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Enhancement of copper resistance and *CupI* amplification in carcinogen-treated yeast cells

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Summary. Carcinogen-induced amplification at the *CupI* locus, coding for a metallothionein protein, was studied in the yeast *Saccharomyces cerevisiae*. Exposure of cells from three different haploid strains, 4939, DBY746 and 320, to chemical carcinogens such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), ethylmethanesulfonate (EMS) and 4-nitroquinoline-N-oxide (4NQO) enhanced the frequency of copper-resistant colonies up to several hundred fold. Copper-resistant clones obtained from strains DBY746 and 320, which contain more than one copy of the *CupI* locus, displayed a four- to eightfold amplification of the *CupI* sequences. In these clones the amplified *CupI* sequences were organized in a tandem array. Carcinogen treatment of strain 4939 in which only one copy of the *CupI* gene is present produced resistant colonies without *CupI* amplification. The possible use of the yeast system to study gene duplication and amplification is discussed.

Key words: *CupI* – Gene amplification – *Saccharomyces cerevisiae*

Introduction

Gene amplification, the increase in the dosage of specific DNA sequences, is known in both prokaryotic and eukaryotic genomes. The amplification includes duplications that are detected when bacteria are plated on a selective medium (Anderson and Roth 1977), amplification of ribosomal DNA (rDNA) during early stages of amphibian and insect embryonic development (Brown and Dawid 1968; Spradling and Manhold 1980), and amplification of genes that confer resistance to drugs and metals in mammalian cells (Schimke 1984; Stark and Wahl 1984) and in lower eukaryotes (Fogel and Welch 1982; Karin et al. 1984; Beverly et al. 1984; Walton et al. 1986). Recently, the amplification of specific oncogenes in tumor cells has been reported (Alitalo et al. 1983; Collins and Groudine 1982; Schwab et al. 1983) suggesting that gene amplification may play an important role in carcinogenesis.

Gene amplification can be enhanced experimentally in mammalian cells by physical and chemical carcinogens. Studies in our laboratory have demonstrated that exposure of SV40-transformed Chinese hamster cells to these agents induces transient amplification of viral (Lavi and Etkin 1981; Lavi 1981, 1982) and nonviral sequences (Lavi et al. 1983). Carcinogens enhance stable amplification of dihydrofolate reductase (DHFR) sequences in 3T6 mouse cells and in CHO cells when selected on methotrexate (Varshavsky 1981; Tlsty et al. 1982; Kleinberger et al. 1986).

The molecular mechanism of gene amplification is as yet unknown. Two mechanisms have been proposed based on the structure of the amplified sequences. One mechanism involves unequal crossing over by mitotic recombination, and the other involves disproportionate replication of the genome during a single cell cycle (Johnston et al. 1983; Schimke 1982). Carcinogen-induced DNA amplification provides a system whereby the initial events associated with the amplification process can be directly controlled and analyzed.

In the search for a simple, well-defined genetic system in which the mechanism of carcinogen-mediated gene amplification could be studied, we examined the ability of various carcinogens to induce gene amplification at the *CupI* locus of the yeast *Saccharomyces cerevisiae*. High frequency of homologous recombination in yeasts and well-characterized origins of replication (ARS sequences) offer an opportunity to study the involvement of origins of replication and/or recombination in gene amplification. Moreover, the discovery of *onc*-like sequences in yeast (DeFeo et al. 1983; Gallwitz et al. 1984; Tamanoi et al. 1984) permits the use of this organism to study the effect of carcinogens on genes involved in carcinogenesis.

The *CupI* gene, encoding a metallothionein-like protein, is an important genetic determinant of copper resistance in yeast. Fogel and Welch (1982) have cloned the *CupI* gene of yeast and demonstrated that copper resistance results from sister chromatid exchange, between chromatids containing more than one copy of this gene (Fogel et al. 1984), leading to *CupI* tandem amplification.

To determine whether carcinogens can induce amplification in yeast, we examined their effect on *CupI* in strains containing single and multiple copies of this gene. The results indicate that chemical carcinogens such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), ethylmethanesulfonate (EMS) and 4-nitroquinoline-N-oxide (4NQO), that cause a variety of DNA lesions, enhance the frequency of copper resistance in treated cells. This resistance results from amplification of the *CupI* locus only in strains containing more than one copy of the *CupI* gene. We discuss the possible implications of these findings on the mechanism of gene amplification and carcinogen action.

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Materials and methods

Yeast strains and plasmids. The following strains were used: 4939 (*a, ade2, leu 2-3, trp 1-289, ura 3-52, his 7, Can I, cupI*^{S} (from L. Hartwell); DBY746, (α, *his 3-1, leu 2-3, leu 2-112, ura 3-52, trp1-289, cupI*^{S} (from D. Botstein); and 320 (*a, rme, ade 2, ura 3, leu 1, Can I, CupI*^{r} (from G. Simhen). Plasmid pXK0.7 carrying the 700 bp *Bam*HI fragment containing the *CupI* sequence (Karin et al. 1984) and plasmid RB128 carrying the 2.5 kb *Sal*I fragment containing the *TUB2-YP2* (Neff et al. 1983) sequences were kindly provided by M. Karin and D. Botstein, respectively.

Growth conditions and carcinogen treatment. The complete (YPD) medium contains 2% Bactopeptone, 1% Bacto-yeast extract and 2% dextrose. Minimal (SD) medium contains 0.67% yeast nitrogen base without amino acids (Difco), 2% dextrose and 50 μg/ml amino acids as required by each of the specific strains. For preparation of solid media 2% Bacto agar (Difco) was added. All the media were prepared in double deionized water. The genotype of each strain was verified, before and after each treatment or after clone isolation, by determination of nutritional requirements on SD plates. Selection of copper-resistant and hyperresistant colonies was achieved by plating cells on SD medium supplemented with 0.5 mM and 1 mM $CuSO_4$, respectively. Cells were grown to a density of 5×10^6 cells/ml in YPD medium and treated with 4 μg/ml MNNG (Sigma), or 4 μg/ml 4NQO (Sigma) dissolved in dimethyl sulfoxide (DMSO), or 3% EMS (Merck). Control cultures were treated similarly with DMSO. At various times, aliquots of cells were removed, washed twice in sterile H_2O and allowed to recover for 100 min at 30° C in YPD medium.

Viable cell counts were determined after incubation at 30° C for ca. 36 h while colonies on the selective medium, with $CuSO_4$, were scored after 72 h. Cell counts were calculated as the average number of colonies appearing in three replicates for each time point. The frequency of copper-resistant colonies is expressed as the number of resistant colonies divided by the number of viable colonies. After treatment, several copper-resistant colonies were transferred twice on SD plates containing copper and were used for further molecular analysis.

Restriction enzyme analysis and quantitation of the amplified sequences. Yeast DNA was isolated from 40 ml cultures according to Sherman et al. (1982) and digested to completion with restriction enzymes according to the manufacturer's instructions (New England Biolabs). DNA samples (10 μg) were electrophoresed on agarose gels and the fragments were transferred to a nitrocellulose filter. Prehybridization was performed for 3 h at 42° C in 50% formamide, 4 × SET (1 × SET is 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.8, 1 mM EDTA) 0.1% sodium dodecylsulfate (SDS), 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrrolidone (Pharmacia) and 0.02% bovine serum albumin (Sigma Fraction V)], 0.1% sodium pyrophosphate and 100 μg/ml denatured salmon sperm DNA. The filters were hybridized at 42° C in the same buffer after the addition of 10^7 cpm ^{32}P-radiolabeled nick-translated DNA probe (specific activity 10^8 dpm/μg). After 18 h hybridization the filters were washed twice at room temperature in 2 × SSC and then with 0.5 × SSC at 50° C.

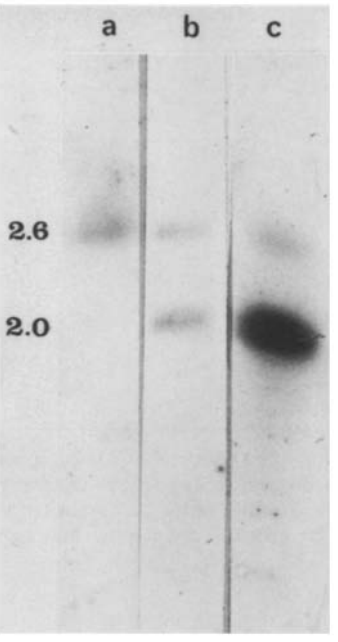

Fig. 1. Determination of *CupI* copy number by *Kpn*I analysis. Samples of DNA (5 μg) were digested to completion with *Kpn*I, separated on a 0.7% agarose gel, transferred to nitrocellulose filters and hybridized to a ^{32}P-labeled *CupI* probe, a 700 bp *Bam*HI fragment derived from pXK0.7 (Karin et al. 1984), as described in Materials and methods. DNA from a, 4939; b, DBY746; c, 320

Quantitation of amplified sequences was performed by comparing the relative hybridization intensities of different ^{32}P-labeled probes to the same filter.

Results

Determination of the copy number of CupI *sequences*

Most yeast strains contain more than one copy of the *CupI* gene. Using Southern blot analysis and a *CupI*-specific probe, the original non-amplified copy appears in a *Kpn*I digest as a 2.6 kb fragment, while a 2.0 kb fragment represents the amplified sequences (Welch et al. 1983). The number of repeats can be calculated from the intensity ratio of the 2.0 and 2.6 kb fragments. Figure 1 shows a Southern analysis of *Kpn*I-digested DNA from the strains used in this study. When grown in SD medium, the strains 4939 (lane a) and DBY746 (lane b) are both sensitive to 0.3 mM $CuSO_4$, while strain 320 (lane c) is resistant to 0.5 mM $CuSO_4$. In strain 4939 only the 2.6 kb band was detected, indicating the presence of a single copy of the *CupI* gene. Both DBY746 and 320 contain amplified *CupI* sequences since the 2.0 kb repeated fragment and the 2.6 kb band are present. However, the intensity ratio of the two bands is 1.5 and 8.5 in DBY746 and 320 indicating a molar ratio of 2 and 11, respectively. Thus, strain DBY746 contains 3 copies of the *CupI* gene while strain 320 harbors 12 copies.

Enhancement of the frequency of copper-resistant colonies by treatment of yeast cultures with carcinogens

To investigate the ability of chemical carcinogens to induce copper resistance, yeasts from two copper-sensitive strains, DBY746 and 4939, and the resistant strain 320 were each

Table 1. The effect of carcinogens on the frequency of copper-resistant colonies

Strain	Treatment	Exposure time (min)	Viable count $\times 10^5$		Viable cells[a] (%)	CUP^r frequency $\times 10^{-5}$[b]		Enhancement[c]
			control	treated		control	treated	
4939	MNNG	20	49	6	12.2	6.8	130.0	19.1
		40	175	2	1.1	1.2	389.0	324.2
		60	165	1	0.6	2.1	455.0	216.7
DBY746	MNNG	40	260	136	52.3	2.6	723.0	278.1
		60	297	67	12.4	1.7	298.0	175.3
	4NQO	60	297	1	0.4	1.7	128.0	75.3
320	MNNG	20	256	32	12.5	12.9	235.0	18.2
		40	383	31	8.1	11.4	549.5	48.2
		60	510	24	4.7	7.7	555.8	72.2

MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; 4NQO, 4-nitroquinoline-N-oxide
[a] Viable count of treated cells divided by viable count of the control culture
[b] Number of copper-resistant colonies divided by the number of viable colonies
[c] Copper resistance frequency in treated cells divided by copper resistance frequency in control culture

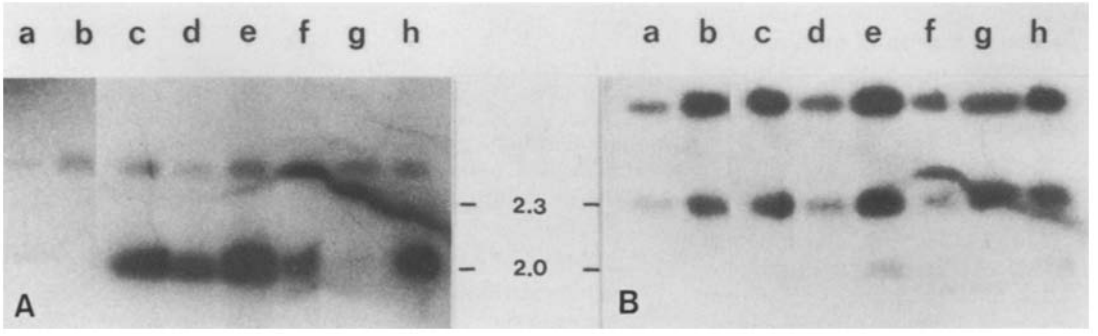

Fig. 2A, B. Southern blot hybridization of *Kpn*I-digested genomic DNA with **A** *CupI*- and **B** *TUB-YP2*-specific probes (after the removal of the *CupI* hybrid). DNA was digested and analyzed as described in the legend to Fig. 1. The hybrids were then removed and the nitrocellulose paper was rehybridized to the radiolabeled 2.5 kb *Sal*I fragment from RB128 (the *TUB-YP2* probe). Lanes a, 4939; b, a copper-resistant derivative of 4939; c, 320; d–f, copper hyperresistant derivatives of 320; g, DBY746; h, a copper-resistant derivative of DBY746. The relative intensity of the 2.0 kb vs the 2.6 kb fragments was determined by densitometric scanning using an LKB densitometer

grown in complete medium, and treated with MNNG. MNNG was chosen since it does not require further activation and the adducts that are formed after its interaction with DNA are well characterized (Montesano 1981). Copper-resistant colonies (growing on 0.5 mM $CuSO_4$) were selected from treated 4939 and DBY746 cells and hyperresistant colonies (growing on 1 mM $CuSO_4$) were selected from treated 320 cells. As shown in Table 1, MNNG treatment was toxic to the cells. The frequency of copper-resistant colonies increased considerably after carcinogen treatment. Maximum enhancement in strain 4939 (324-fold) was achieved in cells exposed to the carcinogen for 40 min. The sensitive strain DBY746 responded similarly with an enhancement of 278-fold (Table 1). The level of resistance could also be affected and from the resistant strain (320) hyperresistant colonies (1 mM $CuSO_4$) were obtained. Hyperresistance to copper reached maximum enhancement (72-fold) after 60 min of exposure to MNNG (Table 1). Enhanced resistance to copper was also obtained with other carcinogenic agents. Exposure of DBY746 to 4 µg/ml 4NQO led to a 75-fold increase in the frequency of copper-resistant colonies (Table 1). Similarly, a 30-fold enhancement of copper resistance was obtained by treating the cells with 3% (vol/vol) EMS (data not shown).

To prove that copper resistance and survival after MNNG treatment are two independent events, the copper-resistant and hyperresistant colonies were tested for their viability following exposure to increasing concentrations of MNNG. The copper-resistant colonies were as sensitive to MNNG as the parental strains.

Detection of amplified CupI *sequences in copper-resistant colonies*

To resolve whether the increased resistance to copper resulted from amplification of the *CupI* sequences, the genomic DNAs of the parental strains and the resistant descendants were subjected to Southern blot analysis.

Figure 2A and B shows the hybridization of the *CupI*- and *TUB-YP2*-specific probes to *Kpn*I-digested DNA from the parental strains 4939 (lane a), 320 (lane c), and DBY746 (lane g) and their copper-resistant derivatives (lane b, for 4939, lanes d–f for 320 and lane h for DBY746). The intensity of hybridization to the *TUB-YB2*-specific probe corresponds to the amount of DNA loaded on each lane.

DNA from the copper-resistant derivative of 4939 did not show any additional bands hybridizing with the *CupI*-specific probe. DNA from the copper hyperresistant deriva-

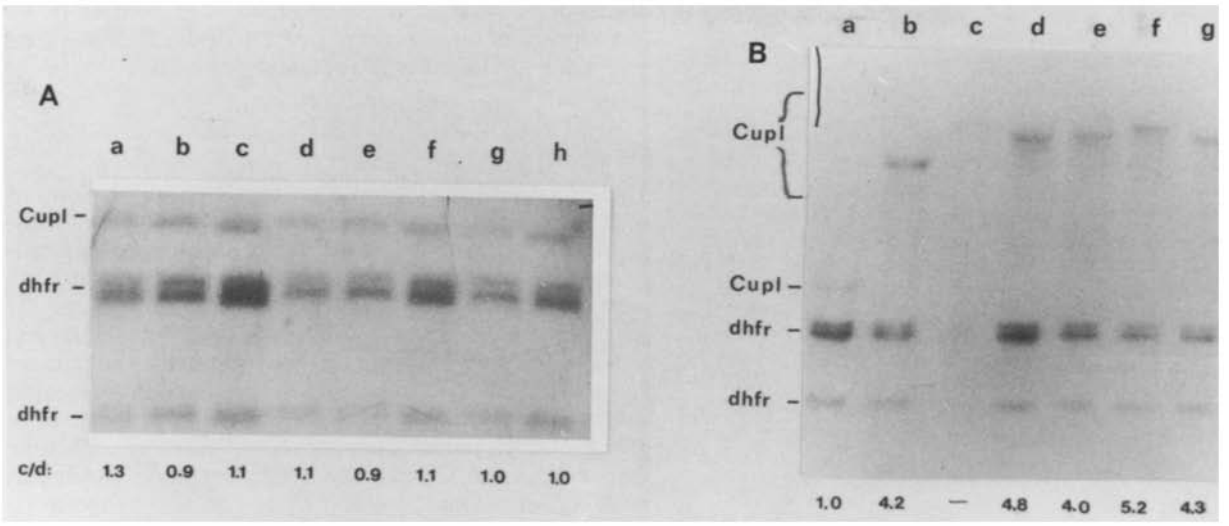

Fig. 3A, B. Southern blot analysis of *Eco*RI-digested genomic DNA. DNA was digested to completion with *Eco*RI, separated on a 0.5% gel, transferred and hybridized to a *CupI*-specific probe and to a dihydrofolate reductase (DHFR)-specific probe (Nath and Baptist 1984). **A** Lanes a–g, copper-resistant derivatives of 4939; h, 4939. **B** Lanes a, DBY746; b–g, copper-resistant derivatives of DBY746. All the resistant clones were derived from carcinogen-treated cultures

tives of 320 shows both the 2.0 and 2.6 kb *CupI* fragments. Densitometric analysis of these fragments indicates amplification of the 2.0 kb fragments corresponding to 17–27 *CupI* copies. Similarly, *CupI* amplification occurred in the copper-resistant clone derived from DBY746; this clone contains 11 copies of the gene. *CupI* was analyzed in 65 copper-resistant clones derived from strain 4939 and 60 DBY746 copper-resistant derivatives.

As the *CupI* gene has no *Eco*RI sites (Fogel and Welch 1982) it was anticipated that tandemly repeated *CupI* sequences would reside within a single *Eco*RI fragment. Indeed the *CupI* sequences appeared as a single *Eco*RI fragment. The same blots were hybridized to a DHFR-specific probe and the ratio between the *CupI* and DHFR hybridizing bands (c/d ratio) was determined. Typical examples of such hybridizations are shown in Fig. 3A and B. The resistant colonies derived from strain 4939 following carcinogen treatment did not show amplified *CupI* sequences (Fig. 3A, a–g). The *CupI* probe hybridized only to the 4.8 kb fragment that was present in the parental 4939 strain (Fig. 3A, h). The intensity of the *CupI* hybridizing bands corresponded to the amount of DNA loaded onto each slot, as was determined by hybridization to the DHFR-specific probe. This pattern was identical in all the 85 copper-resistant clones derived from strain 4939, indicating that in this strain no amplification had occurred in the *CupI* gene.

In all the copper-resistant clones derived from DBY746 (Fig. 3B, b–g) the amplified sequences were localized in new fragments of distinctly higher molecular weight than the fragments containing the *CupI* sequence in the parental strain (Fig. 3B, a). The new fragments hybridized more intensely with the *CupI*-specific probe. Similar analysis of DNA extracted from 54 additional copper-resistant colonies derived from strain DBY746 demonstrated that all clones contained amplified *CupI* sequences (data not shown).

In the absence of selective pressure a fraction of the cells may lose their copper resistance. Some copper-resistant clones from strain DBY746 displayed fainter bands of lower molecular weight hybridizing to the *CupI*-specific probe (i.e., Fig. 3B, d). This heterogeneity in the size of the *CupI*-containing fragments can be attributed to spontaneous variation in the copy number of *CupI* sequences during growth in non-selective conditions. Under these conditions, a considerable fraction of the cells may lose their amplified *CupI* sequences. Indeed, subclones derived from resistant DBY746 colonies grown overnight in YPD medium without copper selection displayed a wide variation in their viability (3%–100%) on copper-containing plates. Heterogeneity in the size of the *CupI*-containing fragment was also detected in DNA preparations derived from cultures that showed stable resistance to copper when grown under non-selective conditions.

Tetrad analyses of crosses between a copper-sensitive strain (4939) and two copper-resistant derivatives of DBY746 containing an amplified *CupI* locus were performed. The diploids showed copper resistance, indicating dominant inheritance. This trait segregated 2:2. All the copper-resistant progeny of these crosses contained an amplified *CupI* locus. Figure 4 shows Southern blot analysis of two representative tetrad progeny, indicating that *CupI* amplification segregates as a single Mendelian locus.

Mapping and quantitation of CupI *sequences in resistant and sensitive yeast strains*

Our findings suggest that the structure of the amplified region might be similar to that described by Fogel and Welch (1982) for spontaneous amplification in the *CupI* locus. Therefore, a detailed restriction analysis was performed on one of the resistant colonies designated DMX (Fig. 5).

A *Kpn*I digest of DBY746 (Fig. 5, a) and DMX (Fig. 5, b) DNA shows that both strains harbor the 2.6 and 2.0 kb *CupI*-containing fragments. The 4.0 kb fragment, representing partially digested doublets of the 2.0 kb repeat, appears only in DMX DNA. Further digestion with either *Eco*RI (Fig. 5, e and f) or *Hin*dIII (Fig. 5, g and h) does not alter this pattern, thus confirming the absence of *Eco*RI or *Hin*dIII restriction sites inside the *CupI* repeats. These sites are also

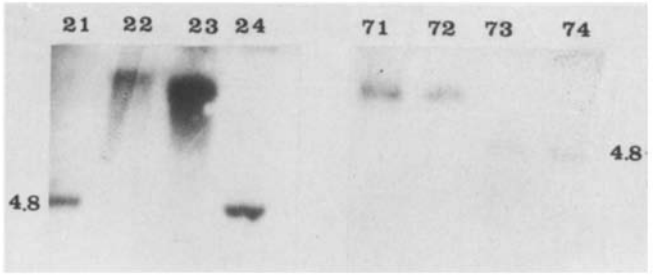

Fig. 4. Southern blot analysis of tetrad progeny: hybridization of *Eco*RI-digested DNA to the *CupI*-specific probe. Left, a tetrad derived from sporulation of a DM09/4939 diploid in which colonies 21 and 24 are copper sensitive and colonies 22 and 23 are copper resistant. Right, a tetrad derived from sporulation of a DM12/4939 diploid in which colonies 71 and 72 are copper resistant whereas 73 and 74 are copper sensitive. DM09 and DM12 are two independently isolated copper-resistant clones derived from DBY746

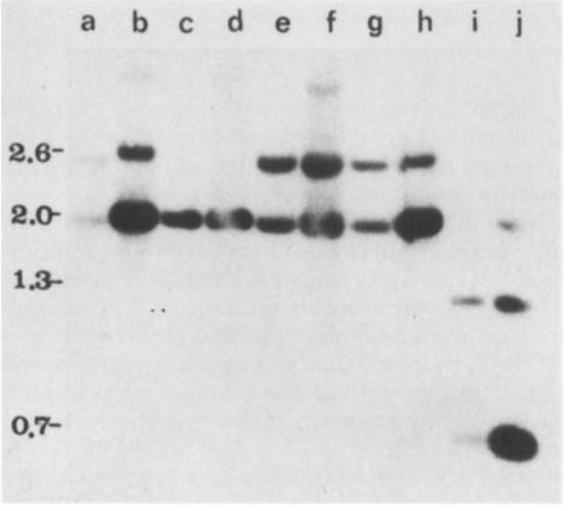

Fig. 5. Mapping and quantitation of the *CupI* locus in the copper-sensitive strain DBY746 (lanes a, c, e, g, i) and its resistant derivative DMX obtained after carcinogen treatment (lanes b, d, f, h, j). DNA preparations were digested by *Kpn*I (lanes a and b), *Xba*I (lanes c and d), *Kpn*I and *Eco*RI (lanes e and f), *Kpn*I and *Hin*dIII (lanes g and h), *Kpn*I and *Xba*I (lanes i and j). (A smaller amount of DNA was loaded in lanes a and d, as revealed by ethidium bromide staining.) The DNA preparations were separated on a 0.7% agarose gel, transferred to nitrocellulose paper and hybridized to the *CupI*-specific probe as described in Materials and methods

absent from the original 2.6 kb fragment. An *Xba*I digest (Fig. 5, c and d for DBY746 and DMX, respectively) shows a single 2.0 kb fragment that hybridized with the *CupI* probe. The partially digested 4.0 kb fragment also appears as a faint band in DMX DNA. The identical size of the *Kpn*I and the *Xba*I fragments clearly indicates that both enzymes cut once within the repeated sequence. This assumption is confirmed by a double digest with both enzymes which yields two fragments, 1.3 and 0.7 kb (Fig. 5, i and j for DBY746- and DMX-derived DNA, respectively). The residual 2.0 kb fragment in lane j represents the product of a partial digest as further incubation with *Xba*I produced a fainter band in this position (data not shown). These results demonstrate that the amplified locus in strain DMX is composed of tandemly repeated 2.0 kb stretches that were originally duplicated in the parental strain DBY746.

Discussion

The enhancement of copper resistance in various strains of *S. cerevisiae* following treatment with chemical carcinogens was studied. Exposure of exponentially growing cells to the DNA damaging agents MNNG, 4NQO and EMS increased the frequency of copper-resistant cells among the survivors. The enhancement ranged between ten and several hundred fold depending on the yeast strain and the carcinogen used. The selected copper-resistant strains displayed similar growth characteristics and sensitivity to the carcinogens as did the parental copper-sensitive strains, indicating that we had not selected for a subpopulation which was both copper resistant and more likely to survive the treatment. Thus, copper resistance was a new phenotype induced by treatment with the carcinogen.

Analysis of the induced copper-resistant colonies revealed the involvement of more than one mechanism in the acquisition of copper resistance. The first mechanism, the amplification of *CupI* sequences, occurred in all the resistant colonies derived from strains DBY746 and 320. These strains initially contained tandemly repeated *CupI* sequences. Tetrad analysis of sporulating diploids generated from crosses between copper-resistant clones derived from strain DBY746 and the copper-sensitive strain 4939 revealed that copper resistance segregated as a single Mendelian locus.

In strain 4939 that harbors one copy of the *CupI* gene, no amplification was detected in the copper-resistant derivatives. In this strain copper resistance was probably acquired by another mechanism. Resistance may have been induced by alterations in the expression or the activity of the copperthionein encoded by the *CupI* locus. As several loci in the yeast genome are known to be associated with copper resistance (Strathern et al. 1982), the amplification independent enhancement of copper resistance may be a result of altered activity or expression of their gene products. Such a mechanism has been suggested (Hamer et al. 1984) to account for the viability of yeast cells in which the *CupI* locus has been deleted.

Carcinogen-induced *CupI* amplification as observed in strains DBY746 and 320 provides a means for the study of the induction of gene amplification in yeast. Our previous studies (Kleinberger et al. 1986; Lavi and Etkin 1981; Lavi 1981, 1982) have shown that transient amplification can be detected at high levels following treatment of mammalian cells with various carcinogens. Nevertheless, stable amplification, which is a less frequent event, can be detected in a small proportion of the treated cells after selective pressure has been applied (Kleinberger et al. 1986; Tlsty et al. 1984). It appears that in those systems, stabilization of the amplified state occurs as a separate process after the additional copies have been generated. Carcinogen treatment may enhance only the initial amplification or may be involved in both initiation and stabilization of the amplified locus. In the yeast system, the frequency of stable amplification is considerably enhanced by carcinogen treatment. Thus, it may be possible to define the role of chemical carcinogens in both stages of gene amplification using yeasts as a model.

Two mechanisms have been proposed for the generation of gene amplification. The first involves recombination and sister chromatid exchange and the second unscheduled replicon firing (Schimke 1982; Stark and Wahl 1984). Carcinogen action is known to be related to both processes since it enhances sister chromatid exchange (Evans 1976) as well as activating amplification of viral sequences in an origin dependent manner (Brown and Dawid 1968; Lavi 1981, 1982). It has recently been suggested that the DHFR gene contains an origin of replication within the amplified unit (Burhans et al. 1986). In yeast cells, Fahrig (1979) have demonstrated that carcinogens enhance the frequency of sister chromatid exchange. Our finding that the enhanced stable amplification at the *CupI* locus occurs only in strains that harbor several copies of this sequence, suggests the involvement of unequal crossing over in this process. This hypothesis is further supported by the fact that the structure of the amplified region is identical to the tandem iterations observed for spontaneously occurring copper-resistant strains of *S. cerevisiae* (Fogel et al. 1984). Our results show that the number of duplications at the *CupI* locus is variable, as some of the resistant strains displayed more than one *Eco*RI fragment hybridizing with the *CupI*-specific probe. Furthermore, a considerable fraction of the cells may lose their resistance to copper following growth in non-selective conditions. Gain and loss of *CupI* sequences probably occurs spontaneously as a result of unequal crossing over between the homologous tandem repeats. The magnitude of this process cannot be determined precisely since a small variation in the copy number of the *CupI* sequences may not lead to a detectable alteration in copper resistance. Carcinogen action may enhance the frequency of this natural process.

Carcinogens may also act on a different level, producing extra copies of the *CupI* gene by unscheduled DNA replication. These amplified sequences will subsequently integrate into the genome by homologous recombination. In the presence of multiple copies of the *CupI* gene, crossing over between the extrachromosomal copies and the genomic tandem repeats may generate an increased copy number of these sequences. In a strain that contains a single copy of the *CupI* gene overreplication will not lead to stable amplification since an integration of each single extrachromosomal copy into the unique parental genome will result in gene replacement, causing no alteration in copy number. Thus, although carcinogens may induce transient amplification in all the treated strains, stable amplification will be detected only in cells that initially contained duplicated sequences. An assay for the detection of amplified copies derived from unique sequences is currently being developed in our laboratory.

The availability of a system in which gene amplification can be induced renders it possible to use yeast in order to study the complex mechanism involved in carcinogen action and gene amplification, to identify the DNA sequences which govern the amplification process and to study their role in the regulation of cell proliferation.

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