



## Yeast Functional Analysis Reports

# Green Fluorescent Protein as a Reporter for the DNA Damage-induced Gene *RAD54* in *Saccharomyces cerevisiae*

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The green fluorescent protein (GFP) of *Aequorea victoria* is now an established marker for gene expression and subcellular localization in budding yeast. Relatively high expression (greater than 2500 copies per cell) of GFP is required for direct microscopic visualization. This report provides a method for studying the expression of less highly expressed genes by the analysis of crude cell extracts—a simple and cheap alternative to the fluorescent activated cell sorter (FACS). The utility of this marker is demonstrated in a study of the expression of the *RAD54* gene. It is shown that the induction of the *RAD54* promoter leads to the accumulation of Rad54p and of GFP and that the fluorescence induction is correctly regulated. This method should allow the screening of large numbers of novel gene disruptants for their effects on *RAD54* expression and so identify *trans*-acting factors involved in the cellular response to DNA damage. © 1997 John Wiley & Sons, Ltd.

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KEY WORDS — DNA repair; GFP; *RAD54*; recombination

### INTRODUCTION

Genetic analysis in *Saccharomyces cerevisiae* has already revealed more than 100 genes directly or indirectly involved in DNA damage repair. Together with Rad51p and Rad52p, Rad54 protein 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). In contrast to its homolog in mammals (Kanaar *et al.*, 1996), the transcription of the *RAD54* gene is up-regulated by DNA damage in *S. cerevisiae*. This occurs as a consequence of exposure to UV and ionizing-radiation as well as to a variety of chemical agents

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including methyl methanesulfonate (MMS) and DNA cross-linking agents (Cole *et al.*, 1987; Averbeck and Averbeck, 1994).

Induction also occurs during meiosis, which is possibly related to the induction of transient DNA double-strand breaks during meiosis. Moreover, *RAD54* expression is induced during the cell cycle at the G1/S border and in response to mating-type heterozygosity. Diploid cells with mating-type heterozygosity show an increased resistance to ionizing radiation when compared with haploids and with diploids homozygous for *MAT* (reviewed in Game, 1993).

*RAD54* is also induced upon expression of the *EcoRI* restriction endonuclease in *S. cerevisiae* (Cole *et al.*, 1987). It is not known how the DNA damage signal is transmitted to the *RAD54* promoter, nor is it known how this signal or the

activity of the repair systems is linked to the cell cycle control. Although responding to a wide variety of DNA damages, *RAD54* does not respond to other forms of stress such as temperature shock (Cole *et al.*, 1987).

The precise role of the Rad54 protein in recombinational repair remains unclear. Its central role in the machinery of recombinational repair is underlined by its interaction with Rad51p (Jiang *et al.*, 1996; Clever *et al.*, 1997). Rad51 protein is a RecA homolog of *S. cerevisiae* (reviewed in Heyer, 1994) which forms a protein:DNA filament active in homology search and strand exchange (Sung, 1994). Sequence comparisons suggest that Rad54p is a member of the Snf2p/Swi2p family of DNA-dependent/activated ATPase (Eisen *et al.*, 1995).

In previous studies of *RAD54* regulation, the promoter region was fused to the  $\beta$ -galactosidase gene and the reporter was shown to be coordinately regulated (Cole *et al.*, 1987; Averbeck and Averbeck, 1994). The constitutive level fluctuates about three-fold in synchronized cells, reaching a maximum at the onset of S-phase. Induction above this level varies depending on the nature and magnitude of the DNA damage. Ionizing and UV irradiation results in levels three to six times above basal level, and expression remains at the induced level for about 8 h following radiation. MMS can induce to an even higher level (up to 12-fold). Despite the cell cycle regulation at G1/S, DNA damage induction is not cell-cycle-dependent. Induction in response to DNA damage still occurs when the *MluI*-box cell cycle control element is removed (Johnston and Johnson, 1995) and cells arrested with mating pheromone or grown to stationary phase show induction kinetics similar to those of growing cells.

The use of enzyme assays in any reporter system is inevitably complex and as a consequence difficult to reproduce. In this work we report the development of a new reporter system for the *RAD54* gene. It is based upon a promoter fusion to the jellyfish green fluorescent protein (GFP) which can be estimated by simple, reagent-free, fluorescence measurement. These properties might allow a more detailed and sensitive dissection of the kinetics of *RAD54* induction. They will also facilitate a screen for new genes involved in the DNA damage repair process by virtue of their impact on the expression of the GFP gene. The systematic cloning and sequencing of the yeast genome has led to the generation of a large collection of strains carrying single gene disruptions.

This new reporter construct can be used to screen these collections for strains in which there is either constitutive over-expression or failure to induce the *RAD54* promoter.

#### *The green fluorescent protein*

The wild-type GFP of the jellyfish *Aequorea victoria* consists of 238 amino acids. It absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm). The fluorescence is very stable, persisting after treatment with formaldehyde, and virtually no photo bleaching is observed (reviewed by Cubitt *et al.*, 1995). It has been suggested that effective measurement of fluorescence requires about one million copies of GFP per mammalian cell or 12 000 copies per yeast cell and this limits its usefulness in whole cells (Niedenthal *et al.*, 1996). The strong visible absorbance and fluorescence comes from a *p*-hydroxybenzylidene-imidazolidinone fluorophore, which is generated by cyclization and oxidation of the Ser-Tyr-Gly amino acid residues at positions 65–67 of GFP. This reaction appears to be less effective at high temperatures, possibly because of misfolding, and for this reason all cell cultures in this work were grown at 25°C. Formation of the final fluorophore is spontaneous. The reaction only requires molecular oxygen and proceeds with a time constant of approximately 4 h at 22°C and atmospheric pO<sub>2</sub>. The S65T derivative (Ser65 to Thr65) used in this study, has an excitation maximum at 488 nm, an emission maximum at 511 nm and the final oxygen-requiring step is completed in about 75 min (Cubitt *et al.*, 1995). The main experimental implications of this are that for optimal fluorescence the *S. cerevisiae* samples need to be stored following the inhibition of protein synthesis or cell killing.

## MATERIALS AND METHODS

### *Strains and media*

In this study we used two haploid *S. cerevisiae* strains (FF18734, *MAT $\alpha$  leu2-3,-112 ura3-52 trp1-289 lys1-1 his7-2* and FF18984, *MAT $\alpha$  leu2-3,-112 ura3-52 lys2-1 his7-1*) and the diploid WDHY669 made from these two haploid strains. The *mec1* strain was TWY308 (*MAT $\alpha$  mec1-1 ura3 trp1*), and the *rad53* (= *mec2*) strain was TWY312 (*MAT $\alpha$  mec2-1 ura3 his7 trp1*) (Weinert *et al.*, 1994). The media YP plus 2% glucose (YPD) and

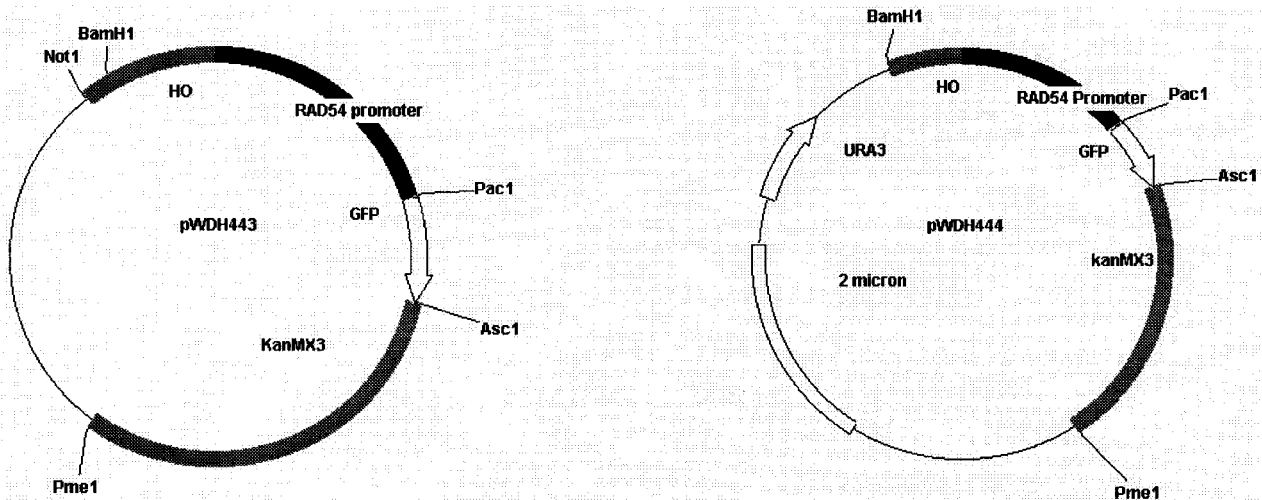


Figure 1. *RAD54-GFP* reporter plasmids. The construction is detailed in the text. pWDH443 can be cleaved at a unique *Bam*HI site to target integration to the *HO* locus. A fragment of the 2  $\mu$  plasmid allows pWDH444 to replicate autonomously in *S. cerevisiae*. Sequences without annotation are for replication and selection in *E. coli*.

synthetic media plus 2% glucose (SD) were prepared as described (Kaiser *et al.*, 1994). G418 (geneticin) was added to a final concentration of 200  $\mu$ g/ml (w/v) to both liquid and solid media where appropriate. MMS was added as a liquid to induce DNA damage at the concentrations indicated. Cells were treated with 0.02% sodium azide and incubated on ice with shaking for 15 min before proceeding with the fluorescence measurement. This treatment prevents further cell growth. Control experiments (data not shown) showed that sodium azide did not affect the fluorescence due to GFP.

#### Construction of *RAD54-GFP* reporter plasmids

Two plasmids were constructed: one integrative and one replicative (Figure 1). Both share a common skeleton consisting of a derivative of the plasmid pFA-kanMX3 (Wach *et al.*, 1994) in which the  $\beta$ -galactosidase gene has been replaced with DNA encoding the S65T derivative of GFP (pFA-kanMX3GFP).

In the first construction step, cosmid DNA (from the clone used to derive the DNA sequence for *RAD54* in the genome sequencing project) was used as a template to amplify a fragment extending from the start codon of *RAD54* upstream to the next gene. The primers were designed to facilitate subsequent cloning steps. The *RAD54*-distal primer added a *Bam*HI site and the *RAD54*-proximal primer included the ATG of *RAD54*

followed by an in-frame *Pac*I site. The PCR fragment was cut with *Bam*HI and *Pac*I and ligated into *Pac*I-*Bam*HI-cut pFA-kanMX3GFP.

In the second step, a fragment of the DNA was added to allow targeted integration. An internal *Dra*I fragment (759–1655) of the *HO* gene was chosen: first, because integration at *HO* only affects homothallic mating ability and second, because the fragment contains a unique *Bam*HI site. The fragment was purified from a *Dra*I-digested PCR product of the *HO* gene, ligated into the plasmid DNA which had been cut at the unique *Bam*HI site and treated with *Poll*I and *CIP* to produce blunt and un-self ligatable ends.

A number of clones with the predicted insert were identified and one, named pWDH443, was used in the subsequent study. Plasmid DNA linearized with *Bam*HI was used to transform FF18984 yeast cells to G418 resistance and eight of eight transformants examined contained correctly integrated plasmid (data not shown).

In the third step, an autonomously replicating derivative was constructed. pWDH443 was cut with *Bam*HI and *Pme*I, liberating a fragment extending from within the *HO* insert, through the *RAD54* promoter/GFP fusion and to the end of the KanMX3 module. This was ligated to a fragment of DNA containing the *S. cerevisiae* 2  $\mu$  plasmid replication origin and the *URA3* gene, together with *Escherichia coli* plasmid replication and ampicillin-resistance factors (derived from

pRDK249; Johnson and Kolodner, 1991). Transformants were co-selected on LB plates containing ampicillin and kanamycin and one, named pWDH444, was used in subsequent studies. Uncut plasmid DNA was used to transform yeast cells to G418 resistance and ten of ten transformants studied contained free plasmid.

#### Measurement of whole cell fluorescence

Cells were grown to late stationary phase ( $OD_{600}=25-30$ ) in YPD (G418 was added when appropriate), harvested by centrifugation and washed twice in distilled water. This step was essential to remove traces of YPD which itself contains material which fluoresces at the emission wavelength optimum for S65T GFP (data not shown). The cells were then diluted to an  $OD_{600}$  of between 0.2 and 0.35. Fluorescence was determined using a 10 nm excitation window at 488 nm and a 5 nm emission window at 511 nm. The brightness units for whole cells used in this paper were calculated as fluorescence at 511 nm divided by the  $OD_{600}$  value.

#### Measurement of cell extract fluorescence

The GFP fluorophore is very resistant to chemical and enzyme attack (Ward and Bokman, 1982). These properties were taken into account in the following modification of the method of Johnson and Kolodner (1991), which was used in all but the first assessment cell extracts. Whole cells were dosed with 0.02%  $NaN_3$  and agitated on ice for 75 min to allow fluorophore development. Following washes with water and with extraction buffer (20 mM-Tris-HCl, pH 7.5, 0.1 M-NaCl), aliquots of cells (between 1 and 10  $OD_{600}$  units) were re-suspended in 0.25 ml crushing buffer (20 mM-Tris-HCl, pH 7.5, 0.1 M-NaCl, 1 mM-EDTA, 1 mM-PMSF) and added to 0.25 ml of 0.45  $\mu$ m diameter glass beads in a 1.6 ml microcentrifuge tube. Cells were broken by vortexing for 1 min, three times, with an intervening period of 1 min on ice between treatments. The cell debris and buffer were then pipetted into a fresh tube. The beads were washed with a further 0.25 ml crushing buffer and the supernatants were combined. The extract was adjusted to 0.1 M-Tris, pH 11 by the addition of 1 M-Tris base and cleared by centrifugation. The absorbance and fluorescent properties of this extract were stable for at least 24 h at room temperature (data not shown). Yield of extract was estimated by measuring  $OD_{280}$ . Control exper-

iments indicated that this gave a figure directly proportional to the yield of protein estimated by the Bradford assay (Bradford, 1976), in the  $OD_{280}$  range 0.0-0.7 (Figure 2). Fluorescence was determined using a 10 nm excitation window at 488 nm and a 5 nm emission window 511 nm. Brightness units for protein extracts in this work are defined as fluorescence at 511 nm divided by absorbance at 280 nm. These units are independent of the amount of protein measured. In this crude extraction protocol, the extent of breakage varies. In one experiment, a single culture was divided into two equal parts and extracts were prepared. The  $OD_{280}$  yield varied by 50% between the extracts but the brightness was identical. The fluorescence due to unidentified autofluorescent components of the cell varies between a value of 1 and 2 brightness units.

#### Western blotting

Proteins were separated on polyacrylamide gels according to the method of Laemmli (1970), immunoblotted with rabbit anti-Rad54p antibodies (Clever *et al.*, in preparation), and visualized using CDP-Star (Boehringer Mannheim) according to the manufacturer's instructions. The specificity of the anti-GFP antibodies is demonstrated in Figure 6, the specificity of the anti-Rad54p antibodies has been confirmed by blotting extracts of *rad54* $\Delta$  cells (Clever *et al.*, in preparation).

## RESULTS

#### *MMS induces weak fluorescence of whole cells containing the RAD54-GFP reporter*

Cells with and without the reporter constructs were inoculated into YPD and allowed to grow overnight in the presence or absence of 0.01% MMS. The cultures were indistinguishable by fluorescence microscopy. Fluorescence was then estimated spectrophotometrically. Four independent transformants of each of the haploid and diploid strains were allowed to grow overnight in the presence or absence of 0.01% MMS. There was no increase in fluorescence from cells (measured as described above) containing the integrative vector. With the replicative plasmid there was a statistically significant increase (40%) in fluorescence associated with MMS-treatment in both of the haploid cultures, and a small increase was seen in the diploid cultures (data not shown).

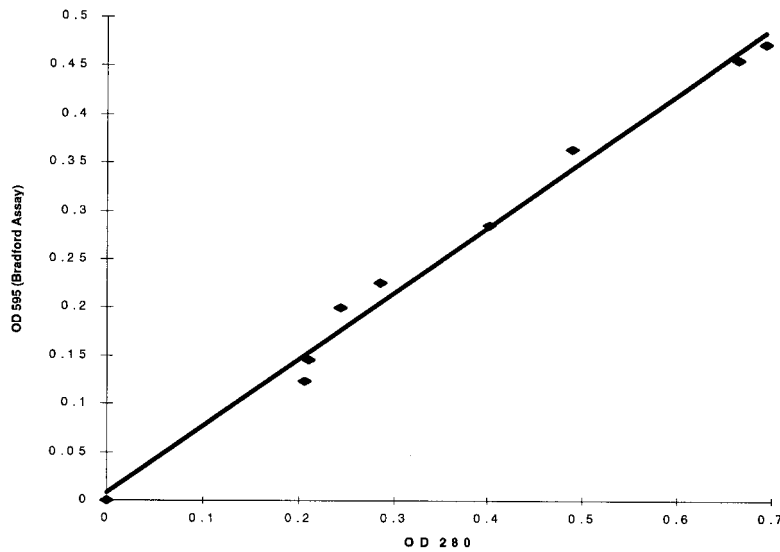


Figure 2. Measurement of optical density at 280 nm provides a reliable estimate of protein yield in cell extracts. Protein extracts were prepared from eight different cultures as described in Materials and Methods. Samples (5  $\mu$ l in a total volume of 1 ml) were used in a Bradford assay and 100  $\mu$ l samples, in total volume of 2.5 ml, were used to measure OD<sub>280</sub> directly.

With such low fluorescence values, light scattering from cells is an important source of error. To avoid this problem, subsequent experiments were performed using cell extracts.

*MMS treatment strongly induces fluorescence in cell extracts from cells containing the RAD54-GFP reporters*

A crude protein extract (Johnson and Kolodner, 1991) was prepared from untransformed cells and from MMS-exposed cells containing the replicative plasmid. There was very much greater fluorescence in the extracts from MMS-treated plasmid-containing cells (seven-fold) and induction was greatest in the haploid cultures (data not shown). The use of extracts effectively reduced signal to noise ratio. The extraction method was then modified to include a period of incubation to allow maturation of GFP and a pH 11 treatment to selectively denature proteins other than GFP. Following the estimation of brightness, the samples were adjusted to 1% SDS and heated for 15 min at 65°C. This treatment is known to abolish fluorescence of GFP (see Figure 3). These modified methods further increased the signal to noise ratio and were then used in a series of experiments

designed to confirm that the fluorescence was due to the production of GFP under the control of the *RAD54* promoter.

*Fluorescence is reduced in diploid cells compared to haploids. GFP is produced at a low level without MMS treatment*

The haploid strains FF18734 and FF18984 and the diploid strain WDHY669, untransformed or transformed with either the integrative or the replicative plasmid were incubated overnight with or without 0.01% MMS. The stability of GFP allows this long incubation and results in accumulation of the protein. The activity of the integrated, single-copy reporter was readily detectable (Figure 3). The result with the replicative reporter reproduced the first experiment. Moreover, the signal to noise ratio was increased giving an enhancement of fluorescence of up to 12-fold in the MMS-treated replicative plasmid containing haploid FF18984. There was consistently less fluorescence from the diploid cells. In addition, all transformants showed a degree of increased brightness even without MMS, with the replicative plasmid containing strains showing the greatest background (Figure 3).

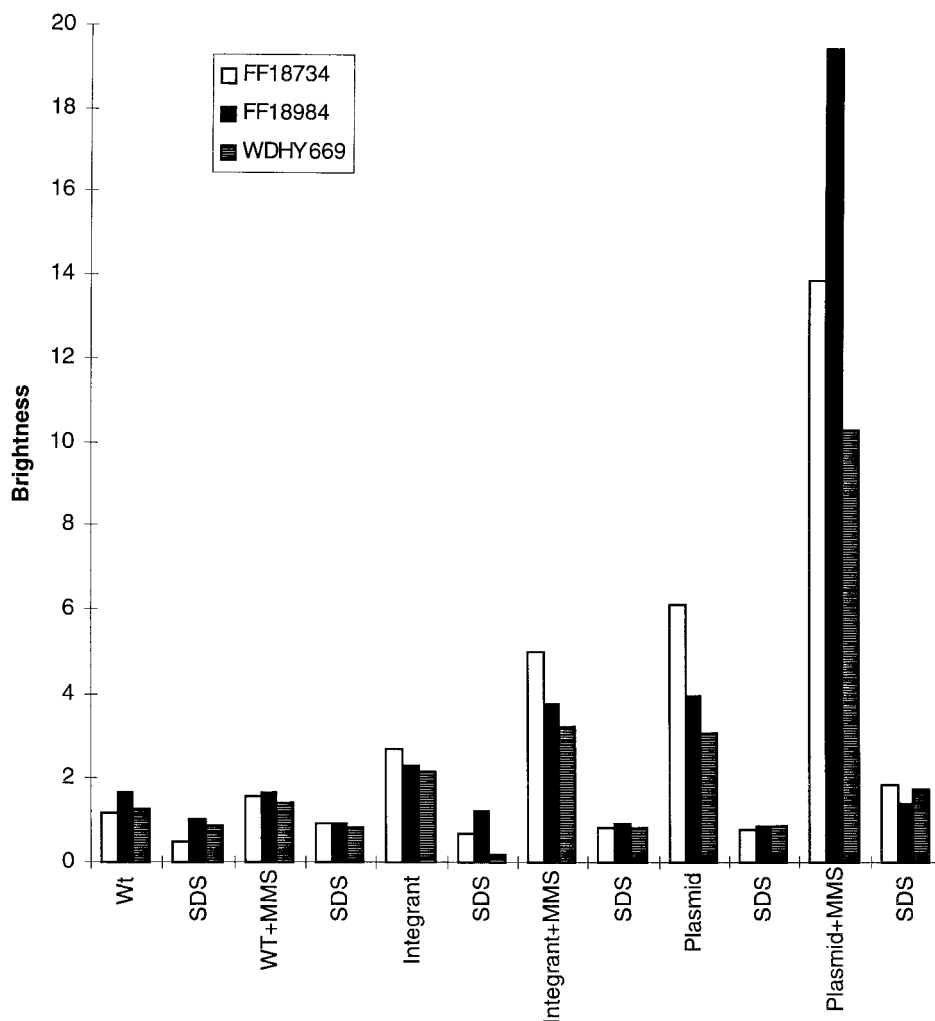


Figure 3. The *RAD54-GFP* reporters increase the fluorescence of cell extracts and are stimulated by exposure to MMS. Cultures of either untransformed cells (wt) or cells containing pWDH443 (integrand) or pWDH444 (replicating plasmid) were each split into two, one half was exposed overnight to 0.01% MMS and the other half was left unexposed. Extracts were prepared as described and brightness was assessed. Each group of samples was then treated with SDS at 65°C and brightness was determined again.

#### *Fluorescence induction by MMS is dose-dependent in cell extracts*

Cultures of the haploid strain FF18984, with and without the replicative plasmid, were incubated overnight either with no MMS, 0.002% MMS or 0.01% MMS. Extracts were prepared and brightness was estimated as described. In this simple dose-response experiment, brightness increased with MMS concentration and the brightness was sensitive to the SDS treatment. As seen

before, there was some fluorescence in untreated plasmid-containing cells (Figure 4).

#### *Fluorescence becomes apparent after 1 h exposure to 0.05% MMS in cell extracts*

The maximum induction of *RAD54* reported for the  $\beta$ -galactosidase gene fusion was achieved using 0.05% MMS (Cole *et al.*, 1987). This concentration of MMS almost completely arrested cell growth

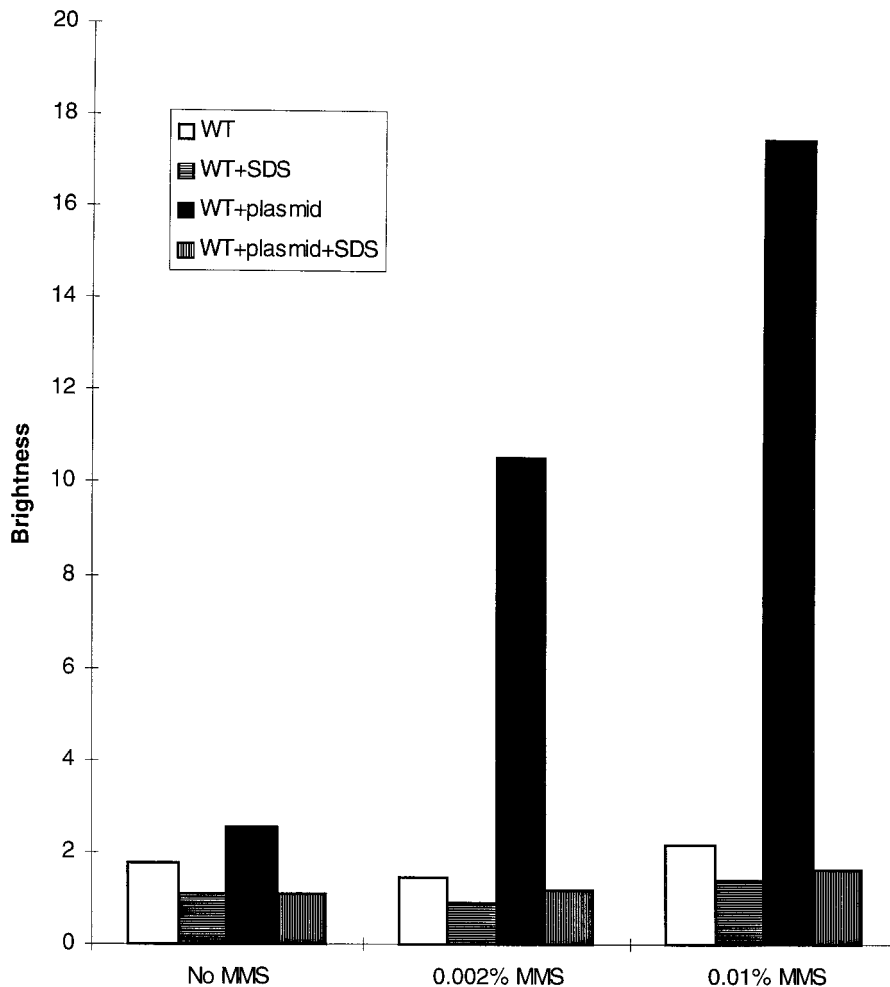


Figure 4. Fluorescence induction by MMS is dose-dependent in cell extracts. Cultures of the haploid strain FF18984, with or without the plasmid-borne *RAD54-GFP* reporter (pWDH444), were split into three parts and treated overnight with the indicated concentration of MMS (0%, 0.002%, 0.01%). Following estimation of brightness, the samples were treated with SDS at 65°C to quench GFP brightness.

in our strains. Brightness was monitored in the haploid strain FF18984 harboring either the integrative plasmid or the replicative plasmid following the addition of MMS (0.05%) to growing cells. There was a continuing increase in brightness beginning 1 h after the addition of MMS (Figure 5b and c) in extracts from both the reporter-containing strains, but not in the control cells (Figure 5a). Taken together, the data shown in Figures 4 and 5 show that induction of brightness occurred in response to DNA damage.

*Induction of fluorescence is associated with induction of GFP protein and Rad54 protein*

To confirm that increase in fluorescence corresponds to the induction of GFP and Rad54 proteins, extracts were prepared from FF18984 cells harboring either the integrative plasmid or the replicative plasmid exposed to 0.01% MMS or as control without exposure to MMS. The proteins were separated using polyacrylamide gel electrophoresis, prepared for immunoblotting and probed concomitantly with anti-GFP antibody (Clontech)

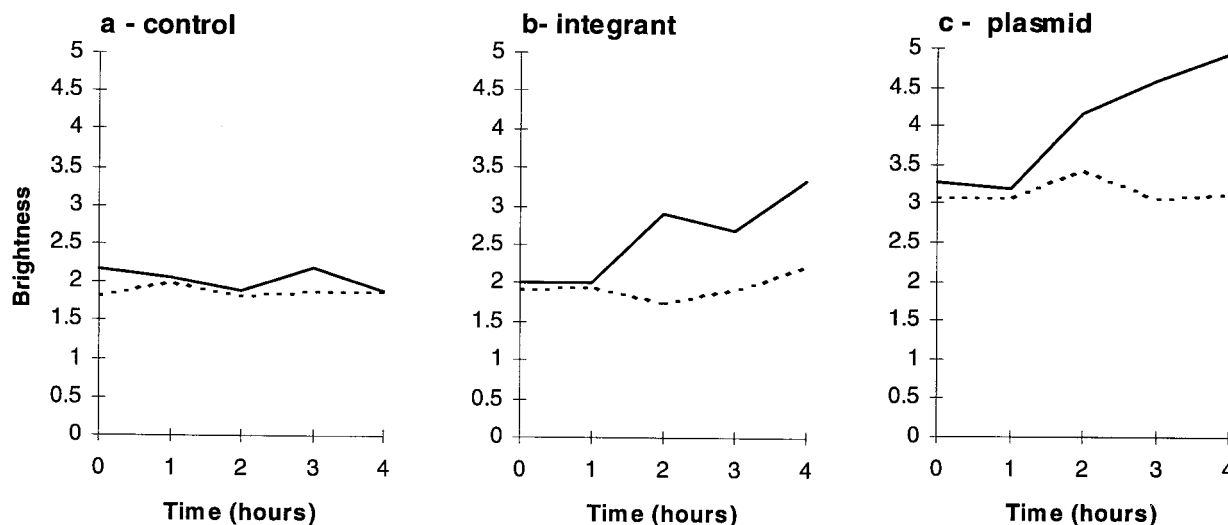


Figure 5. Exposure to MMS causes an increase in fluorescence after 1 h. Cultures of the haploid strain FF18984, with or without the *RAD54-GFP* reporters, were grown to the early exponential phase of growth and split into two. One sample was treated with MMS (0.05%, solid line) at time zero and the other with an equal volume of water (dashed line). (a) Control without reporter, (b) integrated vector (pWDH443), and (c) replicating reporter (pWDH444).

and with anti-Rad54p antibody. In each case, induction of Rad54p by MMS was apparent. GFP became detectable in the strain containing the integrated plasmid following MMS induction. In the strain containing the replicative plasmid, a low level of GFP was seen without MMS and a greatly increased amount following MMS exposure (Figure 6).

#### *Induction of fluorescence is dependent on the DNA damage checkpoint*

In order to determine how effective this reporter might be in identifying new genes involved in the repair pathway, the predicted dependence of *RAD54* induction on two DNA damage checkpoint genes was tested. *RAD53* (also known as *MEC2*, *SPK1*, *SAD1*) encodes a central protein kinase in the DNA damage checkpoint pathway (reviewed in Elledge, 1996). *MEC1*, an ATM homolog of *S. cerevisiae*, encodes another DNA damage checkpoint kinase that acts upstream of Rad53p (Sun *et al.*, 1996; Sanchez *et al.*, 1996; for review see Elledge, 1996). Strains with mutant alleles of *RAD53* (*mec2-1*) and *MEC1* (*mec1-1*) were transformed with the replicative plasmid. These mutant strains are by their nature super-sensitive to DNA-damaging agents and also temperature-sensitive for growth. Two different exposure regimes were followed: overnight expo-

sure to 0.01% MMS or 3 h exposure to 0.01% MMS, both at the permissive temperature (25°C). Extracts were prepared and brightness was measured with or without treatment with MMS. There was only very slight induction in both mutants after 3 h exposure compared to wild-type cells. After overnight exposure the fluorescence of the *mec1-1* extract increased by about 75%, whereas in *mec2-1* extracts no induction was observed (Figure 7). For comparison, extracts from wild-type cells showed a 350% increase in fluorescence under these conditions (Figure 7). Whilst the mutant strains are not isogenic with the strains used in other experiments, the level of induction in different wild-type strains does not vary to this extent (data not shown). From these data we conclude that the response of the *RAD54-GFP* reporter is consistent with its being controlled by the DNA damage checkpoint for MMS-induced gene expression.

#### DISCUSSION

In this work a reporter is described which is activated by the signals which normally induce transcription of the *RAD54* gene in response to DNA damage. Rad54p is not an abundant protein: fluorescence from the reporter is not visible using fluorescent microscopy, and is only detectable in

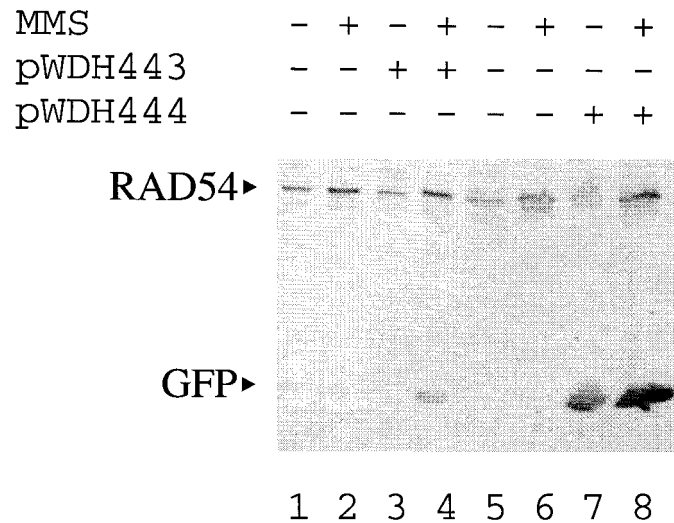


Figure 6. Exposure to MMS causes induction of Rad54p and GFP. Equal amounts (200  $\mu$ g) of protein extracts were analysed by immunoblotting for their content of Rad54p and GFP. Cultures of strain FF18984 either untransformed (lanes 1, 2, 5, 6), or transformed with pWDH43 (lanes 3, 4) or with pWDH444 (lanes 7, 8) were treated with MMS (lanes 2, 4, 6, 8) or left untreated (lanes 1, 3, 5, 7). The positions of Rad54p and GFP are indicated.

whole cells using fluorescent spectroscopy when multiple copies of the reporter are present. The method for assessing brightness associated with GFP production can be used in other instances where weak promoters are studied. Improved versions of the reporters described here are currently being constructed utilizing yeast-enhanced GFP (Cormack *et al.*, 1997) and these should not only increase the sensitivity of assays but also allow assessment in whole cells, rather than requiring the preparation of cell extracts. GFP is relatively stable compared to most proteins, however, and it is important not to draw conclusions from this type of data concerning the amount of protein in a cell. GFP fluorescence is bound to accumulate to a greater extent than the protein of interest as a consequence of its protease resistance. Moreover, post-translational regulation of the protein may occur which cannot be assessed by this system.

It is known from previous studies that, in addition to the DNA damage-induced transcription of *RAD54*, there is a level of cell cycle regulation. The increased fluorescence in strains containing the RAD54-GFP construct, when not induced by MMS (Figures 4 and 5), suggests that the reporter is transcribed at a basal level responding to this control.

Six lines of evidence have been presented to demonstrate that induction of fluorescence was due to the activity of the reporter rather than the induction of other uncharacterized autofluorescent components of the cell. First, induction of fluorescence was dependent on the presence of the reporter constructs. Second, induction shows a dosage dependence on MMS treatment, linking fluorescence to DNA damage (Figure 4). Third, the kinetics of fluorescence induction corresponds to the reported induction of *RAD54* RNA (Figure 5). Fourth, the induction of fluorescence corresponds to both the production of GFP and Rad54p (Figure 6). Fifth, full induction of fluorescence is dependent on an intact DNA damage checkpoint (Figure 7). Sixth, the observed fluorescence had the physico-chemical characteristics of the GFP fluorescence. Taken together, we conclude that the RAD54-GFP reporter construct responds in DNA damage-induced gene expression similar to the native *RAD54* gene. Therefore, we believe that this reporter will be useful in screening for additional functions in this pathway. Any ORF disruptions which affect plasmid stability will appear as false positives, but all positives will be re-screened, initially using the integrating vector.

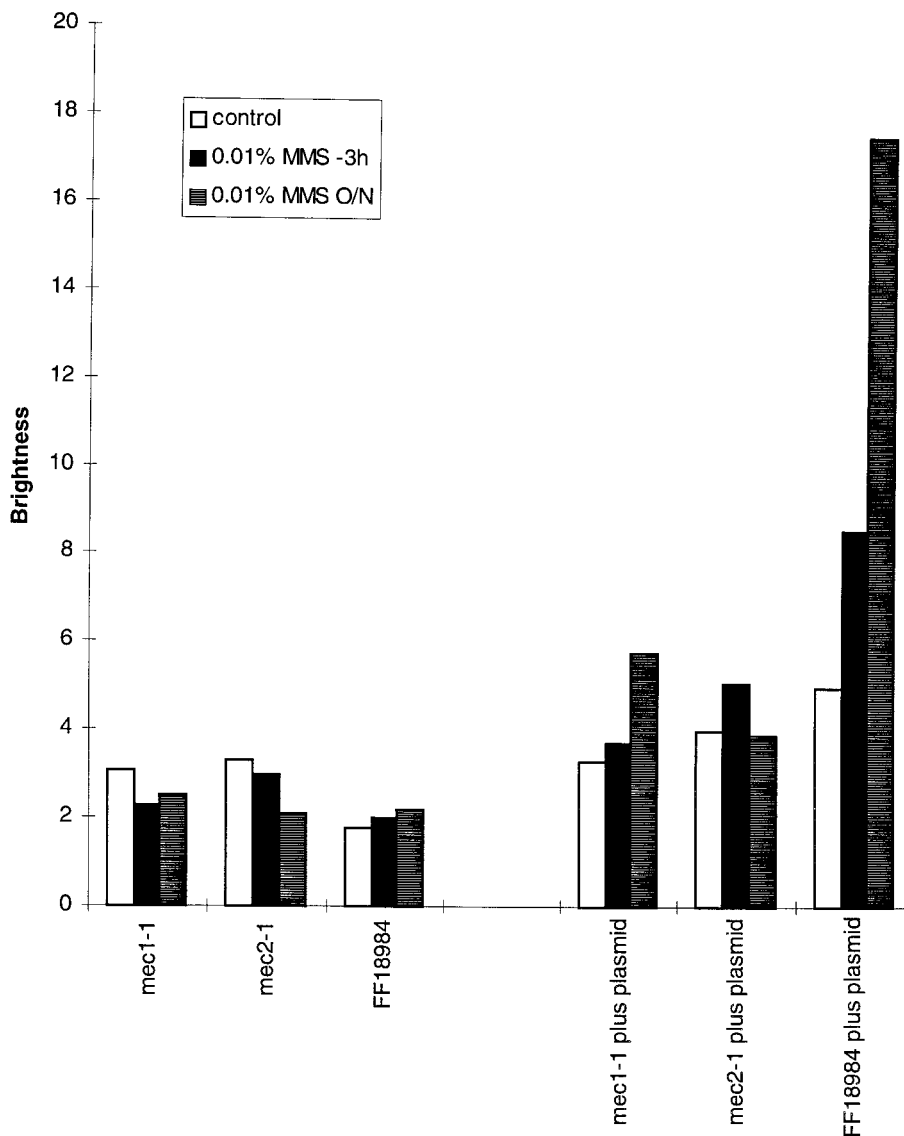


Figure 7. Induction of fluorescence is dependent on DNA damage checkpoints. Strains were wild-type (FF18984), *mec1-1* (TWY308), and *mec2-1* (*rad53*; TWY312). Brightness was measured as described and experimental details are given in the text.

It has been demonstrated by immunoblotting that there is an increase in Rad54 protein following MMS induction (Figure 6). This is predicted from the GFP data presented here as well as from previous data using a *RAD54* promoter  $\beta$ -galactosidase fusion, but has not been reported previously. What was not predictable, however, is the apparent lower level of induction in the diploid cell line compared to the haploid cells. This suggests that there may be post-transcriptional con-

trol of the Rad54 protein level and/or activity, given the increased efficiency of repair in diploids compared to haploids. It will be interesting to discover whether this can be confirmed biochemically when the role of Rad54p is better understood.

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