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SELECTIVE RESPONSE OF Ty1 TRANSPOSITION TEST TO CARCINOGENS

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Abstract

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The Ty1 assay is a short-term test for detection of carcinogens based on induction the transposition of a gene-engineered Ty1 retrotransposon in *Saccharomyces cerevisiae* cells. Previously, we have shown that Ty1 transposition is induced to positive values of the Ty1 test by a number of laboratory carcinogens, including such that are undetectable by *Salmonella*, DEL or other short-term tests. Here, we provide evidence that the Ty1 test responds positively in concentration and kinetics experiments to the carcinogens 3-aminotriazole, formaldehyde, urethane, benz(a)pyrene, benz(a)anthracene, to the carcinogenic heavy metals arsenic, hexavalent chromium and to the carcinogenic free bile acids chenodeoxycholic and taurodeoxycholic acids. However, the Ty1 test gives negative values with the non-carcinogenic mutagens 5-bromuracil, benzo(e)pyrene, benz(b)anthracene and anthracene, with the heavy metals cadmium and trivalent chromium and the non-carcinogenic conjugated bile acids lythocholic and taurodeoxycholic acids. Results obtained by using the *S. cerevisiae* D7 test and the studied compounds make the involvement of enhanced gene conversion in the Ty1 test response not likely, and strongly suggest that the positive answer to treatment with carcinogens is due to enhanced Ty1 transposition. It is concluded that the Ty1 test shows a selective positive response to carcinogens and gives negative values with the studied non-carcinogenic mutagens.

Key words: Ty1 transposition, short-term test, carcinogens

Introduction

Biomonitoring of early genetic effects requires accurate, sensitive, easy and fast methods to assess genome damages. The *Salmonella* mutagenicity short-term test developed by Ames and co-workers (Maron and Ames, 1983) is used to identify chemicals with mutagenic and carcinogenic potential. The subsequent validation of this method and its modifications made it

the most widely used short-term test. Although a considerable overlapping of mutagenic and carcinogenic chemicals, tested in the Ames assay was first reported (Bratsch et al., 1980), recent analyses have concluded that the correlation between carcinogenicity and mutagenicity is lower than the earlier estimations (Ashby and Tennant, 1996; Ramel et al., 1996). A large amount of data established that animal and human carcinogens exist that do not exhibit any apparent

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genotoxic activity. These carcinogens are difficult or impossible to be detected by the Ames test which provoked the development of other short-term tests. The micronucleus expression in peripheral blood lymphocytes is a well established standard method for monitoring chromosomal damage in human populations (Kirsch-Volders, 2001). Alkaline elution, nick translation and single-cell gel electrophoresis (SCGE) were evaluated and the results indicated SCGE as a promising method for monitoring of populations exposed to genotoxic chemicals (Leroy et al., 1996). The restriction site mutation assay appears to be more adaptable than other assays due to its tissue and organism independence and has the potential to provide more molecular information of genotoxicity (Parry et al., 1990). The yeast *Saccharomyces cerevisiae* responds to a vast spectrum of mutagens and carcinogens. The most frequently used genetic end-points in a variety of yeast systems for monitoring mutagens/carcinogens are mitotic gene conversion, mitotic crossing-over, forward and reverse mutations (Zimmermann et al., 1984). *S. cerevisiae* strains have been constructed for simultaneous detection of several end-points (Zimmermann et al., 1975) or chromosomal missegregation (Adler and Parry, 1993). A short-term test for simultaneous detection of cytotoxic and genotoxic effects based on genetically modified *S. cerevisiae* cells was recently reported (Lichtenberg-Frate et al., 2003). There is evidence in the current literature that increased genome lability and substantial genome rearrangements are associated with cancer (Cairns, 1981; Haluska et al., 1987; Varmus, 1984; Bishop, 1987; Mihelman and Heim, 1990; Sandberg, 1991; Bertman, 2001). A system, selective for intrachromosomal recombination that results in genome deletions, has been constructed in *S. cerevisiae* (Schiestl et al., 1989) and has been termed deletion (DEL) assay. The inducibility of DEL recombination with various carcinogens has been studied. The results indicated that the DEL test was inducible with a variety of non-mutagenic carcinogens that were not

detectable with the Ames assay (Kirpnik et al., 2005). The reason for the wider detection spectrum of the DEL assay was explained by the positive response of the test to deletion events inducible by carcinogens which remained undetectable in the other short-term tests.

Recently, the Ty1 transposition assay has been proposed as a short-term test for detection of carcinogens (Pesheva et al., 2005). The test is based on the induction of *S. cerevisiae* Ty1 retrotransposon whose structure and life cycle are very similar to those of the known oncoviruses. The transposition of Ty1 to new places in the genome creates genome instability and a wide spectrum of DNA damages such as point mutations, deletions, insertions, inversions and large DNA rearrangements (Garfinkel, 1992; Bradshaw and McEntee, 1989; Morawetz and Hagen, 1990). The results obtained with the Ty1 test evidenced that the Ty1 transposition is induced by a number of laboratory carcinogens, including such that are undetectable by Ames, DEL or other short-term tests (Pesheva et al., 2005; Staleva and Venkov, 2001).

In this communication we provide evidence for a selectivity of the Ty1 transposition test to carcinogens. The test responded positively to the studied carcinogenic substances, heavy metals and free bile acids and gave negative results with non-carcinogenic mutagens, heavy metals or conjugated bile acids. It is important to note that these results were obtained with chemicals, not chosen for testing at random, but selected to have similar structures, however different – carcinogenic or non-carcinogenic – potentials. This has been made by purpose for a better validation of selectivity and sensitivity properties of the Ty1 test.

Materials and Methods

Yeast strains and media

The *Saccharomyces cerevisiae* strain DG1141ts1 (MAT α , *ura3-167 his3* Δ 200Tym HIS3AI *ts1*) was

used as tester in the Ty1 assay. The DG1141ts1 is obtained from DG1141 strain (Curcio and Garfinkel, 1999) by replacement of the *TS1* gene with the temperature sensitive allele *ts1* (Pesheva et al., 2005). The *ts1* is a mutant allele of *SEC53* gene which increases the cellular permeability to different substances, including mutagens/carcinogens (Teziyska et al., 2000; Pesheva et al., 2005), which in turn enhances the sensitivity of the test.

The strain *S. cerevisiae* D7 (*MAT α /MAT α ade2-40/ade2-119 trp5-12/trp5-27 ilv1-92/ilv-92*) was kindly provided by F.

Zimmermann and was used as a tester in the D7 assay. Standard yeast media were prepared as described (Sherman et al., 1986).

Ty1 transposition test

The Ty1 test was performed as already described (Pesheva et al., 2005). Briefly, cells were cultivated at 30°C in YEPD liquid medium (yeast extract 1%, peptone 1%, dextrose 2%) to a density of 4-6x10⁷ cells/ml. Appropriate concentrations of studied substances were added to culture aliquots for 30 min, except in the experiments with bile acids, where the treatment of cells was for 16h. Cells were washed, suspended in fresh YEPD and cultivated at 20°C for 12h to complete the Ty1 transposition events. Dilutions were made and cells were plated on YEPD to determine the titer of viable cells and onto SC-HIS plates to determine the number of His⁺ transposants. Median transposition rates were determined (Drake, 1998) and the average value \pm S.D. from 5 to 10 different experiments calculated for each concentration. In the supplied tables, results are presented also as "fold increase" (Staleva and Venkov, 2001) of Ty1 transposition. The fold increase = Fts/Ftc where:

$$Fts = \frac{\text{Number His}^+ \text{ colonies (SC-His medium) of treated culture}}{\text{Number colonies (YEPD medium) x rate dilution of treated culture}}$$

$$Ftc = \frac{\text{Number His}^+ \text{ colonies (SC-His medium) of control culture}}{\text{Number colonies (YEPD medium) x rate dilution of control culture}}$$

The presentation of results as fold increase of Ty1 transposition takes into account the dilutions of cultures which are different due to the different toxicity of the chemicals and concentrations studied and relates the number of Ty1 transposants of treated to control cells. The Ty1 transposition in the controls was taken as fold increase of 1.0 and an increase of ≥ 2 was considered as a positive response of the Ty1 test.

Saccharomyces cerevisiae D7 test

The procedure described by Zimmermann et al. (1975; 1984) was used. The strain *S. cerevisiae* D7 was tested for spontaneous revertants and revertants prior to the experiments and cultures with low background (3-10 revertants/10⁵ cells and 0-10 revertants/10⁶ cells) were grown at 30°C in YEPD liquid medium to a density of about 5x10⁷ cells/ml and tested with mutagens or carcinogens for 1h. After treatment washed cells were plated on appropriate media to detect revertants, revertants, mitotic crossingover and survival rate. Five plates in each category were incubated and the actual colony counts determined. For each concentration of compound or control sample, three independent experiments were performed and the data presented in Table 6 are representative for the mean values obtained. A frequency greater than at least 2-fold over the control frequency was judged as a positive response.

Preparation of S9 mix

S9 fraction from rat liver was obtained from Microbiological Associates (Rockville, USA) and S9 mix was prepared as described (Maron and Ames, 1983). S9 mix contained per ml: 0.9 ml of S9 fraction, 8 μ mol of MgCl₂, 33 μ mol of KCl, 5 μ mol of glucose-6-phosphate, 4 μ mol of reduced nicotinamideadenine dinucle-

otide phosphate and 0.5 units of glucose-6-phosphate dehydrogenase with 100 μ mol of sodium phosphate buffer (pH = 7.0).

Materials

All chemicals were obtained from Sigma Ltd (St. Louis, Mo). Analytical grade of arsenic as Na_2HAsO_4 , trivalent chromium as Cr_2O_3 , hexavalent chromium as CrO_3 and cadmium as CdCl_2 were used. Chemicals were dissolved in water or dimethylsulfoxid (Me_2SO) and dilutions made into the appropriate aqueous treatment solution. Me_2SO , added to the treatment flasks, was in no case more than 5% v/v. Each chemical was treated over a concentration range or to the limits of solubility.

Yeast media were prepared with nutritional components from Difco Chem. Co.

Results

The Ty1 test response to carcinogens

In preliminary experiments we studied 3-aminotriazol, formaldehyde and urethane in the Ty1 test and the results obtained evidenced a positive response of the test in a concentration dependent manner (Table 1). The positive responses appeared at relative low doses having moderate killing effect on tester cells and gradually rose with increasing the concentrations of the carcinogens. The studied substances are carcinogens with different mutagenic status. While 3-aminotriazol is classified as carcinogenic for animals and does not have a mutagenic effect (WHO, 1994), formaldehyde and urethane are animal and human carcinogens that induce mutations and DNA damages in bacteria, rodent and human cells (Swenberg et al., 1980; Cham, 1996)

The study of 5-bromuracil, a well known mutagen without carcinogenic properties, showed negative responses in the Ty1 test even at high concentrations, toxic for about 30% of the tester cells (Table 1). The increased Ty1 transposition, e.g. the positive response

of the Ty1 test, is not due to the stress conditions, generated by higher toxicity of 3-aminotriazol, formaldehyde and urethane to the tester cells, since the treatment with 5-bromuracil or the powerful cell poison NaN_3 (Pesheva et al., 2005) had no effect on Ty1 transposition. Our previous results (Staleva and Venkov, 2001; Pesheva et al., 2005) with different carcinogenic or mutagenic substances and the positive response of Ty1 test to 3-aminotriazol, formaldehyde and urethane suggested that the Ty1 test may have a sensitive response to carcinogenic substances. This suggestion was studied further with pairs of substances that are very similar in chemical structure, however the one being strong carcinogen and the other having only mutagenic activity without being a carcinogen.

Benzo(a)pyrene (B(a)P) and benzo(e)pyrene (B(e)P) represent one such pair of substances. B(a)P is considered a "complete carcinogen" because it is both an initiator and a promoter of cancer (Pepera et al., 2005; Bae et al., 2005). B(a)P is one of the few chemicals known to be carcinogenic in single-dose experiments and following prenatal exposure. B(a)P has produced positive results in numerous short-term *in vitro* assays, using bacteria, fruit flies and cultured human or mammalian cells (Bushy, 1995). B(e)P is non-tumorigenic when applied alone (Chang et al., 1981) and may have co-carcinogenic properties only if applied together with B(a)P (Baird et al., 1984). However, B(e)P is mutagenic to *Salmonella typhimurium* and induces sister chromatid exchange in hamster bone marrow cells.

The study of B(a)P in the Ty1 test showed a dose-dependent positive response at concentrations of the carcinogen, enabling substantial survival rate of tester cells (Pesheva et al., 2005; this study Table 2). Contrary to this, B(e)P does not induce Ty1 transposition above the control values even at concentrations, killing about 30% of the tester cells. It should be noted that the Ty1 tester cells contain the *tsI* mutation, which in previous studies was shown (Terziyska et al., 2000;

Table 1
Induction of Ty1 retrotransposition

Concentration, $\mu\text{g/ml}$	Viability ^a , %	Median Rate of Transposition $\times 10^{-7b}$	Fold Increase
Control (H ₂ O)	100 (812)	2.4 \pm 0.7	1.00
Positive control (EMS 0.1 mM)	56 (455)	8.8 \pm 0.9	6.50
Negative control (NaN ₃ 0.1 mM)	47 (382)	1.8 \pm 0.4	1.60
3-aminotriazol			
50.00	87 (706)	4.3 \pm 0.6	2.20
150.00	71 (577)	9.8 \pm 1.1	6.10
250.00	38 (309)	8.7 \pm 0.5	8.20
Formaldehyde			
25.00	54 (439)	3.6 \pm 1.3	1.90
40.00	42 (341)	8.4 \pm 1.0	8.50
50.00	31 (252)	12.2 \pm 0.7	11.10
Urethane			
40.00	79 (642)	6.0 \pm 1.2	3.20
80.00	41 (333)	11.8 \pm 0.9	12.00
120.00	16 (130)	6.9 \pm 0.4	17.90
5-bromuracil			
1.00	85 (690)	1.9 \pm 1.2	0.80
5.00	67 (544)	2.6 \pm 1.1	1.60
10.00	50 (406)	2.2 \pm 0.9	1.80
15.00	34 (276)	1.5 \pm 0.8	1.80

^a Actual number of colonies is given in parenthesis

^b Average \pm S.D. of median frequencies from six experiments

Pesheva et al., 2005) to increase cellular permeability to mutagens/carcinogens, including B(a)P and derivatives. Therefore, the negative results in Ty1 test for B(e)P are not due to permeability problems, typical for wild type *S. cere-visiae* cells and suggest that the Ty1 test responds positively to carcinogenic substances but remains silent to mutagens without carcinogenic properties.

This suggestion was further proved by the study in

Ty1 test of benz(a)anthracene (B(a)A), benz(b)anthracene (B(b)A) and anthracene. B(a)A is assessed as carcinogenic for animals and humans (Seike et al., 2004) and is mutagenic in bacteria, *Drosophila melanogaster* (Fahmy et al., 1973) mammalian and human cell culture assays (Amacher et al., 1980). Anthracene and B(b)A are non-carcinogenic compounds, however, they have mutagenic activity (Salamone, 1981). As shown on Table 2, only B(a)A induced Ty1 transposition, whereas B(b)A and anthracene did not.

The results obtained supply evidence for a relative selectivity of the Ty1 test in detection of the studied carcinogenic substances. Despite the similar structure of compounds, the Ty1 test responded positively only to substances that are carcinogenic and transposition of Ty1 is not activated by mutagens that are not necessarily carcinogens, even at equitoxic doses such as at 30% viability. The sensitivity of Ty1 test to carcinogens is further evidenced by the positive test response only in presence of metabolic activation transforming procarcinogens into carcinogens (Table 2).

Positive response of Ty1 test to carcinogenic heavy metals

Some heavy metals (the so-called trace elements) are essential for the survival of all life forms. Other heavy metals, however, can be quite toxic or carcinogenic. We studied representatives of the latter group with the Ty1 test.

Arsenic (As) and hexavalent chromium (Cr6) are classified as confirmed human carcinogens, whereas the carcinogenic potential of trivalent chromium (Cr3) is uncertain and Cr3 belongs to a group of substances, defined as not classifiable with regard to carcinogenicity (Hu, 2002). Cadmium (Cd) has been categorized as possible carcinogen since indirect data indicate that inhaled as cigarette smoke Cd may have co-carcinogenic action in combination with the other polyaromatic hydrocarbons (Donkin et al., 2002; Hu, 2002). However, cadmium is not considered as car-

Table 2
Response of Ty1 test to carcinogens or mutagens

Concentration ^a , µg/ml	S9 mix	Viability ^b , %	Median Rate of Trans- position x 10 ^{-7c}	Fold Increase
Control	+	100 (683)	1.8 ± 1.0	1.0
(5% Me ₂ SO)	-	100 (754)	1.5 ± 1.2	1.0
Benzo(a)pyrene				
20	+	95 (650)	4.3 ± 0.9	2.4
40	+	88 (600)	11.5 ± 0.7	7.3
80	+	80 (546)	36.2 ± 0.5	25.1
160	+	42 (287)	48.5 ± 0.3	64.1
160	-	87 (656)	3.4 ± 2.1	3.9
Benzo(e)pyrene				
20	+	86 (587)	2.3 ± 1.2	1.5
40	+	72 (492)	1.8 ± 1.0	1.4
80	+	54 (370)	2.5 ± 1.1	2.4
160	+	31 (212)	1.1 ± 1.1	2.0
160	-	76 (573)	1.5 ± 1.2	1.9
Benz(a)anthracene				
150	+	95 (650)	3.1 ± 0.8	1.7
300	+	91 (622)	5.8 ± 0.9	3.5
450	+	86 (587)	10.3 ± 0.8	6.7
600	+	61 (417)	11.1 ± 0.6	10.1
600	-	98 (738)	3.8 ± 1.6	2.5
Benz(b)anthracene				
150	+	93 (635)	2.0 ± 0.9	1.1
300	+	85 (580)	2.2 ± 1.2	1.4
450	+	71 (485)	2.1 ± 1.1	1.6
600	+	37 (252)	0.8 ± 0.3	1.2
600	-	91 (686)	1.7 ± 0.7	1.2
Anthracene				
150	+	89 (608)	1.5 ± 1.1	0.9
300	+	87 (594)	2.3 ± 0.9	1.3
450	+	80 (546)	2.7 ± 1.2	1.8
600	+	68 (464)	1.7 ± 1.0	1.4
600	-	95 (716)	1.0 ± 0.8	0.7

^a Substances were dissolved in dimethylsulfoxide (Me₂SO) and the response of the Ty1 test measured in presence or absence of S9 mix.

^b Actual number of colonies is given in parenthesis

^c Average ± S.D. of median frequencies from six experiments

cinogen, if applied alone (Groten and Bladeren, 1994). The results obtained in the study of these heavy metals with the Ty1 test (Table 3) showed that As and Cr6 are strong inducers of Ty1 transposition with a fold increase of 30 to 70, respectively.

As comparison, the effect of ethylmethanesulfonate (EMS), a well known carcinogen, is more moderate and treatment of Ty1 tester cells with equitoxic doses at 50% viability gave a fold increase, ranging from 6 to 8 (Table 1).

The positive response of Ty1 test to As and Cr6 appeared at low concentrations, having negligible killing effect and increased by dose dependent manner. The data on Table 4 confirmed the positive response of the Ty1 test to As and Cr6 in kinetics experiments: with increasing the time of exposure, the tester cells responded with an increase of the rate of Ty1 transposition, reaching saturation levels at longer periods of treatment. Contrary to these results, treatment with Cr3 or Cd did not enhance the rate of Ty1 transposition and background values were obtained even at high concentrations or long exposures (Tables 3 and 4)

Although some cells are non-permeable to Cr3, the uptake of chromium through the zinc transport system was recently demonstrated in *S. cerevisiae* cells (Jianglong et al., 2003; Gitan, 2003). Similar intramolecular amounts of Cr6 and Cr3 were found after treatment of yeast cells with chromium (Ksheminska et al., 2003). The uptake of Cr3 in our experiments was evidenced by the decrease of the rate of cell survival (Table 3), which was similar to the survival rate of cells, treated with Cr6. Therefore, the opposite responses of the Ty1 test to Cr6 and Cr3 are not due to low permeability of tester cells to Cr3. The obtained results evidence the applicability of the Ty1 test for detection of carcinogenic heavy metals. Surprisingly, the test can differentiate carcinogenic properties of the tested heavy metals even at the level of different valency of the same element.

Table 3
Concentration dependence of Ty1 test to heavy metals

Concentration, mM	Viability ^a , %	Median Rate of Transposition x 10 ^{-7b}	Fold Increase
Control (H ₂ O)	100 (525)	2.2 ± 1.0	1.0
As			
0.04	95 (500)	2.9 ± 1.1	1.3
0.08	83 (434)	5.3 ± 0.9	2.9
0.16	78 (410)	9.0 ± 0.7	5.2
0.33	65 (341)	18.7 ± 0.3	13.1
0.50	62 (326)	36.2 ± 0.4	26.5
1.00	39 (205)	30.5 ± 0.2	36.7
Cr6			
0.5	92 (483)	6.2 ± 0.8	3.0
1.0	85 (446)	13.4 ± 0.5	7.2
10.0	72 (387)	32.6 ± 0.6	20.6
5.0	59 (310)	59.3 ± 0.3	45.7
10.0	33 (173)	56.1 ± 0.4	77.4
Cr3			
0.5	95 (500)	2.9 ± 0.9	1.3
1.0	90 (473)	2.6 ± 1.1	1.2
10.0	76 (400)	2.0 ± 1.2	1.2
5.0	65 (340)	2.4 ± 0.8	1.6
10.0	41 (215)	1.8 ± 0.7	2.0
Cd			
0.1	98 (515)	2.0 ± 0.9	0.9
0.5	56 (294)	2.6 ± 1.4	2.1
1.0	41 (215)	2.4 ± 1.3	2.6
2.0	17 (90)	0.8 ± 1.1	2.1

^a Actual number of colonies is given in parenthesis

^b Average ± S.D. of median frequencies from six experiments

Positive response of Ty1 test to free bile acids

Bile acids were first regarded as cancer promoters rather than as carcinogens. In a study, using the *S. cerevisiae* D6 test (Ferguson and Parry, 1984) it was found that free, but not conjugated, bile acids are potent inducers of mitotic chromosome aneuploidy. In view of the observed correlation between the ability of a chemical to induce chromosomal aneuploidy and tumor promotional activity, this and other results (Bernstein et al., 2005) indicated that the levels of free bile acids in the colon may be significant factors in the etiology of colonic cancer. Recently, considerable evidence is accumulated in support of the view that free bile acids are carcinogenic in humans. The stimulation of COX-2 expression in human colonic adenomic cells was completely specific for the free bile acids (deoxycholic and chenodeoxycholic acids) and was not achieved with the conjugated taurodeoxycholic acid (Jurek et al., 2005). Free but not conjugated bile acids were found to induce MUC2 expression in human colon carcinoma cells by differential activation of PKC isoenzymes (Looby et al., 2005). We took advantages from these studies to characterize further the ability of the Ty1 test to specifically respond to carcinogens and studied the effect of the free chenodeoxycholic and lithocholic bile acids and the conjugated taurodeoxycholic and glycodeoxycholic bile acids.

The results obtained (Table 5) show that the carcinogenic free bile acids induced positive response in the Ty1 test, whereas conjugated bile acids, which are defined as non-carcinogenic, gave values for the Ty1 transposition close to the controls. For the two free bile acids studied, chenodeoxycholic and lithocholic acids, the data indicate that the positive induction of Ty1 transposition (fold increase > 2.0) does not take place until toxicity was observed. With increasing the concentrations of free bile acids, toxicity rates of Ty1 transposition are enhanced. Similar relationship was found between toxicity and induction of mitotic chromosome aneuploidy in previous studies of free bile acids in *S. cerevisiae* (Ferguson and Parry, 1984).

Table 4
Kinetics of Ty1 response to heavy metals

Heavy Metal	Time, min	Median Rate of Transposition x 10 ^{-7a}	Fold Increase
Control (H ₂ O)	15	2.0 ± 0.8	1.0
	30	1.9 ± 1.1	1.0
	45	2.3 ± 1.2	1.0
	60	2.4 ± 0.8	1.0
As 0.33 mM	15	6.9 ± 0.9	3.6
	30	15.6 ± 0.5	8.1
	45	20.8 ± 0.7	10.8
	60	31.0 ± 0.2	16.1
Cr6 5.0 mM	15	29.6 ± 0.8	14.2
	30	68.3 ± 0.2	37.6
	45	98.3 ± 0.3	47.2
	60	98.2 ± 0.6	43.4
Cr3 5.0 mM	15	3.2 ± 0.8	1.4
	30	1.8 ± 1.1	0.8
	45	2.5 ± 1.4	1.1
	60	2.3 ± 1.3	1.0
Cd 1.0 mM	15	1.9 ± 1.1	1.1
	30	2.4 ± 1.3	1.4
	45	2.6 ± 1.0	1.5
	60	1.9 ± 1.4	1.1

^a Average ± S.D. of median frequencies from six experiments

While the two free bile acids increase the frequency of Ty1 transposition over a ten fold concentration range, the conjugated acids showed no such activity, even though toxicities of up to 40% were produced. The positive response of Ty1 test to free bile acids was found only in presence of metabolic activation (Table 5) evidencing the procarcinogenic status of this compounds, suggested previously (Ferguson and Parry, 1984; Bernstein et al., 2005). On the bases of these results we concluded that the Ty1 test responds

positively to treatment of tester cells with the carcinogenic chenodeoxycholic and lithocholic acids and gives negative results with the non-carcinogenic taurodeoxycholic and glycodeoxycholic acids.

The D7 test response

An increased response of the Ty1 test can be due to either enhanced transposition of the marked Ty1 element, or to its gene conversion with other Ty1 elements (Garfinkel, 1992). In separate experiments we studied the latter possibility using the *S. cerevisiae* D7 test developed to measure simultaneously several genetic end-points, including gene conversion (Zimmermann et al., 1975; Zimmermann et al., 1984). The D7 tester cells were treated with representatives of the carcinogens and mutagens studied in the Ty1 test and the induced frequencies for mitotic crossingover, gene conversion and reverse mutations were determined (Table 6). All tested compounds with the exception of the bile acids induced reverse mutations. The mutagenicity of As and Cd studied on D7 cells after 1h treatment was markedly lower compared to the mutagenic effect of B(a)P, B(e)P, or MMS. It has been reported that these heavy metals induced genetic instability (Sciandrello et al., 2003) or inhibition of the DNA repair mechanisms (McMurray and Tainer, 2003) which give rise to frame shift, base substitution mutations and rearrangements that are rarely seen following acute exposure. In our experiments the tester cells were treated for 1h only which can explain there lower response in induction of reverse mutation following treatment with As or Cd compared to B(a)P, B(e)P and MMS for which a direct DNA damage has been evidenced (Busby, 1995; Chang et al., 1981). The negative responses found with lithocholic and taurodeoxycholic bile acids for the induction of reverse mutations confirmed previous results (Ferguson and Parry, 1984) obtained with another yeast strain.

The data found in our study for the induction of gene conversion showed similar activity for all studied compounds (Table 6). The obtained values evidenced

Table 5
Effect of free and conjugated bile acids on induction of Ty1 transposition

Concentration, mM	S9 mix	Viability ^a , %	Median Rate of Transposition x 10 ⁻⁷ ^b	Fold Increase
Control (5% Me ₂ SO)	+	100 (490)	2.5 ± 0.5	1.0
	-	100 (535)	2.1 ± 0.8	1.0
Chenodeoxycholic acid ^c				
100	+	98 (480)	5.0 ± 0.6	2.0
250	+	72 (353)	8.3 ± 0.8	4.6
500	+	53 (260)	10.5 ± 0.4	7.9
1000	+	32 (157)	12.8 ± 0.5	16.0
1000	-	87 (466)	3.2 ± 1.0	1.8
Lithocholic acid ^c				
100	+	81 (397)	5.3 ± 1.0	2.6
250	+	58 (284)	11.5 ± 0.8	7.9
500	+	44 (216)	17.0 ± 1.0	15.5
1000	+	32 (157)	19.3 ± 1.2	24.1
1000	-	80(428)	4.0 ± 1.1	2.3
Taurodeoxycholic acid ^d				
250	+	89 (436)	3.5 ± 0.9	1.4
500	+	80 (397)	2.8 ± 1.0	1.4
1000	+	58 (284)	3.0 ± 0.8	2.0
2000	+	39 (191)	2.3 ± 1.2	2.1
2000	-	93(498)	1.8 ± 0.9	0.9
Glycodeoxycholic acid ^d				
250	+	103 (505)	3.3 ± 0.8	1.3
500	+	93 (456)	2.0 ± 1.0	0.8
1000	+	85 (417)	4.5 ± 0.9	2.1
2000	+	52 (255)	2.8 ± 0.9	2.1
2000	-	81 (433)	3.3 ± 1.0	1.9

^a Actual number of colonies is given in parenthesis

^b Average ± S.D. of median frequencies from six experiments

^c Free and ^d conjugated bile acids were dissolved in Me₂SO and the response of Ty1 test measured in presence or absence of S9 mix

a moderate induction of gene conversion for both, Ty1 test positive and Ty1 test negative compounds, thus making the involvement of enhanced gene conversion in the Ty1 test response to carcinogens not likely.

Discussion

In this study we provide evidence for the response of the Ty1 test to several carcinogenic and non-carci-

Table 6
Response of *S. cerevisiae* D7 test to mutagens and carcinogens

Chemicals, mM	S9 mix	Viability ^a , %	Crossingover ^a , %	Gene conversion ^a , per 10 ⁵ cells	Reversion per 10 ⁶ cells
Controls					
H ₂ O	-	100 (5150)	0	0.2 (8)	0
MMS, 100mM	-	68 (3502)	1.8 (62)	13.4 (467)	9.1
(positive control)					
Me ₂ SO, 5%	+	100 (3720)	0	1.9 (71)	0
Benzo(a)pyrene ^b					
80	+	66 (2455)	3.0 (74)	2.2 (54)	3.4
160	+	38 (1414)	5.4 (76)	4.8 (68)	5.1
Benzo(e)pyrene ^b					
80	+	52 (1934)	0	3.1 (60)	4.2
160	+	30 (1116)	0.1 (1)	4.4 (49)	5.8
Arsenic ^c					
0.33	-	58 (2987)	0	1.4 (42)	0.5
1.00	-	31 (1597)	0.2 (3)	3.2 (51)	0.9
Cadmium ^c					
0.50	-	66 (3400)	0	1.9 (65)	0.8
1.00	-	35 (1805)	0	3.8 (69)	2.0
Lithoholic acid ^b					
500	+	64 (2380)	0	3.2 (76)	0
1000	+	42 (1562)	0	4.1 (64)	0
Taurodeoxycholic acid ^b					
1000	+	55 (2046)	0	4.3 (88)	0
2000	+	32 (1190)	0	5.9 (70)	0

^a) The number of actual colony counts is given in parenthesis

^b) Results related to control Me₂SO 5% + S9 mix

^c) Results related to control H₂O – S9 mix

nogenic substances. The results obtained with carcinogens or mutagens that are not carcinogens, with heavy metals and with free or conjugated bile acids have shown that the Ty1 test responds positively only to carcinogens and gives negative values with non-carcinogenic substances.

In the tester DG1141ts1 cells, the *HIS3 AI* tagget Ty1 element is transcribed as a whole in polyA containing Ty1-RNA and after splicing the artificial intron

AI, the spliced Ty1-RNA is encapsulated in virus-like particles where it is reverse-transcribed into cDNA and the latter is integrated into new places of the genome (Garfinkel, 1992). The Ty1-cDNA is inserted into the genome either by integrase-mediated transposition to a new locus, or by recombination with another genomic Ty1 element.

All these processes might be influenced by the compounds tested in our study. The results obtained with

the *S. cerevisiae* D7 strain (Table 6) showed that both, Ty1 positive and Ty1 negative substances induced gene conversion at similar frequencies, thus making the involvement of an enhanced gene conversion in the Ty1 test response to carcinogens highly unlikely. The Ty1 transposition can be strongly induced by increased transcription when the Ty1-*HIS3* construct is placed under the *GAL1* promoter and cells are grown in galactose containing media (Garfinkel, 1992; Scholes et al., 2001). However, previous studies (Rolfe and Banks, 1986; Bradshaw and McEntee, 1989; Staleva and Venkov, 2001) have shown that both, mutagens and carcinogens induced the transcription of Ty1 to comparable rates. Our preliminary results also indicate similar transcription levels of Ty1-*HIS3 AI* after treatment the DG1141ts1 cells with compounds that are positive or negative in the test. Therefore, the reasons for the higher transposition rates of Ty1 upon treatment with carcinogenic substances are most likely of post-transcriptional nature. Recently *S. cerevisiae* cells have been screened for mutations that result in elevated Ty1 mobility and 21 *RTT* (Regulation of Ty1 Transposition) genes have been characterized (Scholes et al., 2001). The *RTT* gene products play a role in genome maintenance, DNA recombination repair, suppression of DNA recombination and DNA-damage response pathways. A linkage between the amount of Ty1-cDNA and the frequency of transposition has been found in characterization of *rtt* mutants evidencing the key role of the reverse transcription in determination the transposition rate. Further experiments (now in progress) will show if the synthesis and stability of the Ty1-cDNA in cells treated with carcinogens or mutagens can help the molecular explanation of the results found in our studies.

A special interest represents the negative responses of the Ty1 test to B(e)P, B(b)A and anthracene, which induce mutations that are not necessarily carcinogenic. This observation suggests that not every DNA lesion is an activator of Ty1 mobility. Our results strongly suggest the activation of Ty1 transposition by DNA

damages induced by carcinogens, which in turn activate signal transduction pathways (Rolfe and Banks, 1986; Bradshaw and McEntee, 1989; Scholes et al., 2001). Previous experiments using quantitative assays and Northern blot analysis demonstrated that the induction of Ty1 transposition by methylmethane sulfonate is dependent on the function of *RAD9* and independent of *DUN1* gene (Staleva and Venkov, 2001). Recently, Ty1 has been shown to be induced by an arm of the DNA damage response pathway, which includes *RAD9*, *RAD24*, *RAD17*, *MEC1*, *RAD53* but not *DUN1* genes (Scholes et al., 2001).

The response of the Ty1 test to carcinogens seems to be very sensitive and the test differentiates Cr6 from Cr3. The hexavalent chromium causes mainly lung carcinomas and to a lesser extent cancers in respiratory and gastrointestinal tracts of humans. Although the mechanism by which it converts a normal cell to a tumor cell is not known in details, the current view is that once inside the cells Cr6 is reduced to Cr3 via the intermediate Cr5 and Cr4 forms, which are highly genotoxic. Cr3 itself does not induce DNA damages and is accumulated into cells as a stable form (Arslan et al., 1987; Liu et al., 1995). Recent studies (Kaszycki et al., 2003; Ksheminska et al., 2003) suggested the existence of the same scenario in yeast cells, which can explain the positive results of Ty1 test with Cr6 and the negative response with Cr3, the latter being not a donor of the highly active Cr5 and Cr4 intermediates.

Several explanations can be supposed for the selective response of Ty1 test to carcinogens. First, Ty1 is a retrotransposon with structure and life cycle very similar to those of the known retroviral oncoviruses (Garfinkel, 1992). The similarity in structure and life cycle may be considered as precondition for selective responses to carcinogens of Ty1 retrotransposons and oncoviruses. The carcinogen-induced Ty1 transposition depends on the function of *RAD9* gene and transit through G1 phase of the life cycle (Staleva and Venkov, 2001). *RAD9* is a component of a DNA dam-

age signaling pathway which monitors the integrity of the genome and can arrest the cell cycle to allow DNA repair to occur. The *RAD9* checkpoint gene is the yeast functional counterpart of the human tumor suppressor gene *TP53* which has among its functions the ability to monitor the integrity of the genome and the property to delay DNA replication in G1 phase until repair has been completed (Bertram, 2001). These data suggest the existence of similarities in certain steps of Ty1 retrotransposition and neoplastic differentiation of cells which is in favor of a specific response of Ty1 test to carcinogens.

Second, it had been shown (Garfinkel, 1992) that the integration of Ty1 into new places of the genomic DNA creates genome instability and is accompanied by the appearance of different DNA damages. While most of the short-term tests were developed to detect the induction of a specific genetic end-point, the Ty1 test will respond to a number of DNA damages, which can explain to some extent its relative selectivity to carcinogens, known to induce genome instability and different DNA damages (Bertram, 2001).

Third, results published by other authors evidence the induction of increased levels of reactive oxygen species (ROS) by the carcinogens which are positive in the Ty1 test. For instance, B(a)P and B(a)A induced *in vitro* oxidative DNA damage on fragments of the human *TP53* tumor suppressor gene and *in vivo* formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine, an indicator of oxidative DNA damage (Lowndes and Murguia, 2000). In human cell cultures and primary hepatocytes the deoxycholic acid enhanced the production of ROS, an effect that was abolished in cells with defective mitochondria or by prior incubation with ROS scavengers (Fang et al., 2004). At least 15 reports from 1980 through 2003 indicated that free bile acids cause induction of oxidative stress and enhanced production of ROS that lately damage DNA (Bernstein et al., 2005). The strong carcinogen Cr6, which is positive in the Ty1 test (Tables 3 and 4), was found to induce the formation of ROS in Chinese hamster cul-

ture cells (Ueno et al., 1995). The generation of reactive oxygen and nitrogen species has been proposed also for As, (Valko and Morris, 2005). These literature data suggest that the positive response of the Ty1 test to carcinogens may depend on increased production of ROS. Our preliminary experiments with mitochondrial rho⁻ mutants of the tester strain indicated that the selectivity of the Ty1 test to carcinogens was lost in cells with mitochondrial dysfunctions. We found the same loss of selective response to carcinogens in cells with disrupted *SCO1* nuclear gene coding for a component of mitochondrial oxidative phosphorylation or in tester cells cultivated with ROS scavengers (data to be published). Since the oxidative phosphorylation in mitochondria, which is lacking in rho⁻ mutants or in Δ *sco1* cells is the main source of ROS production, these results suggest the role of elevated levels of ROS in determination the response of the Ty1 test to carcinogens. Further molecular biology studies, are needed for a better understanding the mechanism of selectivity of the Ty1 test to carcinogens.

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