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INDUCTION IN THE GENE *RNR3* IN *SACCHAROMYCES CEREVISIAE* UPON EXPOSURE TO DIFFERENT AGENTS RELATED TO CARCINOGENESIS

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Abstract—The induction of the gene *RNR3* was investigated in yeast *Saccharomyces cerevisiae* using *RNR3/lacZ* fusion. Gene induction was monitored by measuring β-galactosidase activity. Various drugs that cause DNA damage effectively induced *RNR3* expression; alkylating agents (cisplatin, mitomycin C and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), a radical producer (bleomycin), and an intercalator (actinomycin D) induced *RNR3*. When yeast expressing rat CYP1A1 was exposed to 2-aminofluorene, a concentration-dependent induction of *RNR3* was observed. Aflatoxin B_1 also induced the expression of *RNR3* in the same yeast strain concomitant with inhibition of cell growth. In control yeast, no induction of *RNR3* was observed upon exposure to 2-aminofluorene or aflatoxin B_1. Exposure to 2-acetylaminofluorene or benzo[*a*]pyrene did not lead to induction of *RNR3* in yeast expressing CYP1A1. These results indicate that DNA damage by chemicals related to carcinogenesis induces *RNR3*, and that activation of these procarcinogens was required for DNA damage-dependent induction of *RNR3*.

Key words: RNR3; RNR3/lacZ; Saccharomyces cerevisiae; DNA damage; cytochrome P450; carcinogenesis

RNR§ (EC 1.17.4.1) catalyzes the rate-limiting step in the production of deoxyribonucleotides needed for DNA synthesis. Deoxyribonucleotide levels are critical to many cellular functions and are also important for DNA repair processes. RNR expression is cell-cycle regulated and is inducible by DNA-damaging agents in all organisms, including humans, *Saccharomyces cerevisiae*, and *Escherichia coli*, because excision repair, recombinational repair, and post-replicational repair all require DNA synthesis [1]. RNR is composed of two dissimilar subunits, a large subunit, R1, containing the allosteric regulatory sites, and a small subunit, R2, containing a binuclear iron center and a tyrosyl free radical [2]. In *S. cerevisiae*, the large subunit R1 is encoded by two genes, *RNR1* and *RNR3*. The *RNR3* gene is inducible at the level of transcript accumulation by agents, such as HU, that block DNA replication or by agents, such as 4NQO, that damage DNA [3, 4].

Cytochrome P450s play important roles in metabolic activation and detoxication of chemical carcinogens [5]. Some metabolites may be active intermediates in the carcinogenic process. Heterologous expression of human and rodent P450s in mammalian cells and yeast facilitates the investigation of the mechanisms in chemical carcinogenesis. We recently reported the formation of DNA adducts derived from carcinogenic aromatic amines in yeast expressing recombinant P450s [6]. Both 2-AF and 2-AAF have been used as models of the metabolic activation of arylamine/arylamide carcinogens. CYP1A1 is the major isozyme induced by 3-methylcholanthrene in rat liver, and it preferentially metabolizes 2-AAF and 2-AF to the mutagenic *N*-hydroxy-AAF and *N*-hydroxy-AF, respectively [7, 8]. The mutagenicity of *N*-hydroxy-AAF, however, is only one-tenth that of *N*-hydroxy-AF in bacteria due to lack of a deacetylating enzyme [9, 10]. It has been shown that *N*-hydroxy-AF binds non-enzymatically to DNA and forms *N*-(deoxyguanosin-8-yl)-2-AF. This adduct is a critical lesion for the initiation of hepatic tumorigenesis [10]. Intracellular toxic effects by P450-dependent activation of 2-AF have not been demonstrated.

As part of our carcinogenesis studies, we have constructed a fusion plasmid of the 5′ promoter region of *RNR3* and *lacZ*, and measured the β-galactosidase activity in order to detect induction of *RNR3*. The *lacZ* gene is commonly used for gene fusion since an assay for the β-galactosidase activity in cell extracts is quantitative, easy, and extremely sensitive. In this paper, we report the induction of *RNR3* in yeast upon exposure to various DNA-damaging agents. We also show that activation of procarcinogens in yeast expressing mammalian P450 causes *RNR3* induction.

MATERIALS AND METHODS

Materials

S. cerevisiae strain JE1003.1E (*MAT a, his 3-11, ade 2-1, ura 3-1, leu 2-3, 112, can 1-100*) and the plasmid YEp353 were gifts from Dr. K. Mizuta, Hiroshima University, and the yeast AH22/pACCD1 expressing rat CYP1A1 was obtained as described previously [6]. The yeast strains were grown to a cell density of 5×10^6/mL, as described by Oeda *et al.* [11]. MMC was purchased from the Kyowa Hakko Kogyo Co., Ltd. Cisplatin was from Bristol-Myers Squibb K.K. Actinomycin D, 2-AF,

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§ Abbreviations: RNR, ribonucleotide reductase; HU, hydroxyurea; 4NQO, 4-nitroquinoline-1-oxide; 2-AF, 2-aminofluorene; 2-AAF, 2-acetylaminofluorene; MMC, mitomycin C; B[a]P, benzo[*a*]pyrene; AFB_1, aflatoxin B_1; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; and PCR, polymerase chain reaction.

and 2-AAF were from the Sigma Chemical Co. Bleomycin hydrochloride, 4NQO, B[a]P, AFB_1, and HU were from Wako Chemical Ind. Ltd., and MNNG was from Nakarai Tesque. Oligonucleotides were synthesized on an Applied Biosystem model 380B DNA synthesizer. All other chemicals used were of analytical grade.

DNA probes

The DNA fragments of the *RNR3* and *URA3* genes used for RNA blots were isolated as follows: two primers, 5′-TACGGTTTAGACCCAAACCG-3′ corresponding to nucleotides 781-800 of the *RNR3* gene and 5′-ACCAGGTCTCTTGTTACCAC-3′ complementary to nucleotide 1561–1580 of the gene [4], and two primers, 5′-ATGTCGAAAGCTACATA-3′ corresponding to nucleotides 227–243 of the *URA3* gene and 5′-TGTGCAATAAAGCCGAT-3′ complementary to nucleotides 764–780 of the gene [12], were synthesized. The DNA was amplified by PCR for 30 cycles, using the corresponding two primers and *Bam* HI-digested yeast genomic DNA as a template. The DNA fragments of the correct sizes were isolated, ligated into the pGEM-T vector (Promega Co.), and sequenced. The resulting nucleotide sequences coincided with those of *RNR3* [4] and *URA3* [12].

Northern blot analysis

Total RNA from yeast was isolated by the method of Carlson and Botstein [13]. RNA preparations were electrophoresed in a 1% agarose gel under denaturing conditions, and then transferred onto a nylon membrane. Conditions of hybridization and washing were as described previously [14].

Plasmids

To facilitate the analysis of *RNR3* induction, a fusion between the 5′-promoter region of *RNR3* and the *lacZ* gene was constructed on a high copy-number yeast vector YEp353 to form pYE3 (Fig. 1). Namely, the 5′ regulatory region of *RNR3* was amplified by PCR using *Bam* HI-digested yeast genomic DNA as a template. Synthetic primers, 5′-GGATCCAGAAGGAAACACTC-3′ (sense) and 5′-CTGCAGGTCATTTGTGTGGGAGTATTTG-3′ (antisense) [4], were used for production of the DNA fragment. The amplified DNA fragment was digested with *Bam* HI and *Pst* I and cloned into the *Bam* HI-*Pst* I site of the YEp353 vector [15] in frame to form plasmid pYE3. The constructed vector, pYE3, was used for transformation of *S. cerevisiae* strain JE1003.1E by the lithium acetate method [16], creating strain YYE3. Yeast YYE3 cells were transformed with pACCD1, an expression vector of rat CYP1A1 [6], to create the strain YYE3-1A1. The CYP1A1 activity in YYE3-1A1 was measured using 7-ethoxycoumarin *O*-deethylation activity in the whole cell, and the activity was calculated to be 3.3×10^{-7} nmol/cell/hr.

β-Galactosidase assay

Liquid β-galactosidase assay was carried out with yeast strains according to the method of Guarente [17]. Briefly, the cells were grown overnight in selective minimal medium and diluted into fresh selective medium; then the cells were grown to mid-log phase ($O.D._{600} \cong$ 0.4 to 0.5). At this point, the DNA-damaging agents were added to the culture (2 mL), which was incubated

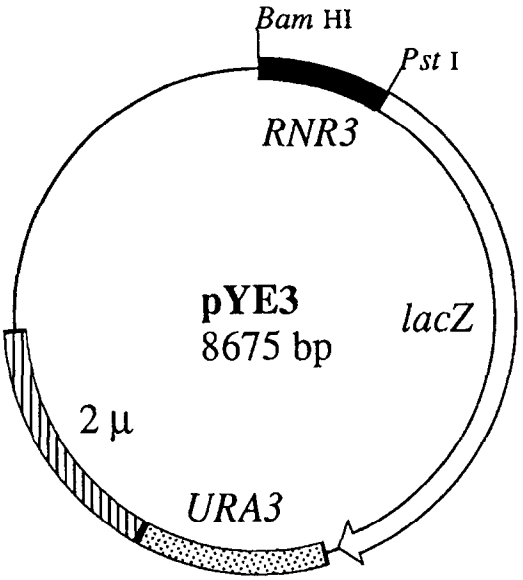

Fig. 1. Construction of the plasmid pYE3 containing the fusion of *RNR3* and *lacZ* gene.

at 30° for various periods of time. Then, the yeast cells were pelleted and resuspended in 1 mL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol, pH 7.0). An aliquot of the cell suspension (50 μL) was added to 1 mL of H_2O, and the cell density was measured at 600 nm. Twelve microliters of 0.1% sodium dodecyl sulfate and 15 μL of chloroform were added to 950 μL of the solution. After 15 min of preincubation at 30°, 0.2 mL of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/mL) was added, and the mixture was incubated at 30° for 30 min [17]. The reaction was stopped by the addition of 0.5 mL of 1 M Na_2CO_3, and the absorbances at 420 and 550 nm were measured. Units of the β-galactosidase activity were calculated according to the method of Miller [18]. Assays were carried out in triplicate.

Other methods

Cytochrome P450 hemoprotein was measured by reduced CO-difference spectra, according to the method of Oeda *et al.* [11]. The 7-ethoxycoumarin *O*-deethylation activity, using yeast microsomal fractions or whole yeast cells, was measured as described previously [6].

RESULTS

Induction of RNR3 *in yeast by treatment with DNA-damaging agents*

We examined the effects of various DNA-damaging chemicals on the induction of *RNR3* in the yeast transformed with a reporter plasmid pYE3 (Fig. 1). The yeast strain thus obtained (YYE3) was incubated with 50 mM HU or 1 μM 4NQO for 4 hr, and the β-galactosidase activity was measured. As shown in Table 1, the activity increased about 7-fold with HU and 11-fold with 4NQO as compared with that in untreated cells. Two alkylating agents, MMC and cisplatin, markedly induced expression of *RNR3* in a concentration-dependent manner. A monofunctional alkylator, MNNG, also induced *RNR3*. A radical producer, bleomycin, induced *RNR3*, and the

Table 1. Induction of the *RNR3* gene on the yeast YYE3 strain upon exposure to various DNA-damaging agents

Chemicals	Concentration (μM)	β-Galactosidase activity* (unit)
None		1.1 ± 0.2
HU	50,000	8.2 ± 0.4
4NQO	1.0	12.3 ± 2.6
MMC	10	1.7 ± 0.0
	50	1.9 ± 0.1
	100	4.6 ± 0.3
	200	6.7 ± 0.8
Cisplatin	10	1.2 ± 0.1
	50	2.5 ± 0.2
	100	4.6 ± 0.3
	200	5.3 ± 0.1
MNNG	0.1	1.0 ± 0.1
	0.5	1.9 ± 0.1
	1.0	3.5 ± 0.3
	5.0	8.8 ± 0.2
Bleomycin	0.5	1.9 ± 0.7
	5.0	3.3 ± 1.1
	25	4.1 ± 0.7
	75	8.7 ± 2.0
Actinomycin D	0.5	1.3 ± 0.1
	1.0	1.7 ± 0.1
	5.0	2.2 ± 0.4
	10.0	3.0 ± 0.7

Cells were exposed to the indicated DNA-damaging agents for 4 hr, and β-galactosidase activity was measured.
* Values are means ± SD of triplicate experiments.

induction level was higher than with cisplatin and MMC. An intercalator, actinomycin D, slightly induced expression of *RNR3*. These results indicated that exposure to various DNA-damaging agents leads to the induction of *RNR3* in yeast.

Induction of RNR3 *by treatment with procarcinogens in yeast expressing CYP1A1*

To examine whether the expression of *RNR3* can be induced by P450-dependent activation of procarcinogens, a yeast strain AH22/pACCD1 expressing rat CYP1A1 was exposed to 2-AF, and RNA blots were performed. As shown in Fig. 2, *RNR3* mRNA in the AH22/pACCD1 cells was induced by exposure to 50 μM 2-AF for 4 hr, while no induction was observed in control yeast (AH22/pAAH5). The level of mRNA for *URA3* remained unchanged by any treatment.

In the next experiment, the yeast strain YYE3-1A1 was exposed to 2-AF, and the β-galactosidase activity was measured. As shown in Fig. 3, the activity in the yeast exposed to 2-AF increased with time up to 6 hr and decreased slowly thereafter. The maximal activity was concentration related. No induction was observed when control yeast (YYE3) was exposed to 2-AF. Another aromatic amine, 2-AAF, is reported to be N-hydroxylated mainly by CYP1A1. In yeast YYE3-1A1 exposed to 100 μM 2-AAF, no induction of *RNR3* was observed. Yeast YYE3-1A1 was also exposed to polycyclic aromatic hydrocarbons, which are activated metabolically by the CYP1 family. As shown in Table 2, exposure of yeast YYE3-1A1 to AFB_1 (>1 μM) led to marked induction of *RNR3* concomitant with marked inhibition of cell growth. An environmental carcinogen, B[a]P, did

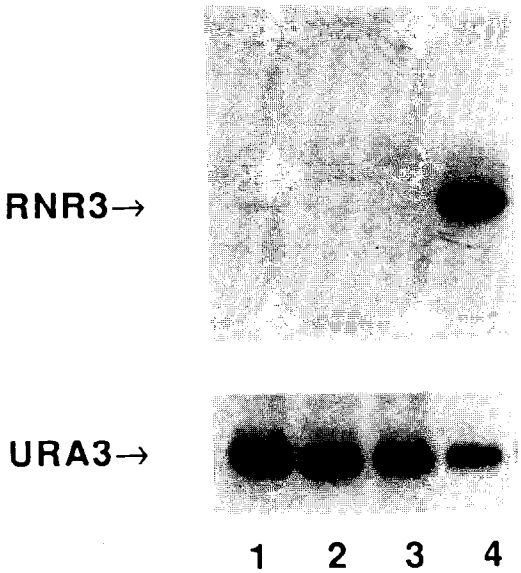

Fig. 2. Induction of *RNR3* mRNA in yeast expressing P4501A1 upon exposure to 2-AF. Yeast AH22/pAAH5 (lanes 1 and 2) and AH22/pACCD1 (lanes 3 and 4) were incubated without (lanes 1 and 3) or with 50 μM 2-AF (lanes 2 and 4) for 4 hr. Hybridization was carried out, using as probes fragments of the *RNR3* gene (upper panel) or the *URA3* gene (lower panel).

not induce *RNR3* at all. These results indicate that expression of *RNR3* is induced by procarcinogenic chemicals activated by P450 in yeast cells, but not by those requiring further activation by other drug-metabolizing enzymes.

DISCUSSION

The present study demonstrates the induction of the gene *RNR3* in *S. cerevisiae* in response to various DNA-

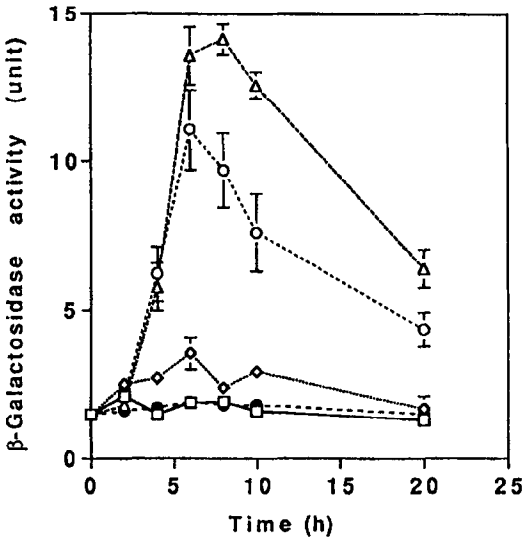

Fig. 3. Induction of *RNR3* in the yeast YYE3-1A1 strain upon exposure to 2-AF or 2-AAF. Yeast YYE3-1A1 expressing rat CYP1A1 was treated without (●) or with 5 μM (◇), 25 μM (○), or 100 μM (△) 2-AF. After the exposure for the indicated period, cells were collected, and β-galactosidase activity was measured. The yeast was also treated with 100 μM 2-AAF (□). Each data point is the mean ± SD of three separate experiments.

Table 2. Effect of CYP1A1 on the induction of *RNR3* gene by AFB_1

Chemicals	Concentration (μM)	β-Galactosidase activity* (unit) Yeast strain YYE3	YYE3-1A1
None		0.9 ± 0.0	0.8 ± 0.1
AFB_1	1.0	0.8 ± 0.0	3.5 ± 0.1
	5.0	1.0 ± 0.2	6.9 ± 0.1
	10	1.1 ± 0.1	7.0 ± 1.1
	50	0.8 ± 0.4	11.8 ± 0.5
B[a]P	10	ND†	0.7 ± 0.2
	50	ND	0.9 ± 0.1
	100	ND	0.9 ± 0.1
	200	ND	1.0 ± 0.2

Cells were exposed to AFB_1 or B[a]P for 4 hr, and β-galactosidase activity was measured.

* Values are means ± SD of triplicate experiments.

† ND: not determined.

damaging agents. Alkylating agents, which form bulky DNA adducts, such as MMC, 4NQO, and MNNG, were effective in inducing *RNR3* (Table I). The formation of DNA adduct is generally considered to be a critical event in chemical carcinogenesis [19] and the cytotoxic effect of anticancer agents [20]. Expression of *RNR3* was also induced by treatment with different types of DNA-damaging agents, which causes DNA strand breaks by producing radicals (bleomycin), interstrand cross-link (cisplatin), or fragmentation (actinomycin D) [20–22]. Since the induction of *RNR3* in yeast was concentration dependent, it might reflect the DNA damage caused by these agents. In yeast, several genes such as *RNR2, CDC9, CDC8, POL1, RAD2* and *RAD54* are known to be DNA-damage inducible, but most of them are also regulated by the cell cycle [2]. Since induction of *RNR3* transcription by DNA-damaging agents is independent of cell cycle and expression level of *RNR3* is low in the absence of DNA-damaging agent [23], the measurement of the *RNR3* induction will certainly be a useful tool in helping to find the stress of DNA damage more precisely.

When yeast expressing CYP1A1 was exposed to 2-AF, *RNR3* mRNA increased within 4 hr (Fig. 2). Onset of the induction of the *RNR3* by 2-AF was similar to that in yeast exposed to HU or 4NQO [4]. Since the level of *RNR3* transcript remained unchanged in the absence of CYP1A1 or 2-AF, the appearance of the transcript seems to be induced by the activation of 2AF. The present study showed that the induction of *RNR3* occurred within 3 hr, reached a maximum at 6 hr, and then decreased. The rapid and transient induction was similar to that seen in the case of other DNA damage-inducible genes in response to 4NQO or UV-irradiation [24], and is a ubiquitous characteristic associated with DNA damage.

It is reported that various kinds of P450s, such as rat CYP1A1, and 2C, and human CYP1A, 2A, 2B, and 3A, can be involved in activation of AFB_1 [25, 26]. In our study, exposure of yeast expressing rat CYP1A1 to AFB_1 resulted in the induction of *RNR3* gene expression, implying that CYP1A1 activates AFB_1. Simultaneously, the cell density of yeast exposed to AFB_1 markedly decreased, which indicated that expression of CYP1A1 led to the formation of an AFB_1-DNA adduct. These findings are consistent with the findings of Sengstag and Würgler [27] that yeast expressing human CYP1A1 or 1A2 activates AFB_1, followed by the induction of mitotic recombination. Thus, measurement of the induction of DNA damage-responsive genes using yeast expressing various kinds of P450 appears to be a sensitive and accurate test system for metabolism-related procarcinogens. Other systems based on mammalian cell lines have been developed previously for similar purposes [28, 29]. Compared with systems using other cells, however, measurement of *RNR3* gene induction using yeast is less expensive, faster, and simpler to use.

Exposure of yeast expressing rat CYP1A1 to B[a]P did not lead to the induction of *RNR3*, which is consistent with our previous data that a DNA adduct was not formed in yeast AH22/pACCD1 upon exposure to B[a]P [6]. Other investigators have shown that both microsomal epoxide hydrolase and P450s are required to produce the ultimate mutagen [30]. Yeast used in this study is free from epoxide hydrolase activity [31], and thus cannot produce the postulated ultimate carcinogen. Similarly, 2-AAF did not induce *RNR3* in yeast expressing CYP1A1. Johnson *et al.* [24] reported that activation of 2-AAF occurred with CYP1A and CYP2C. It is known, however, that acyltransferase- or sulfotransferase-mediated reaction, which is lacking in yeast, can be essential for subsequent activation of 2-AAF [10]. Co-expression of these enzymes in addition to P450s in yeast would make this method applicable to the assessment of the DNA-damaging potential of compounds requiring multiple activation steps.

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