Mol Gen Genet (1988) 211:260–265

MGG

Genetic characterization of hyperresistance to formaldehyde and 4-nitroquinoline-N-oxide in the yeast *Saccharomyces cerevisiae*

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Summary. The hyperresistance to 4-nitroquinoline-N-oxide (4-NQO) and formaldehyde (FA) of yeast strains transformed with the multi-copy plasmids pAR172 and pAR184, respectively, is due to the two genes, *SNQ* and *SFA*, which are present on these plasmids. Restriction analysis revealed the maximal size of *SFA* as 2.7 kb and of *SNQ* as 2.2 kb, including transcription control elements. The presence of the smallest 2.7 kb subclone carrying *SFA* increased hyperresistance to formaldehyde fivefold over that of the original pAR184 isolate. No such increase in hyperresistance to 4-NQO was seen with the smaller subclones of the pAR172 isolate. Disruption of the *SFA* gene led to a threefold increase in sensitivity to FA as compared with the wild type. Expression of gene *SNQ* introduced on a multi-copy vector into haploid yeast mutants *rad2*, *rad3*, and *snm1* did not complement these mutations that block excision repair.

Key words: Mutagen resistance – Yeast – Formaldehyde – 4-Nitroquinoline-N-oxide – Multi-copy plasmids

Introduction

The phenomenon of acquired resistance to agents that interfere with DNA structure or function is of genetic and medical importance. Mutants hyperresistant to radiation have been isolated in bacteria (Davies and Sinskey 1973) as well as in yeast (Moustacchi 1965) and bacterial mutants with enhanced resistance to chemical mutagens as diverse as nitrosoguanidine, formaldehyde (FA) and psoralen plus near ultraviolet light have been selected and partially characterized (Chao and Tillman 1982; Kaulfers and Laufs 1985; Ahmad and Holland 1985). Recently, it was shown that yeast transformants exhibiting hyperresistance (HYR) to mutagens could be selected for (Ruhland et al. 1986) using a yeast genome library based on the multi-copy vector plasmid YEp13 (Nasmyth and Tatchell 1980). By transfer to *Escherichia coli* and subsequent retransformation of yeast a number of these plasmids were shown to carry stably the genetic information for HYR either to FA or to the UV-mimetic chemical 4-nitroquinoline-N-oxide (4-NQO). Since it was our aim to elucidate the mechanisms that lead to the observed HYR phenotypes we began to investigate more closely how size and orientation of the insert DNA and the type of vector (single or multi-copy) containing it influence its expression in the yeast transformants and whether disruption of this genetic information has any consequences for the viability and resistance status of the yeast mutants thus generated. We also began complementation studies with yeast mutants with known defects in the repair of damaged DNA to find out whether the observed HYR phenotype can be attributed to our cloning of DNA repair genes or to the cloning of genes that normally would not fit into that category.

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Materials and methods

Yeast strains, bacteria, and plasmids. The genotypes of the seven haploid strains of *Saccharomyces cerevisiae* used in this study are shown in Table 1. Diploid MM5108 was constructed by mating of the two haploid leucine prototrophic strains AH22 *sfa-0:LEU2* and EH3605-4D. *E. coli* strain JF1754 (Schultz and Friesen 1983) was used for transformation and amplification of vectors. The yeast-*E. coli* shuttle vectors YRp17 and YCp50, carrying the yeast *URA3* gene, were kindly provided by R. Fleer, Stanford University; shuttle vector YEp13, carrying the yeast *LEU2* gene, was kindly provided by E. McIntosh, York University, Toronto. YEp13-derived plasmids pAR172 (stably conferring HYR to 4-NQO; identical by restriction analysis to another original isolate pAR173) and pAR184 (conferring HYR to FA) were isolated and first characterized by Ruhland et al. (1986). The three plasmids are shown in Fig. 1. The single copy plasmid pMC1 (not shown in Fig. 1) was derived from centromere vector YCp50 by ligation of the *Sal*I-*Hin*dIII fragment conferring HYR into the plasmid digested with *Sal*I and *Hin*dIII. The construction of integrating plasmid pMI1 is shown in Fig. 4.

Preparation of DNA and transformation procedures. Plasmid DNA was isolated from *E. coli* by alkaline lysis (Birnboim and Doly 1979). Purification, restriction, ligation, and analysis on agarose gels of plasmid DNA were performed as described by Maniatis et al. (1982). Electro-eluted DNA was further purified by Elutip columns (Schleicher and Schüll). *E. coli* was transformed according to Dagert and Ehrlich (1979), and yeast according to the method of Ito et al. (1983) with modifications described by Rodriguez and Tait (1983). Isolation of yeast plasmid DNA was also described by Rodriguez and Tait (1983).

Table 1. Genotypes and source of yeast strains employed

Strain	Genotype	Source
AH22	*MATa leu2–3, 112 his4–513 can1*	Hinnen et al. (1978)
EH3605-4D	*MATα LEU2 ura3–52 his5-2 lys1-1 ade2-1*	This study
AH22/sfa	*MATa leu2–3, 112 his4–513 can1 sfa-0::LEU*	This study
EH3606-1D	*MATα leu2–3, 112 ura3–52 his5-2 ade2-1 RAD SNM*	This study
EH3606-2B	*MATα leu2–3, 112 URA his5-2 ade2-1 RAD snm1-1*	This study
EH3259-1D	*MATa leu2–3, 112 ura3–52 hisx ADE rad3 SNM*	This study
EH3250-2D	*MATα leu2–3, 112 ura3–52 hisx ADE rad2–20 SNM*	This study

hisx, gene(s) conferring auxotrophy to histidine not determined

Gene transplacement. Insertion mutants sensitive to FA were constructed according to the one-step gene transplacement method of Rothstein (1983). Plasmid pMI1, containing the wild-type *LEU2* gene inserted into the fragment conferring HYR, was linearized with *Pst*I and *Hin*dIII and used for yeast cell transformation.

Subcloning of DNA fragments conferring HYR. The DNA inserts of pAR172 and pAR184 were partially digested by *Sau*3AI and the resulting fragments separated by preparative agarose gel electrophoresis (Maniatis et al. 1982). Fragments sized between 1 and 4 kb were isolated via electroelution and ligated into the vector YEp13 which had been cut by *Bam*HI and treated with alkaline phosphatase. The mixture was transformed into *E. coli* JF1754 and transformants sensitive to tetracycline and resistant to ampicillin were selected. 300 clones were washed off the agar plates using 10 ml $MgCl_2$, (20 mM) and grown for 3 h in liquid culture (40 ml LB + ampicillin). Isolated plasmids were used to transform yeast strain AH22 and ca. 100 transformants were tested for HYR to FA or 4-NQO. Plasmid DNA was prepared from transformants which demonstrated the appropriate phenotypes and after retransformation and amplification in *E. coli* JF1754 was used for further restriction analysis (cf. Fig. 2).

Determination of HYR phenotype. Mutagen resistance was determined in the diffusion test as described by Ruhland et al. (1981). Survival curves were calculated as described by Ruhland and Brendel (1979). Resistance to FA was measured by determining the minimal inhibitory concentration in growth medium. Cells in logarithmic phase were inoculated into fresh growth medium at 10^6 cells/ml and incubated at 30° C for 24 h with the appropriate FA concentration. Growth was monitored by microscopic counting. Figure 5 depicts the relative growth of cells in the presence of FA on a logarithmic scale. Since the inoculum of 10^6 cells/ml can grow maximally to 10^8 cells/ml a relative growth of 1% implies no growth at all (total inhibition by FA).

Results

Expression of HYR in different vector plasmids

YRp17 and YCp50 received the fragments conferring HYR from episomal vectors pAR172 and pAR184. A *Sal*I-*Cla*I fragment of pAR172, containing the 4.8 kb insert, was ligated into the *Sal*I-*Cla*I site of replicating vector YRp17 (Fig. 1) and yeast strain EH3255-6A was transformed to

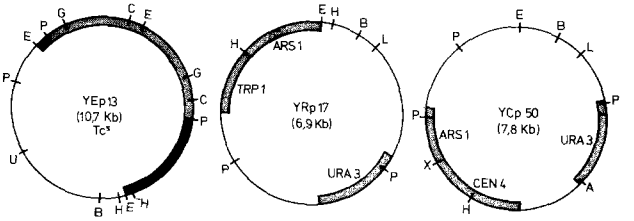

Fig. 1. Plasmid vectors used in this study. YEp13, yeast episomal plasmid; YRp17, yeast replicating plasmid (multi-copy vector); YCp50, yeast centromere plasmid (single copy vector). *Stippled bar*, yeast chromosomal DNA; *closed bar*, 2 μm DNA; *thin line*, pBR322 plasmid DNA. A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; L, *Sal*I; N, *Nru*I; P, *Pst*I; U, *Pvu*II; X, *Xho*I

uracil prototrophy. All transformants exhibited the same HYR to 4-NQO as the original isolate ARi172. A similar result was obtained when a *Sal*I-*Eco*RI fragment from pAR184, including the 7.3 kb insert, was cloned into the *Sal*I-*Eco*RI site of YRp17, i.e. all resulting transformants were hyperresistant to FA like the original ARi184 (Mack 1986). No HYR phenotypes, however, were obtained when the *Sal*I-*Hin*dIII or the *Sal*I-*Cla*I fragments were cloned into the *Sal*I-*Hin*dIII or *Sal*I-*Cla*I sites of single copy centromere vector YCp50 (Fig. 1) and yeast strain EH3255-6A was transformed (data not shown).

Physical mapping and subcloning

A large part of the original insert DNA – 4.8 kb in size in pAR172 (HYR to 4-NQO) and 7.3 kb in pAR184 (HYR to FA) – may not contain genetic information for the observed resistance phenotypes. Restriction of the two DNA inserts with 13 endonucleases yielded the maps depicted in Fig. 2. The relative scarcity of cleavage sites for some of the enzymes and the absence of any for other enzymes necessitated the strategy of limited *Sau*3AI digestion for subcloning of the DNA inserts of pAR172 and pAR184. Approximately 100 YEp13 subclones of both insert DNAs were isolated and were used for transformation of yeast.

Figure 2 shows that the smallest subclone still conferring HYR to FA in yeast is 2.7 kb and conferring HYR to 4-NQO is 2.2 kb. Expression of HYR to FA does not require the 3 kb sequence on the insert DNA between the *Pst*I cleavage sites, whereas the DNA sequence between *Nru*I and *Bgl*II is important for this (Fig. 2). Likewise, HYR to 4-NQO does not require the DNA between *Eco*RI and the right *Bam*HI/*Sau*3AI site while the fragment between the *Pst*I and *Hin*dIII sites is mandatory (Fig. 2). The

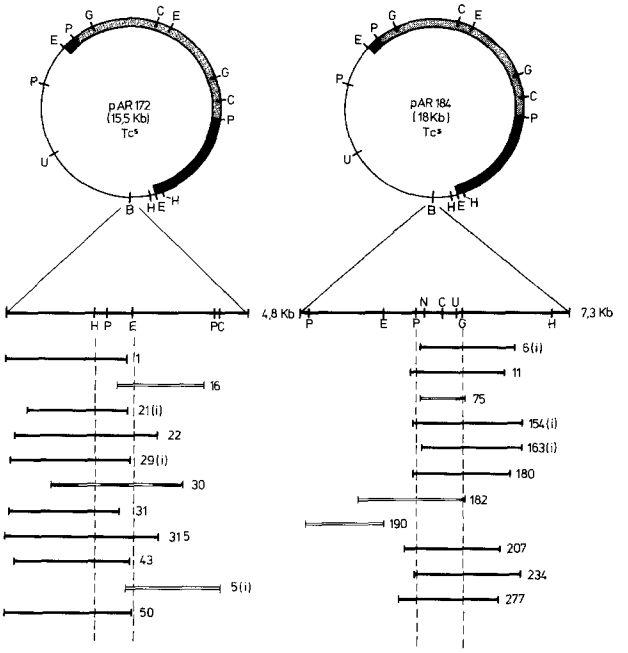

Fig. 2. Physical mapping and subcloning of inserts that confer hyperresistance (HYR) to formaldehyde (FA) (pAR184) and 4-nitroquinoline-N-oxide (4-NQO) (pAR172). *Stippled bar*, yeast chromosomal DNA; *closed bar*, 2 μm DNA; *thin line*, pBR322 plasmid DNA; *thick line*, insert DNA and its subcloned fragments that still confer HYR to FA or to 4-NQO; *open bar*, subcloned fragments that do not confer HYR to FA or to 4-NQO; *open/closed bar* (isolate 30), fragment conferring partial HYR to 4-NQO; inverse orientation of subcloned inserts is indicated by (i) after the isolation number. For abbreviations for cleavage sites, see Fig. 1

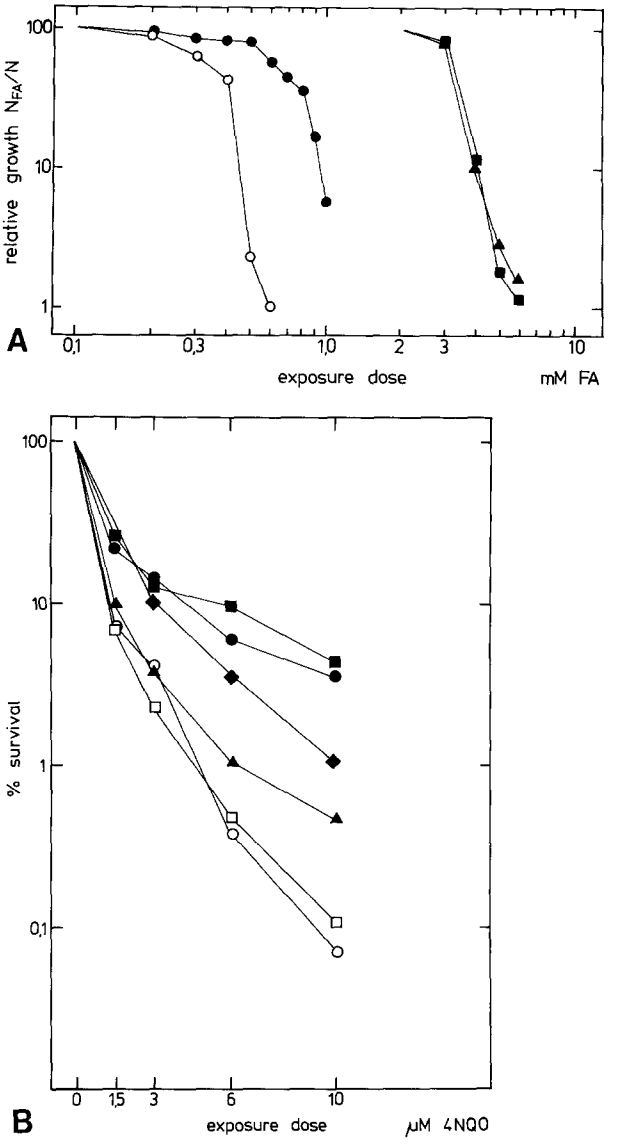

Fig. 3A, B. Response to formaldehyde (FA) and 4-nitroquinoline-N-oxide (4-NQO) treatment of yeast transformed with subclones of the insert DNAs conferring hyperresistance (HYR). **A** Relative growth in the presence of FA of haploid wild-type AH22 YEp13 relative to strains carrying the original pAR184 or subclones thereof. ○—○, AH22YEp13; ●—●, AH22pAR184; ■—■, AH22pME180; ▲—▲, AH22pME207. **B** Survial of haploid yeast strain AH22 transformed with yeast episomal vectors carrying the original DNA fragment conferring HYR to 4-NQO and subclones. ○—○, AH22; □—□, AH22YEp13; ■—■, AH22pAR172; ●—●, AH22pGE31; ▲—▲, AH22pGE30; ◆—◆, AH22pGE1

orientation of the subcloned DNA insert has no influence on expression of the phenotype. Some of the smaller subclones, e.g. pME207, pME180, and pME163i (the latter not shown in Fig. 2) conferred a fivefold higher resistance to FA than did the original pAR184 (Fig. 3). This phenomenon was not found in smaller subclones of pAR172: they either conferred the same resistance to 4-NQO as the original isolate or, as in the case of pGE30, led to expression of intermediate HYR to 4-NQO, i.e. a response between that of the wild type and the original HYR isolate ARi172. This indicates that part of the DNA sequence between *Hin*dIII and the left *Bam*HI/*Sau*3A site is necessary for full expression of HYR to 4-NQO (Fig. 2).

Gene disruption results in sensitivity to FA

HYR to FA is apparently achieved by overexpression of a gene cloned on a multi-copy plasmid. When the function of this gene is blocked by gene disruption in a haploid wild-type strain an FA-sensitive phenotype results (Fig. 5). The construction of the integrating vector is shown in Fig. 4. The integration of the *LEU2* gene at the site of the gene for FA resistance is demonstrated by the segregation pattern of leucine prototrophy and FA sensitivity in the tetrad analysis of a diploid strain constructed by crossing wild-type EH3605-4D *LEU2* with an FA-sensitive transformant. FA sensitivity and FA resistance segregate in a 2:2 fashion while each tetrad splits off at least one spore auxotrophic for leucine in a cross involving as one parent a haploid strain transformed to leucine prototrophy with the linear plasmid pMI1 carrying the disrupted gene locus for FA resistance (6 tetrads with 3 *LEU2*:1*leu2* and 2 tetrads with 2 *LEU2*:2*leu2*; all FA-sensitive transformants are leucine prototrophic). We designate the gene that confers wild-type resistance to FA as *SFA* (sensitivity to FA) and the mutant allele which was derived by gene disruption *sfa-0*:*LEU2*.

Transformation of strain MM5108-2C *sfa-0*:*LEU2* with the single copy vector pMC1 that contains the original insert DNA of pAR184 complemented the FA sensitivity to the level of wild-type resistance to FA (Fig. 5).

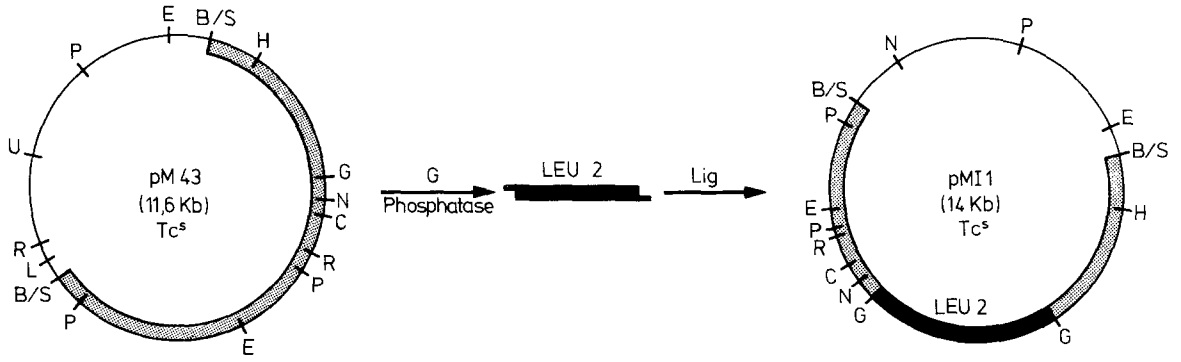

Fig. 4. Construction of the integrating plasmid pMI1. Plasmid pM43 was constructed by *Eco*RI digestion of pAR184. The resulting 11.6 kb *Eco*RI fragment was separated by agarose gel electrophoresis and religated after purification. Plasmid pM43 thus contains the fragment conferring hyperresistance (HYR) and pBR322 DNA and may be propagated in *Escherichia coli*. The HYR fragment was disrupted by restriction at the unique *Bgl*II site, treated with alkaline phosphatase, and ligated to a 2.4 kb *Bgl*II fragment containing the *LEU2* gene. The resulting integration plasmid pMI1 has the yeast *LEU2* gene as a selective marker. pMI1 was linearized with *Pst*I and *Hin*dIII before transforming yeast. *Stippled bar*, HYR fragment; *closed bar*, yeast selective marker LEU2; *thin line*, pBR322 DNA; Lig, ligation; B/S, *Bam*HI/*Sau*3AI; other abbreviations for cleavage sites as in Fig. 1

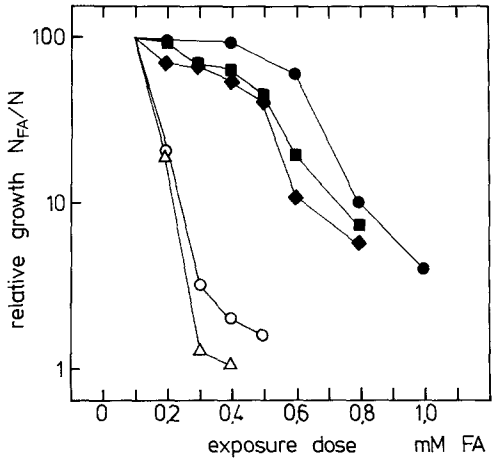

Fig. 5. Relative growth of haploid yeast in the presence of formaldehyde (FA). Segregation of the FA-sensitive phenotype after sporulation of diploid MM5108 derived from the cross: wild type × FA-sensitive pMI1 transformant. △—△, MM5108-2A *sfa-0:LEU2*; ○—○, MM5108-2C *sfa-0:LEU2*; ◆—◆, MM5108-2D *SFA*; ■—■, MM5108-2B *SFA*. ●—●, MM5108-2C *sfa-0:LEU2* pMC1: complementation of *sfa-0:LEU2* by insert DNA on a centromere vector

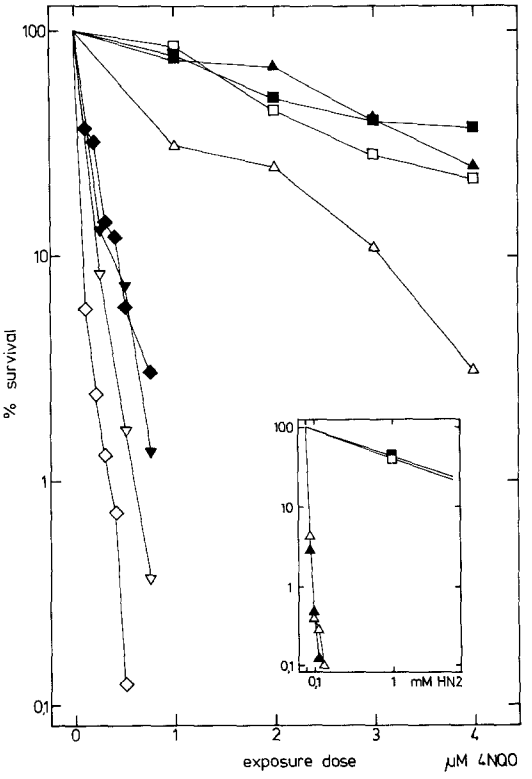

Fig. 6. pAR172-mediated resistance to 4-nitroquinoline-N-oxide (4-NQO) in mutants deficient in excision repair. □—□, *RAD SNM* YEp13; ■—■, *RAD SNM* pAR172; △—△, *RAD snm1-1* YEp13; ▲—▲, *RAD snm1-1* pAR172; ◇—◇, *rad3 SNM* YEp13; ◆—◆, *rad3 SNM* pAR172; ▽—▽, *rad2-20 SNM* YEp13; ▼—▼, *rad2-20 SNM* pAR172. Insert depicts sensitivity to nitrogen mustard

Expression of HYR phenotype in mutants deficient in excision repair

Transformation of the three haploid yeast mutants *rad2*, *rad3*, and *snm1*, deficient in different steps of excision repair of damaged DNA (Reynolds and Friedberg 1981; Siede and Brendel 1982), with the original pAR172 plasmid, conferring the 4-NQO HYR phenotype in transformed wild-type yeast, yielded an increase in 4-NQO resistance in all three repair mutants (Fig. 6). Neither of the highly UV-sensitive *rad2* and *rad3* mutants, however, reached the level of HYR witnessed in wild-type transformants. Mutant strain *snm1-1* which is highly sensitive to nitrogen mustard (Ruhland et al. 1981) and only weakly UV and 4-NQO sensitive was transformed to at least the wild-type 4-NQO resistance level. This, however, was not seen when applying HN2 to the pAR172 transformant where increase in mutagen resistance was minimal compared with that of the non-transformed control strain (inset of Fig. 6).

Discussion

We have presented evidence that the two genes *SNQ* and *SFA* of *S. cerevisiae*, when incorporated into a multi-copy plasmid, confer HYR to 4-NQO and FA, respectively. The presence of these genes in either episomal or replicating multi-copy vectors yielded the same level of HYR but no increase in mutagen resistance was observed when the corresponding genes were introduced into *S. cerevisiae* on the

single copy centromere vector YCp50 (Fig. 1). We may thus assume that only high copy numbers of the genes *SNQ* and *SFA* lead to the HYR phenotypes. The addition of a single copy of the genes to the haploid yeast genome with the help of the YCp50 vector would not be expected to result in an HYR phenotype since it is known that haploid and diploid yeast exhibit identical resistance to FA (Chanet et al. 1976) and to 4-NQO (P. Gömpel-Klein, unpublished results).

Restriction mapping and subcloning of the two original insert DNAs showed that the minimal size of the DNA fragments still conferring HYR to FA or to 4-NQO was 2.7 kb and 2.2 kb, respectively (Fig. 2). These DNA sequences must contain transcriptional control elements since the HYR phenotype was expressed regardless of orientation within the vectors (Fig. 2). Some of the subclones conferred an HYR to FA fivefold higher than that of the original pAR184 isolate (Fig. 3). This was only observed, however, when the 3 kb sequence between the *PstI* cleavage sites had been eliminated.

Gene transplacement with linearized plasmid pMI1 carrying the disrupted gene locus for FA resistance resulted in a mutant allele that conferred an FA-sensitive phenotype to the haploid transformants (Fig. 5). The single copy vector pMC1 containing the original insert DNA of pAR184 could complement this FA sensitivity to the level of wild-type resistance (Fig. 5) while transformation with a multicopy plasmid containing the *SFA* wild-type allele (based on replicating vector YRp17) conferred the phenotype of HYR to FA. A functional *SFA* gene, unlike e.g. gene *RAD3* of the excision repair pathway, the deletion of which leads to recessive lethality (Higgins et al. 1983), is apparently not essential for yeast cell growth and, when blocked, the only phenotypic change seen so far has been a threefold increase in FA sensitivity over that of the wild type. On the other hand amplification of the *SFA* gene yields the HYR phenotype but does not lead to an increased removal of FA-induced DNA-protein cross links nor to a significant reduction of these DNA lesions in the hyperresistant transformants as compared with the wild type (Sander and Brendel 1988).

Upon retesting of the subcloned DNA fragments conferring HYR to 4-NQO we could not find any that conferred an HYR phenotype exceeding that of the original pAR172 isolate. Some of the shorter subclones, however, conferred a weaker HYR than that of the original isolate and the larger subclones (Fig. 3b). These subclones with intermediate HYR to 4-NQO all lacked some DNA in the fragment flanked by the left *Bam*HI/*Sau*3AI and the *Hin*dIII site (Fig. 2). Also, a deletion of the pAR172 *Hin*dIII fragment led to a reduced HYR to 4-NQO (Fig. 3b). All inactivation curves of 4-NQO-treated wild-type yeast or transformants with HYR phenotype showed the tailing phenomenon, i.e. indicated that the cultures contained a 4-NQO-resistant subpopulation (Fig. 3b); this type of resistance was of physiological rather than genetic origin since cultures derived from these subpopulations again showed the expected survival after treatment with 4-NQO and also again the tailing effect (data not shown).

The metabolic steps which result in HYR to 4-NQO are unclear at present. It is known that two nucleotide excision mechanisms are capable of removing 4-NQO-DNA adducts (Regan and Setlow 1974; Ikenaga et al. 1977) and that DNA lesions may be recognized by specific enzymes (Gagliègue et al. 1982) but our results with excision-deficient mutant strains *rad2, rad3*, and *snm1* that show a decreased sensitivity to 4-NQO after transformation with multi-copy plasmid pAR172 in all three mutants (Fig. 6) clearly indicate that resistance to 4-NQO is achieved by metabolic processes other than those of excision repair. It also shows that none of the genes is complemented by *SNQ*. Thus, we presently favour a role for the *SNQ* gene in metabolic steps that result in an increased availability of mutagen deactivators, i.e. 4-NQO scavengers, or alternatively, in steps that may inhibit the activation of the promutagen into its active form (Sugimura et al. 1965; Tada and Tada 1975).

Acknowledgements. We wish to thank Dr. P. Petrasch for developing and updating the LIT-EDITOR program and Ms M. Marcovic and Ms M. Niesen for excellent technical assistance. This research was supported by a grant from the Deutsche Forschungsgemeinschaft.

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Communicated by C.P. Hollenberg

Received August 17, 1987