living cells. Relatively simple technology is needed to fully exploit the RAD54-GFP reporter in a biosensing device.

Work is currently in progress to develop an automated, portable instrument which will assess the total genotoxicity of a test sample by combining the sample with the genetically modified yeast cells, and estimating the induced GFP concentration by fluorescence in a dedicated spectrophotometric cell. In the first instance, this is taking the form of a continuous flow-through instrument with analysis on-line, for applications in environmental monitoring. The development of a batch system or immobilisation of the cells is also being considered for discrete measurements.

Whilst we envisage the primary applications for this biosensor to be in environmental monitoring and the screening of novel pharmaceutical products (downstream from a combinatorial-chemistry drug-synthesis facility, for example), the generic technology of the reporter can be tailored to more specific sensing applications.

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