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DNA-damage induction of *RAD54* can be regulated independently of the *RAD9*- and *DDC1*-dependent checkpoints that regulate *RNR2*

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Abstract DNA damage checkpoints regulate a number of physiological responses after DNA damage. The transcriptional level of many genes is specifically induced in response to genotoxic stress in a checkpoint-dependent manner. The regulation of DNA damage-induced transcription of *RAD54* and *RNR2* by *RAD9*, *DDC1*, *DUN1*, *CRT1* and *MBP1* was investigated in *Saccharomyces cerevisiae*, using green fluorescent protein reporter assays and Northern blots. *RAD54* and *RNR2* reporter activity in response to the DNA damaging agent, methyl methanesulphonate, was measured in *ddc1-Δ*, *rad9-Δ*, *ddc1-Δ/rad9-Δ*, *dun1-Δ*, *crt1-Δ* and *mbp1-Δ* mutants and was compared with that of the wild type. *RAD9* and *DDC1* were shown to be required for a full *RNR2* transcriptional response, although with the double mutant, *ddc1-Δ/rad9-Δ*, no additive effect on *RNR2* induction was observed. *RAD54* promoter activity was not significantly reduced in either *rad9-Δ* or *ddc1-Δ* mutants and was only partially reduced in the *rad9-Δ/ddc1-Δ* strain, suggesting that DNA damage induction of *RAD54* must depend on other genes, in addition to *RAD9* and *DDC1*. In the *dun1-Δ* mutant, *RNR2* promoter activity was lowered, whilst that of *RAD54* was increased, confirming that *DUN1* is

required for transcriptional induction of *RNR2*, but is not required for damage-induced transcription of *RAD54*. Analysis of the *crt1-Δ* strain confirmed that *RNR2* is regulated via the *CRT1* repressor pathway, downstream of *DUN1*, but *RAD54* is not. *MBP1* was shown to be required for transcription of *RNR2*, but was not needed for transcription of *RAD54*. These results indicate that *RNR2* and *RAD54* are regulated in different ways.

Keywords DNA damage · *RAD54* · *RNR2* · *Saccharomyces cerevisiae*

Introduction

Exposure of *Saccharomyces cerevisiae* to DNA-damaging agents activates cellular stress responses that result in specific alterations in patterns of gene transcription and an active inhibition of cell division. This response is designed to allow the cells to survive and maintain genetic integrity and is regulated by DNA damage checkpoint genes (for reviews, see Elledge 1996; Weinert 1998; Lowndes and Murguia 2000). Genes known to be transcribed in response to DNA damage in *S. cerevisiae* include *RAD54* (Cole et al. 1987; Cole and Mortimer 1989) and *RNR2* (Elledge and Davis 1987, 1989a; Elledge et al. 1993). Rad54 protein exhibits a double-stranded DNA-dependent ATPase activity and this is thought to facilitate recombinational repair mediated by *RAD51* (Petukhova et al. 1998). Rad51 protein carries out homologous pairing and DNA strand exchange (Sung 1994). It is the eukaryotic equivalent of *Escherichia coli* RecA protein. *RNR2* encodes one of the three subunits of ribonucleotide reductase which catalyses the rate-limiting step in dNTP synthesis that is necessary for replication and repair (Reichard 1988).

In order to measure transcription in response to DNA damage, we developed an assay using green fluorescent protein (GFP) as a reporter for either *RAD54* or *RNR2* promoter activity in intact, growing yeast cells. In earlier studies, we showed that both promoters are

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activated by the alkylating agent methyl methanesulphonate (MMS) in a dose-dependent manner (Billinton et al. 1998; Afanassiev et al. 2000) and both have been used to assess the genotoxic potential of a range of chemical agents (Afanassiev et al. 2000). Gasch and co-workers (2001) studied the global transcriptional response to DNA-damaging agents using DNA microarrays and found that these genes did not respond to other stresses, such as heat shock, oxidative stress, reductive stress, osmotic shock or amino acid starvation. This confirmed the specific regulation of *RAD54* and *RNR2* by DNA damage. Averbek and Averbek (1994) assayed induction of *RAD54* and *RNR2* by various DNA-damaging agents, using a β -galactosidase based assay in *RAD54* and *RNR2-lacZ* fusion strains.

This study focuses on the control of DNA damage-induced transcription of *RAD54* and *RNR2* by key genes involved in the DNA damage response. DNA lesions are believed to be sensed at the G1 and G2 cell-cycle checkpoints by gene products that act in two additive pathways. These are defined by: (1) *RAD9* and (2) the *RAD24* subclass, which includes *DDC1*, *RAD17* and *MEC3* (Lydall and Weinert 1995; De la Torre-Ruiz et al. 1998; Lowndes and Murguia 2000). Once damage is detected, signals are transduced through four protein kinases, encoded by *MEC1*, *RAD53*, *CHK1* and *DUN1* (for reviews, see Weinert 1998; Sanchez et al. 1999). This ultimately prevents or delays the cell's entry into mitosis.

We investigated DNA damage-induced transcription of *RNR2* and *RAD54* in strains deleted at the following loci: *RAD9*, *DDC1*, *DUN1*, *CRT1* and *MBP1*. *RAD9* and *DDC1* were chosen to represent the two proposed parallel branches of the sensory pathway, whilst *DUN1* encodes a protein kinase. *CRT1* is an effector gene, which acts downstream of *DUN1*. *RAD9* is essential for G2 checkpoint arrest (Weinert and Hartwell 1988) and G1 delay (Siede et al. 1993) and has been reported as being required for DNA damage-induced transcription of both *RAD54* and *RNR2* (Aboussekhra et al. 1996). The *DDC1* checkpoint gene is required to delay cell cycle progression when DNA is damaged during G1, G2 and S (Longhese et al. 1997). *DUN1* appears to function in the transcriptional induction of ribonucleotide reductase (Zhou and Elledge 1993) and is also involved in cell cycle arrest at G2/M (Gardner et al. 1999). However, *DUN1* does not appear to play a major role in the damage-specific induction of *RAD54* or *RAD51* (De la Torre-Ruiz and Lowndes 2000). *CRT1* is a DNA-binding protein that recruits general repressors to the promoters of damage-inducible genes, e.g. the Ssn6/Tup1 complex to the *RNR2* promoter (Huang et al. 1998). *MBP1* codes for a transcription factor which, together with the *SWI6* gene product, forms the MBF protein complex. MBF binds to *Mlu* I cell-cycle boxes (MCBs) found in the promoter regions of a number of genes regulated at G1 (Koch et al. 1993). The sequence upstream of *RAD54* contains two MCB elements (Saccharomyces genome database; <http://genome-www.stanford.edu/Saccharomyces>), but there are none upstream of *RNR2*.

In this study, we show that *RAD9*, *DDC1* and *DUN1* are all required for normal DNA-damage induction of *RNR2*, and we confirm that the *CRT1* repressor regulates *RNR2*. In contrast, we show that *RAD9*, *DDC1*, *DUN1* and *CRT1* do not play a major role in DNA damage-induced transcription of *RAD54*. *MBP1* is shown to be required for a normal *RNR2* DNA-damage response, but is not required for DNA damage-induced transcription of *RAD54*.

Materials and methods

Strains and media

The isogenic *S. cerevisiae* yeast strains FF18984 *MAT α* and FF18985 *MAT α* were kindly provided by Dr. F. Fabre. A list of all the strains used and generated in the course of this study are shown in Table 1. Strains were routinely grown at 30 °C on YPD medium (1% yeast extract, 2% peptone, 2% glucose) and/or minimal SD medium (0.67% yeast nitrogen base, 2% glucose), supplemented with the relevant auxotrophic requirements. For the selection of cells transformed with GFP-reporter plasmids, cells were grown on SD medium lacking uracil. For isolation of kanamycin-deletion mutant strains, cells were grown on YPD agar plates containing 200 μ gml⁻¹ geneticin (G418). Reporter assays were carried out using cells grown in the minimal defined medium, F1 (Walmsley et al. 1983).

Construction of deletion mutant strains

The single-gene-deletion mutants (see Table 1) were generated by the PCR-mediated short-flanking homology gene-replacement method of Wach et al. (1994). The *rad9- Δ /ddc1- Δ* double-deletion mutant was generated by crossing single mutant strains, followed by tetrad analysis.

Reporter plasmids

The *RAD54* reporter, pWDH444, was constructed by fusion of the promoter sequence to the yEGFP gene (codon optimised for yeast) in a 2 μ -based plasmid (Billinton et al. 1998). The *RNR2* reporter, pUMGP5, was constructed by replacing the *RAD54* promoter sequence with that of *RNR2* (Afanassiev et al. 2000). Both plasmids carry the KanMX cassette, *URA3* and *Amp^r* genes for selection purposes.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype (all strains were <i>leu2-3,112 ura3-52 lys2-1 his7-1</i>)
FF18984	<i>MATα</i>
FF18985	<i>MATα</i>
LW308	<i>MATα rad9-Δ::KanMX4</i>
LW322	<i>MATα rad9-Δ::KanMX4</i>
JJY164	<i>MATα rad9-Δ::URA3</i>
JJY95	<i>MATα ddc1-Δ::KanMX4</i>
JJY189	<i>MATα crt1-Δ::KanMX4</i>
LW316	<i>MATα/MATα DDC1/ddc1-Δ::KanMX4 rad9-Δ::KanMX4/RAD9</i>
LW331	<i>MATα rad9-Δ::KanMX4 ddc1-Δ::KanMX4</i>
LW318	<i>MATα dun1-Δ::KanMX4</i>
LW317	<i>MATα mbp1-Δ::KanMX4</i>

Transformation of yeast cells with the reporter plasmids

To construct reporter strains, approximately 1 µg of plasmid DNA was used to transform yeast cells by the lithium acetate method (Gietz and Woods 1994). Transformants were selected by plating on SD agar lacking uracil.

GFP reporter assays

Reporter induction was assayed by the direct fluorescence method developed by Walmsley et al. (1997) and Billinton et al. (1998). There are several properties of GFP that make it attractive as a reporter (Cubitt et al. 1995). Measurement of fluorescence is simple and reagent-free, compared with the assay of commonly used enzyme reporters. GFP protein is not fluorescent in its nascent form and requires internal post-translational covalent bonding between three amino acids to form a fluorophore. In the yeast-enhanced GFP used in this study, fluorophore development takes approximately 75 min (Cormack et al. 1997). Once formed, GFP is extremely resistant to proteases (Cubitt et al. 1995) and this is reflected in its high stability. The brightness values recorded remain stable for several weeks (data not shown). We have previously reported the use of a flow-through detector to study the expression of GFP from the *RAD54* promoter on-line in situ and in real time (Knight et al. 1999). In this experimental protocol, where the cells are limited by glucose to only about three generations, GFP accumulates in a linear fashion to a maximum. In effect, the end-point GFP signal (brightness) represents an integrated history of GFP expression in the culture.

The *MATa* strain background was always used for induction assays. Starter cultures of yeast cells were grown overnight in F1 medium. Cells transformed with the reporter were selected by omission of uracil from the medium. Untransformed cells were used as controls. An alternative control strain, containing a derivative of the pWDH444 plasmid with a stop codon introduced just after the start codon of the *RAD54* gene, gave similar results. Overnight starter cultures were used to inoculate 1.5 ml volumes of fresh F1 medium. For each yeast strain, two sets of media were prepared, MMS was added to one set of test tubes and the other set was prepared without MMS. In these experiments, sub-lethal concentrations of MMS were used; and thus checkpoints were active (unless deliberately mutated) and there were no complicating factors introduced by other exogenous cell-cycle effectors, such as alpha factor or nocodazole. Cells were incubated for 18 h at 30 °C with shaking and reached the early stationary phase of growth. Induction of the reporter in growing cells was detected by measurement of fluorescence (excitation at 490 nm, emission at 520 nm). To correct for variations in cell number, the fluorescence signal was then expressed as a brightness value (fluorescence signal divided by light scatter at 600 nm). All assay readings were made using a Perkin Elmer LS50B luminescence spectrometer. Scatter readings in the range 50–500 indicated an acceptable level of growth. The constitutive signal was calculated by subtracting the brightness value for the untransformed strain (without MMS) from that of the corresponding transformed strain (without MMS). Similarly, induced signal was calculated by subtracting the brightness value for the untransformed strain (with MMS) from that of the corresponding transformed strain (with MMS).

Northern blot analysis

RNA was isolated using the protocol of Bang et al. (1995). Logarithmically growing cells were induced with 0.1% MMS for 80 min at 30 °C in liquid YPD. Control cells were grown without MMS. Total RNA was extracted and the amount was estimated by absorbance at 260 nm. RNA was denatured by the glyoxal method and 50 µg of total RNA was loaded on a 1% agarose gel containing 10 mM sodium phosphate buffer, pH 6.85. This buffer was also used as the electrophoresis buffer and was permanently circulated during electrophoresis. All apparatus and stock

solutions were treated with diethyl pyrocarbonate to inhibit RNase activity.

The RNA was blotted overnight to a Biotodyne Nylon membrane (Pall) using 20× SSC. The RNA was covalently linked to the membrane by UV irradiation (1,200 J m⁻² in a Stratalinker). The membrane-bound RNA was prehybridised for at least 30 min at 42 °C in the hybridisation solution (ULTRAhyb; Ambion). After blotting, the membrane-bound RNAs were hybridised with the radioactively labelled DNA probes, *RAD54* (JJO82, JJO83), *ACT1* (JJO80, JJO81) and *RNR2* (JJO50, JJO51; Table 2). Probes were generated by PCR and were labelled using the random prime method and the High Prime kit (Roche Molecular Biochemicals). Hybridisation with the *RAD54* and *ACT1* probes was performed overnight at 42 °C. After several washing steps (2× SSC 0.1% SDS; 0.1× SSC 0.1% SDS) at 45 °C, the signal was quantified using a phosphorimager (Molecular Dynamics). Filters were stripped in 0.1% SDS at 90 °C for 2×15 min. Removal of the hybridised probes was confirmed by exposing the stripped filter to an X-ray film overnight. A second hybridisation with the *RNR2* probes was then performed and the signal was quantified as above. The *RAD54* and *RNR2* signals were then normalised against *ACT1*.

Statistical methods

The statistical facility of Microsoft Excel software (1997 version) was used to calculate mean and standard deviation values for the raw data generated. Microsoft Excel was also used to carry out the ANOVA test for statistical significance.

Results

Reporter assay results

RNR2 or *RAD54* promoter activity was monitored in logarithmically dividing cells transformed with the relevant reporter plasmid. Mutants and wild-type controls were exposed to concentrations of MMS judged to be generally sub-lethal (0.005%, 0.01%): viability was measured by drop tests, in which serial dilutions of overnight cultures were plated on SD medium supplemented with MMS (data not shown). The ability of the mutants to form colonies was comparable with the wild type, using 0.005% MMS. With 0.01% MMS, growth was comparable, except in the case of the *rad9-Δ/ddc1-Δ* double mutant, when there was some reduction in the level of viability. Fluorescence and light scatter were measured and the induced reporter signal was calculated and plotted as a brightness value. The constitutive reporter signal was measured in parallel experiments without MMS (Figs. 1, 2, 4). To complement and validate this approach, constitutive and MMS-induced

Table 2. Sequence details for probes used in Northern blot analysis

Probe	Gene	Sequence
JJO80	<i>1ACT1</i>	cgtgataagtgatagtgatattc
JJO81	<i>2ACT1</i>	caactctcaattcgttgtagaag
JJO82	<i>LRAD54</i>	gtgaaccgactaacgaaaccg
JJO83	<i>RRAD54</i>	cgtctgtagggtcatgtagag
JJO50	<i>RNR2</i> start	ccctccaagctgctgccgatgc
JJO51	<i>RNR2</i> end	cccattcggccttctcaccgatctc

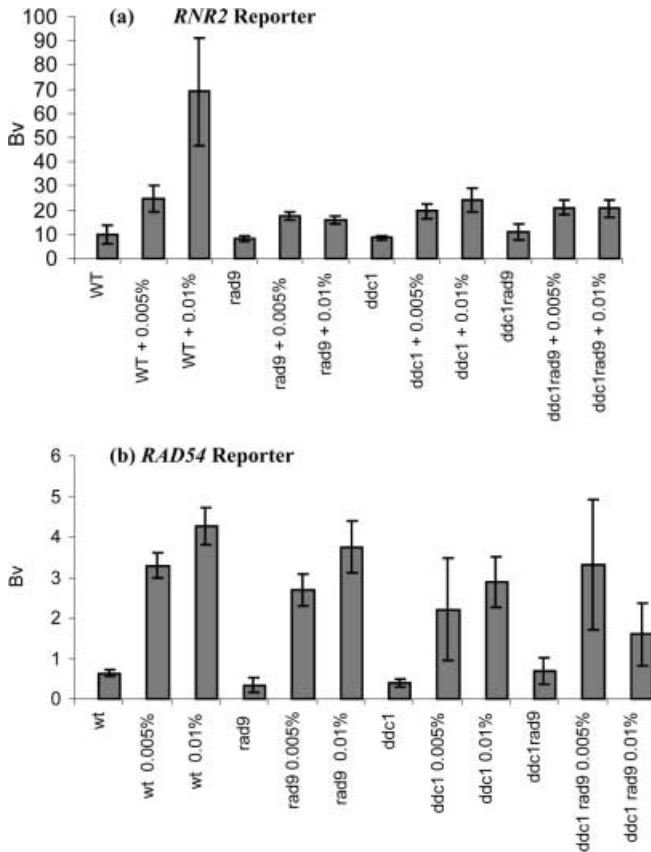


Fig. 1. Induction of **a** *RNR2*- and **b** *RAD54*- green fluorescence protein (GFP) reporters in *rad9*- Δ ::*KanMX4* and *ddc1*- Δ ::*KanMX4* single mutants and the *rad9*- Δ ::*KanMX4* *ddc1*- Δ ::*KanMX4* double mutant. Each strain was grown in the presence or absence of methyl methanesulphonate (MMS; 18 h induction) at the concentrations indicated. Error bars represent mean (\pm SD) values for at least three independent transformants. Bv Brightness value (see Materials and methods), WT wild type

RNR2 and *RAD54* RNA levels were measured by Northern blots for some of the deletion strains studied (Fig. 3).

DNA damage induction of *RNR2* depends on *RAD9* and *DDC1*

With both the *rad9* and *ddc1* deletion mutants, the constitutive *RNR2* signal (the basal or background promoter activity in the absence of MMS) was similar to that of the wild type. However, with both mutants, the induced reporter signal was lower than in the wild type (Fig. 1). For example, using 0.01% MMS, there was a 2-fold induction in the *rad9*- Δ mutant, a 3-fold induction in the *ddc1*- Δ mutant, compared to a 7-fold induction in wild-type cells. Northern blots confirmed that MMS-induced *RNR2* transcription was reduced in the *rad9*- Δ mutant (2.3-fold induction, vs 3.3-fold in the wild type) and the *ddc1*- Δ mutant (1.9-fold induction, vs 2.3-fold in the wild type; Fig. 3). With the *rad9*- Δ /*ddc1*- Δ double

mutant, the level of reporter induction was similar to that seen in either single mutant. These results show that deletion of either *RAD9* or *DDC1* results in a partial *RNR2* transcriptional response, although deletion of both these “sensor” genes does not have any additive effect on reporter induction.

RAD9 and *DDC1* are not required for DNA damage induction of *RAD54*

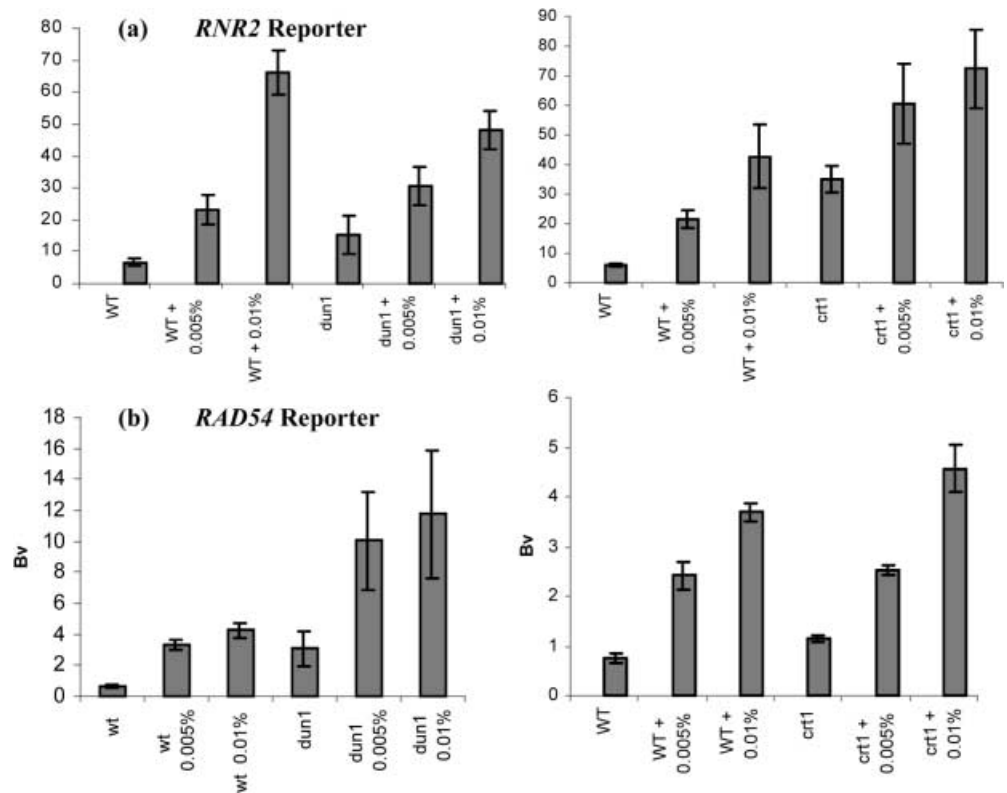
Studies carried out with *rad9*- Δ and *ddc1*- Δ mutants show that transcription of *RAD54* does not appear to rely on either *RAD9* or *DDC1*. For example, using 0.01% MMS, there was an 11-fold reporter induction in the *rad9*- Δ strain, and a 7-fold induction in the *ddc1*- Δ strain (7-fold in the wild type) (Fig. 1). It was noted that actual levels of reporter activity were slightly lower in both *rad9*- Δ and *ddc1*- Δ mutants (due to a lower constitutive signal). However, statistical analysis of the data showed that MMS-induced brightness for the *rad9*- Δ and *ddc1*- Δ single mutants was not significantly different from that observed in corresponding wild-type experiments ($P > 0.05$; ANOVA test). Northern blots confirmed that deletion of *RAD9* or *DDC1* had little effect on *RAD54* transcription (Fig. 3): there was a 5-fold induction in the *rad9* strain (vs 5-fold in the wild type) and a 6-fold induction in the *ddc1* strain (7-fold in the wild type).

Deletion of both *RAD9* and *DDC1* did not affect the constitutive *RAD54* reporter signal, indicated by a similar brightness value to the wild type in the absence of MMS in the *rad9*- Δ /*ddc1*- Δ double mutant (Fig. 1). After induction with 0.005% MMS, the *RAD54* reporter signal was also similar to that of the wild type (approximately 5-fold increases in signal). However, with 0.01% MMS, the reporter signal was reduced (3-fold induction, vs 7-fold induction in the wild type). Therefore, although the single mutations have no major effect on *RAD54* reporter activity, if both *RAD9* and *DDC1* are deleted, there is a reduction in promoter activity, but this is only apparent with higher concentrations of MMS ($> 0.005\%$).

DUN1 is required for DNA damage-induced transcription of *RNR2* but not *RAD54*

Deletion of *DUN1* resulted in lower levels of *RNR2* reporter induction (Fig. 2). For example, using 0.01% MMS, the *dun1*- Δ strain showed only a 3-fold induction (10-fold in the wild type). This marked decrease in reporter induction was partially due to the increased basal levels of *RNR2* reporter signal seen in the *dun1*- Δ strain. In contrast, both basal and MMS-induced levels of *RAD54* reporter signal were increased in the *dun1*- Δ strain: both constitutive and MMS-induced *RAD54* reporter signals were approximately 3-fold higher in the *dun1*- Δ strain than in the wild type.

Fig. 2. Induction of **a** *RNR2*- and **b** *RAD54*-GFP reporters in *crt1*- Δ ::*KanMX4* and *dun1*- Δ ::*KanMX4* mutants. Each strain was grown in the presence or absence of MMS (18 h induction) at the concentrations indicated. Error bars represent mean (\pm SD) values for at least three independent transformants



CRT1 regulates transcription of *RNR2* but not *RAD54*

Deletion of *CRT1* did not affect *RAD54* transcription. Reporter induction in the *crt1*- Δ strain was similar to that of the wild type (Fig. 2) and the result was confirmed by Northern blots (Fig. 3). In contrast, with the *RNR2* reporter, both constitutive and MMS-induced levels of signal were higher than in the wild type (Fig. 2) and, again, Northern blots confirmed this result. *RNR2* transcription was completely de-repressed in the *crt1*- Δ strain: the constitutive level of *RNR2* RNA transcribed was 2.5-fold higher than the wild type, although the MMS-induced signal was similar to that of the wild type (Fig. 3). Negative regulation of *RNR2* would therefore appear to operate predominately through *CRT1*.

MBP1 is required for transcription of *RNR2* but not *RAD54*

In the *mbp1*- Δ mutant, the overall *RNR2* reporter signal was reduced, compared with corresponding wild-type experiments (constitutive by 30%, induced by 50%; Fig. 4). The calculated level of induction seen was also lower than in the wild type (*mbp1*- Δ 3-fold, wild type 4-fold). Conversely, both constitutive and induced *RAD54* reporter signals were higher in the *mbp1*- Δ strain than in the wild type. The calculated reporter induction was also higher: there was a 4-fold induction with the *mbp1*- Δ strain (3-fold in the wild type). These results indicate that *MBP1* plays a role in both basal and DNA

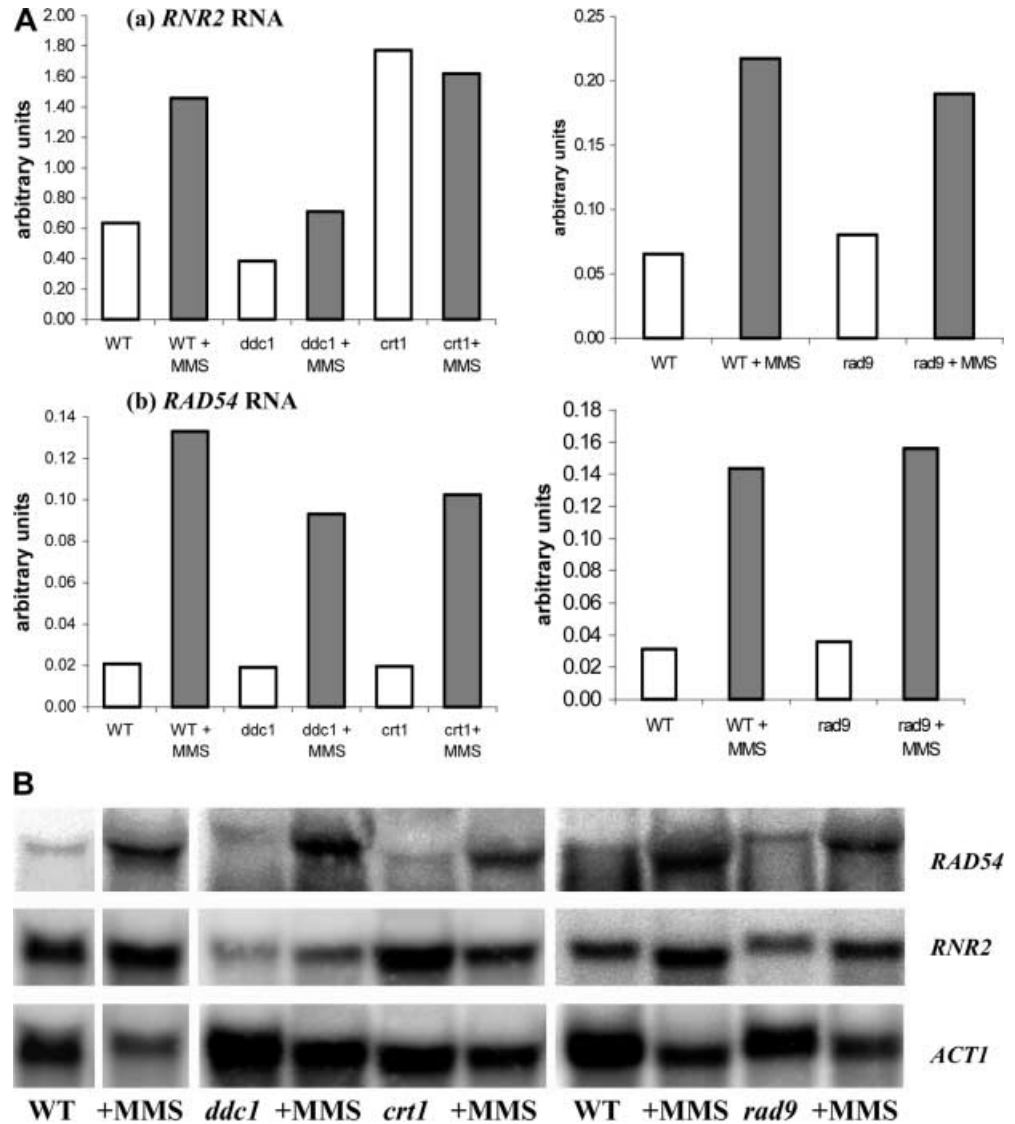
damage-induced transcription of *RNR2*. However, *MBP1* is not required for DNA damage-induced transcription of *RAD54* and instead may function in its down-regulation.

Discussion

Regulated transcription is an important physiological response to DNA damage that is co-ordinated by DNA damage checkpoints. However, there is not yet a complete description either of the regulation of the transcriptional response or of the participating gene products. In the present study, we sought to distinguish the transcriptional regulation of *RAD54* and *RNR2* in response to DNA damage and chose MMS as a suitable inducer of both promoters. This was based on a previous study, in which our *RAD54* and *RNR2* GFP reporter strains were used to assess the genotoxicity of a range of DNA-damaging agents (Afanassiev et al. 2000). Three of the compounds tested (MMS, *N*-nitroso-*N*-methyl urea, cisplatin) all gave similar high induction signals with both reporters.

Deletion of *RAD9*, *DDC1* or *RAD9/DDC1* resulted in a reduction in the *RNR2* reporter output. *RAD9* mutations were previously shown to have a similar effect on *RNR2*. For example, Aboussekhra et al. (1996) showed a reduced level of induction in a *rad9*- Δ strain, using a *RNR2*.damage response element (DRE)-*lacZ* reporter system in exponentially growing cells. With both GFP reporter assays and Northern blot experiments, we

Fig. 3a, b. Phosphorimager quantifications. **a** Northern blot analysis of *a* *RNR2* and *b* *RAD54* RNA in *rad9-Δ::URA3*, *ddc1-Δ::KanMX4* and *crt1-Δ::KanMX4* mutants. Logarithmically growing cells were induced with 0.1% MMS for 80 min at 30 °C. Total RNA was extracted and 50 μg were loaded onto an agarose gel. After blotting, the filters were hybridised with radioactively labelled *RAD54*, *ACT1* and *RNR2* probes. The signals were normalised against *ACT1*. **b** Northern blot image (composite) of *RAD54*, *RNR2* and *ACT1* RNA in *rad9-Δ::URA3*, *ddc1-Δ::KanMX4* and *crt1-Δ::KanMX4* mutants. Logarithmically growing cells were induced with 0.1% MMS for 80 min at 30 °C. Total RNA was extracted and 50 μg were loaded onto an agarose gel. After blotting, the filters were hybridised with radioactively labelled probes



confirmed this general pattern of reduced transcription. Our *RNR2* induction data for *rad9-Δ* and *ddc1-Δ* mutants agrees with the current model for cellular detection of DNA damage, which proposes two sensory pathways upstream of *MEC1* and *RAD53*, i.e. *RAD9* and the *RAD24* subclass, including *DDC1* (Lowndes and Murguia 2000). Paciotti et al. (1998) proposed that the Rad9 and Ddc1 proteins might function in separated branches of the DNA damage checkpoint pathway. However, our results do not correspond with this, as we did not see any additive effect on *RNR2* response in the double *rad9-Δ/ddc1-Δ* mutant.

In contrast with the *RNR2* data, deletion of *RAD9* or *DDC1* did not significantly reduce the *RAD54* reporter signal. This suggests that, when DNA is damaged, *RAD54* is normally transcribed despite the lack of a functional *RAD9* or *DDC1* gene. However, only with the *rad9-Δ/ddc1-Δ* double mutant (and after induction with 0.01% MMS) was the *RAD54* reporter signal reduced (to about 33% of the corresponding wild-type signal).

This suggests that *RAD54* transcription is affected to some degree by deletion of both *RAD9* and *DDC1*. This additive effect was only seen with higher MMS levels and might be due to an accumulation of additional DNA damage, or a reduction in signal simply due to the additional cytotoxic effect of MMS at 0.01%. The *RAD54* GFP reporter data obtained with our *rad9-Δ* strain, i.e. no significant reduction in the level of induction, was paralleled by Northern blot analysis. However, these results differ from the previously published data of Aboussekhra et al. (1996): DRE-*lacZ* reporter induction using 0.05% MMS was less than half that of wild-type cells. MMS concentration may be a factor in explaining this difference, since (as with our strain and assay conditions) the concentration of MMS (0.05%) used by these authors is extremely toxic to the cells. It may also be significant that the promoter sequence used in the current study extends from the start codon of *RAD54* upstream to the next gene. In contrast, the previous study used a 28-nucleotide DRE sequence

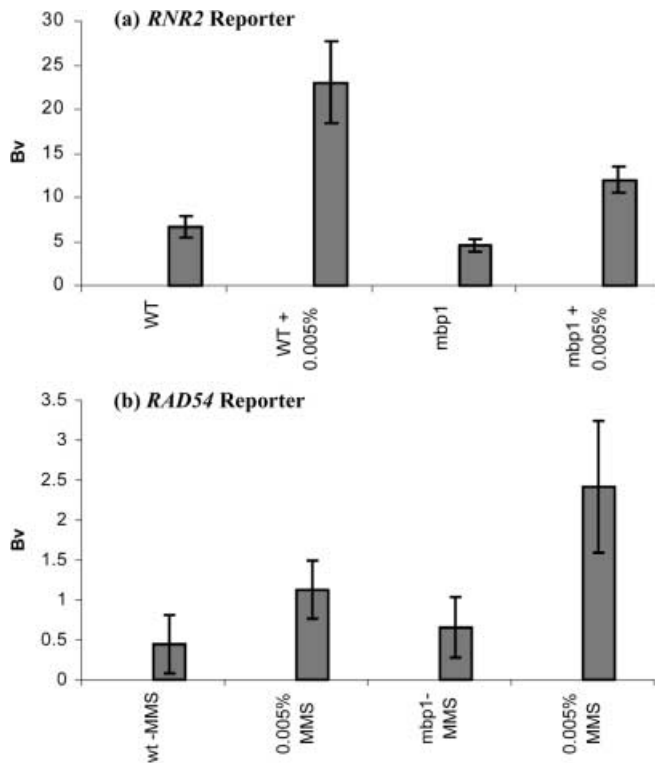


Fig. 4. Induction of **a** *RNR2*- and **b** *RAD54*-GFP reporters in the *mbp1-Δ::KanMX4* mutant. The cells were grown in the presence or absence of MMS (18 h induction) at the concentrations indicated. Error bars represent mean (\pm SD) values for at least three independent transformants

(–255 to –227, relative to the first ATG of *RAD54*). We conclude that the differences between the results from different laboratories should lead to caution in accepting hypothetical regulatory pathways of the transcriptional response to DNA damage.

The previous finding that *DUN1* is required for a normal DNA damage-induced *RNR2* response (Zhou and Elledge 1993) was confirmed by our studies. Transcriptional induction of *RNR2* appears to be mediated downstream of *DUN1* by relief of repression by the Ssn6, Tup1 and Crt1 proteins (Huang et al. 1998). The data generated with our *crt1-Δ* strain corroborates this: the constitutive level of *RNR2* transcription was more than twice that of wild type, confirming that *RNR2* transcription is down-regulated via the *CRT1* repression pathway. The *RAD54* reporter data generated with our *dun1-Δ* mutant confirms the recent finding that *DUN1* does not play a major role in the damage-specific induction of *RAD54* (De la Torre-Ruiz and Lowndes 2000). It may be that *DUN1* regulates other genes which, in turn, are involved in the down-regulation of *RAD54* transcription; and, in the absence of a functional Dun1 product, this regulation is absent, leading to higher basal levels of transcription. The data generated with the *crt1-Δ* strain supports the finding that *DUN1* is not required for DNA damage-induced transcription of *RAD54*. Both constitutive and MMS-induced levels of transcript

were similar to the wild type, suggesting that *RAD54* is not regulated by the *CRT1* repressor pathway regulated by *DUN1*.

Deletion of *MBP1* resulted in a decrease in *RNR2* reporter signal. This was surprising, as a MCB sequence is not present in the region upstream of *RNR2* (in contrast to *RNR1* and *RAD54*, which do contain MCB sites in their promoter regions). This suggests that the effect is indirect; and deletion of *MBP1* might block the transcription of other genes that have a more direct involvement in the regulation of *RNR2* transcription. Several DREs have been identified in the region upstream of *RNR2* (Elledge and Davis 1989b). It may be that transcription factors, which bind to activating sequences in this region, are themselves under MCB control. Another possibility is that *MBP1*-dependent *RNR2* transcription is related to the control of *DUN1* transcription. *DUN1* was recently identified as a genomic target for MBF (Iyer et al. 2001) and inspection of the region upstream of *DUN1* does reveal an *Mlu* I site –161 relative to the start codon. If Mbp1 protein was not available to bind to the MCB element in the *DUN1* promoter region, this could result in transcriptional repression of *DUN1*. This would lead to a failure to relieve repression mediated by Crt1/Ssn1/Tup1 and ultimately down-regulation of *RNR2*. Another explanation could be that *RNR2* is down-regulated in a *MBP1*-dependent manner by the activity of replication protein A (Rpa). Rpa has been shown to be present in the DNA–protein complexes that bind to upstream promoter elements of several DNA repair and metabolism genes (Singh and Samson 1995). Regulatory sites (repressing) for Rpa have been identified in the upstream region of *RNR2* (Xioa et al. 1993). In *S. cerevisiae*, Rpa is a complex of three subunits: Rfa1, Rfa2 and Rfa3 (Wold 1997). *RFA1* and *RFA2* contain *Mlu* I sites in their upstream regions. Therefore it is conceivable that, in the absence of Mbp1, transcription of Rpa is up-regulated, which could then result in down-regulation of *RNR2*.

Deletion of *MBP1* had an effect on the *RAD54* signal similar to that seen with the *dun1-Δ* strain, i.e. an increase in constitutive and induced reporter signal. *RAD54* contains an MCB element in the promoter region, hence its transcription would be expected to be affected by deletion of *MBP1*. Our data confirm the earlier finding that *RAD54* is cell-cycle regulated at G1, but that MCB promoter elements have no essential role in the DNA damage response (Johnston and Johnson 1995). Aboussekhra et al. (1996) suggested that MCBs act synergistically with DREs found in the promoter regions of genes such as *RAD54*. Our results suggest that the Mbp1 product functions directly or indirectly in the down-regulation of *RAD54* transcription.

We used MMS to induce DNA damage, but the results are clearly not particular to this agent, as we detected effects on both induced and constitutive levels of transcriptional activity. The use of the GFP reporter system is consistently corroborated by Northern blot analysis and, with its ease of use, will allow a more

extensive study of genetic control of the transcriptional response to DNA damage.

In summary, this study shows that *RAD9* and *DDC1* play an important role in the DNA damage-induced transcription of *RNR2*, but the two genes do not appear to function in separate branches of the proposed sensory pathways. However, DNA damage-induced transcription of *RAD54* is different: it occurs in the absence of either *RAD9* or *DDC1*. The study shows that there is a gap in the knowledge of the regulation of *RAD54* transcription: clearly other genes in addition to the main sensory pathways (*RAD9*, *DDC1*) must be involved. In our hands, the major role of *DUN1* is in the damage-specific induction of *RNR2* and not *RAD54*. *MBP1* seems to be required for both basal and DNA damage-induced transcription of *RNR2*, but does not appear to be required for *RAD54* transcription and might function in the negative regulation of *RAD54*.

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