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GENOTOXICITY AND CARCINOGENICITY

The response of Ty1 test to genotoxins

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Abstract The Ty1 assay is a short-term test for detection of genotoxins based on induction of the transposition of a gene-engineered Ty1 retrotransposon in *Saccharomyces cerevisiae* cells. Here, we provide evidence that the Ty1 test responds positively in concentration-dependent manner to the carcinogenic genotoxins benz(a)anthracene, benzo(a)pyrene, chenodeoxycholic and taurodeoxycholic free bile acids and to environmental soil samples polluted with carcinogenic substances. The Ty1 test gives negative results with the noncarcinogenic mutagens benz(b)anthracene, benzo(e)pyrene, lithocholic and taurodeoxycholic conjugated bile acids and to soil samples not polluted with carcinogens. Presence or absence of genotoxins in soil samples was evidenced by chemical analysis. Several explanations for the sensitive differential test's response to genotoxins are proposed and discussed. It is concluded that the Ty1 test can complement existing assays in laboratory and environmental studies showing high sensitivity to a wider spectrum of carcinogenic genotoxins.

Keywords Ty1 test · Carcinogens · Soil samples

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Introduction

Biomonitoring requires accurate sensitive easy and fast methods to assess cellular damages. Presently, the *Salmonella* mutagenicity short-term tests (Maron and Ames 1983) are used to identify genotoxins with mutagenic and carcinogenic potential. Although a considerable overlapping of mutagens and carcinogens tested in the Ames assay was first reported (Bartsch et al. 1980), more recent validations have concluded that the correlation between carcinogenicity and mutagenicity is lower than earlier estimations (Ashby et al. 1991; Ramel et al. 1996). Animal and human carcinogens without apparent genotoxic activity are difficult or impossible for detection by the Ames test, which provoked the development of other short-term tests, such as alkaline elution, nick-translation, single-cell gel electrophoresis (Lerry et al. 1996), the restriction site mutation assay (Parry et al. 1990), the micronucleous expression (Kirsch-Volders and Fenech 2001). Some of these assays were evaluated as promising methods for monitoring exposure to genotoxic chemicals; however, few of them can be applied to large-scale environmental studies.

Saccharomyces cerevisiae strains have been used for construction of short-term tests for detection of mitotic gene conversion and crossingover, forward and reverse mutations (Zimmerman et al. 1975) as well as chromosomal missegregation (Alder and Parry 1993). A system selective for deletions has been constructed in *S. cerevisiae* (Schiestl et al. 1989) and has been termed DEL assay. The results obtained showed that the DEL test has a wider detection spectrum compared to Ames test, as explained by the positive response of the test to deletions inducible by genotoxins (Kirpnick et al. 2005). Although *S. cerevisiae* cells respond to a vast spectrum of genotoxins, the short-term tests based on yeasts show lower sensitivity compared

to bacterial tests (Ramel et al. 1996) due to the lower permeability of *S.cerevisiae* cells (Morita et al. 1989; Staleva et al. 1996; Kirpnick et al. 2005). Recently, the Ty1 transposition assay has been proposed as a short-term test for detection of carcinogenic genotoxins (Pesheva et al. 2005). The test is based on the activation for transposition of the *S. cerevisiae* Ty1 retrotransposon, whose structure and life cycle are very similar to those of the known oncoviruses. The sensitivity of Ty1 test was increased by the introduction into the genome of the tester cells of the mutation *ts1/sec53*, which increases the permeability of yeast cells (Staleva et al. 1996). The results obtained evidenced that Ty1 transposition is induced by a number of laboratory carcinogens, including those that are undetectable by Ames or DEL tests (Pesheva et al. 2005).

In this communication, the sensitivity of Ty1 test was studied using laboratory genotoxins of similar chemical structure having either carcinogenic potential or mutagenic potential, as well as environmental soil samples. A positive Ty1 test response was found only for the carcinogenic genotoxins and for soil samples polluted with carcinogens.

Materials and methods

Sample collection and processing

Soil (S) samples were collected from regions in Bulgaria declared by the National Executive Environmental Agency (NEEA) as the regions with low (odd number) or high (even number samples) pollution levels. Sample collection was in June 2006 (S1, S2), November 2006 (S3, S4), May 2007 (S5, S6) and October 2007 (S8). Samples extractions were made according to published procedure (Rossi et al. 1991). Samples, each one representing an average taken in 10 m^2 area, were extracted with toluene in Soxhlet apparatus for 24 h; the solvent was evaporated and the residual dry matter was dissolved in dimethyl sulphoxide (Me_2SO). Samples processed in this way can be directly analyzed chemically and studied in Ty1 test, which gives the advantage to compare the results obtained in the short-term test with the amount of genotoxins found in samples. The reproducibility of Ty1 test response was tested by repetitions in the following 15 days with aliquots of samples stored at −20°C. During this period, changes in their activity were not observed.

Ty1 transposition test

The Ty1 test was performed essentially as described (Pesheva et al. 2005) with *Saccharomyces cerevisiae* DG1141ts1 as tester strain. DG1141ts1 cells (*MATα ura3-167 his3Δ200 Tym HIS3AI ts1*) have a Ty1 element marked with the indicator gene *HIS3AI* developed by Curcio and Garfinkel (1999). The successive transposition of this Ty1 requires transcription and splicing of Ty1-RNA to remove the artificial *AI* intron and encapsulating the spliced RNA in virus-like particles, where it is reverse-transcribed into Ty1-cDNA with intact *HIS3* gene. Every integration of this Ty1-cDNA into new places of the genome gives rise to a HIS^+ colony on minimal medium lacking histidine. Tester cells that did not undergo Ty1 transposition remain HIS^- because of the *his3Δ200* deletion and cannot grow on the selective medium. Thus, Ty1 is a quantitative test for determination of the Ty1 transposition induced by genotoxins.

Tester cells were cultivated at 30°C in YEPD liquid medium to a density of 4–6 × 10^7 cells/ml. Laboratory genotoxins dissolved in Me_2SO were added to culture aliquots for 30 min in presence or absence of metabolic activation by S9 microsomal hepatic fraction. Processed soil samples were tested in presence of S9 fraction. Washed cells were further cultivated in fresh YEPD at 20°C for 12 h to complete the initiated Ty1 transpositions. Dilution of cells were plated on YEPD to determine the titer of survived cells and on SC-HIS for counting the HIS^+ transposants. Median rates of transposition were determined (Drake 1998), and the average value ± SD from five to ten repetitions were calculated. In tables, the results are also presented as "fold increase" of Ty1 transposition. The Ty1 transposition in the controls was taken as 1.00, and by analogy with other short-term tests, a fold increase in treated cultures equal or higher than 2.0 is considered as positive response of the Ty1 assay.

Quantitative chemical analysis

Processed soil samples were studied in NEEA using analytical methods that are internationally recognized in all Reference Centers for the European Environmental Agencies. A short excerpt of the chemical analysis of soil samples is given in Table 4. The chemicals that were analyzed but not included in Table 4, because the concentration of these substances were below the ecological standards for all studied samples, are pesticides such as alpha-HCH, beta-HCH, delta-HCH, epsilon-HCH, heptachlor, aldrin, isodrin, dieldrin, eudrin, *cis*-heptachlor epoxide, *trans*-heptachlor-epoxide, oxy-clodran, *cis*-chlodran, endo sulfanI, endo sulfanII, o.p.DDE, p.p.DDE, a.p.DDD, p.p.DDD, a.p.DDT, p.p.DDT, methoxychlor, merix, ACB, and polychlorinated biphenols such as PCB28, PCB52, PCB101, PCB105, PCB118, PCB138, PCB153, PCB156 and PCB180.

Materials

All chemicals used were of analytical grade and obtained from Sigma Ltd. They were dissolved in Me_2SO and dilutions were made into the appropriate aqueous treatment

solutions. Me_2SO added to the treatment flakes was in no case more than 5% v/v. Fraction S9 from rat liver was obtained from Microbiological Associates (Rockville, USA). Nutritional media components were from Difco Chem. Co. (St Louis, USA) and all media were prepared as described (Sherman et al. 1996).

Results

The Ty1 test response to laboratory genotoxins

Previously, a positive Ty1 test response was found for some carcinogenic genotoxins that were not detectable in other short-term tests, suggesting an increased sensitivity of the test to a wider spectrum of genotoxins (Pesheva et al. 2005). Here, we investigated the sensitivity of Ty1 test to carcinogens by studying pairs of laboratory genotoxins that are very similar in chemical structure, however, one being a strong carcinogen and the second one having only mutagenic activity without being a carcinogen. Benz(a)anthracen [B(a)A] and benz(b)anthracen [B(b)A] are such pair of substances. B(a)A is considered carcinogenic for animals and humans (Seike et al. 2004), while B(b)A is a noncarcinogenic mutagen (Salamone 1981). As presented on Table 1, the Ty1 test showed a positive response to B(a)A at concentrations enabling substantial survival of tester cells. The Ty1 transposition increased in concentration-dependent manner reaching a plateau at about 600 μg/ml B(a)A (data not shown). Contrarily, B(b)A did not induce Ty1 transposition above the control value even at concentrations killing 70% of tester cells. The negative test responses are not due to permeability problems typical for wild type *S. cerevisiae* cells, since the tester cells containing the *ts1/sec53* mutation proved to increase cellular permeability to genotoxins (Staleva et al. 1996; Pesheva et al. 2005). The permeability of cells to studied genotoxins is also evidenced by the positive answer of the test to B(a)A only in presence of S9 mix (Table 1). Similar results were obtained with another pair of genotoxins: benzo(a)pyrene [B(a)P], being strong carcinogen (Perera et al. 2005) and positive in Ty1 test with a fold increase of 19 at 60 μg/ml, and benzo(e)pyrene [B(e)P], a noncarcinogenic mutagen (Chang et al. 1981) giving negative results in the test (fold increase of 1.7 at 130 μg/ml). Recently, considerable evidence is accumulating in support of the view that free but not conjugated bile acids are carcinogenic in humans (Jurek et al. 2005; Looby et al. 2005). We took advantage from these studies to further characterize the Ty1 test response to genotoxins and studied the effect of the free chenodeoxycholic and litocholic bile acids and the conjugated taurodeoxycholic and glycodeoxycholic bile acids. The results obtained (Table 1) showed that the carcinogenic free bile acids induced positive response in Ty1 test, whereas conjugated bile acids defined as noncarcinogenic gave values close to the controls. While the two free bile acids increase the frequency of Ty1 transposition over a 10-fold concentration range, the conjugated acids showed no such activity, even though toxicities of up to 60% were produced (not shown). The positive response of Ty1 test to free bile acids was found only in presence of metabolic activation, evidencing the procarcinogenic status of these compounds suggested previously (Bernstein et al. 2005).

Study of environmental soil samples in Ty1 test

Samples collected from clean regions showed low toxicity and gave negative responses in Ty1 test (Table 2). These

Table 1 Response of Ty1 test to laboratory genotoxins

Concentration	S9 mix	Survival %[a]	Median rate of transposition per10^8 cells[b]	Fold increase
Control (5% Me_2SO)	+	100 (732)	35 ± 9	1.0
	−	100 (816)	28 ± 8	1.0
Benz(a)anthracene (350 μg/ml)	+	89 (652)	206 ± 11	6.6
	−	95 (775)	39 ± 10	1.5
Benz(b)anthracene (350 μg/ml)	+	85 (622)	38 ± 6	1.3
	−	93 (760)	25 ± 12	1.0
Chenodeoxycholic acid (300 mM)	+	67 (490)	193 ± 15	8.2
	−	87 (710)	32 ± 5	1.3
Litocholic acid (200 mM)	+	69 (505)	211 ± 16	8.7
	−	82 (670)	41 ± 8	1.8
Taurodeoxycholic acid (400 mM)	+	87 (637)	29 ± 6	1.0
	−	97 (790)	25 ± 5	0.9
Glycodeoxycholic acid (400 mM)	+	98 (717)	31 ± 7	0.9
	−	107 (873)	33 ± 9	1.1

[a] Actual number of colonies is given within parentheses

[b] Average ± SD of medium frequencies from seven experiments

Table 2 Response of Ty1 test to soil samples

Sample	Survival %[a]	Median rate of transposition per 10^8 cells[b]	Fold increase	Mutagens/carcinogens[c] found in chemical analysis
Control (5% $MeSO_4$)	100 (567)	28 ± 8	1.0	
Clean regions				
S1	92 (521)	22 ± 10	0.9	
S3	90 (510)	27 ± 8	1.1	
S5	97 (550)	31 ± 6	1.2	
Polluted regions				
S2	78 (442)	131 ± 16	6.0	**As**, **B(a)P**, **B(a)A**, **pyrene**, **crysene**, **benzo (ghi)perylene**, B(e)P, fluorenthene, Pb
S4	67 (380)	127 ± 18	6.8	**As**, **B(a)P**, **pyrene**, **crysene**, B(e)P, fluorenthene, Pb
S6	79 (450)	99 ± 9	4.5	**As**, **B(a)A**, **pyrene**, **crysene**, fluoranthene, Pb
S8	42 (240)	141 ± 21	11.9	**B(a)P**, **B(a)A**, **crysene**, **petrol products**, B(e)P, fluoranthene, Pb

[a] Actual number of colonies is given within parentheses

[b] Average values ± SD from 10 experiments made in presence of S9 mix

[c] Carcinogens are shown in bold

results evidence that the method chosen for sample processing is adequate, and Ty1 test does not give false positives with extracts of nonpolluted soils. Samples collected from polluted regions showed different toxicity on yeast cells with a higher toxic effect for samples containing petrol products (S8). All samples from polluted regions gave positive responses in Ty1 test with a fold increase ranging from 4 to 12. The positive answer was confirmed in concentration (=volume) dependent and kinetics experiments. As shown in Table 3, the positive responses (fold increase ≥ 2) appeared at relatively low doses having moderate killing effect on tester cells and gradually rise with increasing the volume of studied sample. No such dose-dependent effect was observed with samples from the clean region. With increasing the time of exposure (15–90 min), the tester cells responded to treatment with samples from polluted regions with an increase in the rate of Ty1 transposition reaching saturation levels at longer periods (not shown). Contrary to these results, treatment with S samples from clean regions did not enhance Ty1 transposition, and background values were obtained even for long exposures.

The excerpt of the chemical analysis given in the last column of Table 2 shows absence of carcinogenic genotoxins in samples from clean regions and presence of carcino-

Table 3 Dose dependence of Ty1 test response to soil samples

Sample	Sample extract (µl/test)	Survival %[a]	Median rate of transposition per 10^8cells[b]	Fold increase
Controls (Me_2SO)	50	100 (612)	16 ± 8	1.0
	100	100 (451)	19 ± 10	1.0
	200	100 (480)	21 ± 7	1.0
	300	100 (555)	26 ± 9	1.0
Clean region (S1)[c]	50	98 (600)	21 ± 9	1.3
	100	99 (445)	20 ± 7	1.1
	200	91 (435)	22 ± 6	1.2
	300	93 (515)	21 ± 10	0.9
Polluted region (S2)[c]	50	89 (545)	45 ± 6	3.2
	100	82 (370)	81 ± 13	5.0
	200	76 (365)	107 ± 18	6.7
	300	61 (340)	156 ± 22	9.8

[a] Actual number of colonies is given within parentheses

[b] Average values ± SD from six experiments made in presence of S9 mix

[c] Median rates and survival values for S1 and S2 were related to the corresponding controls of Me_2SO volumes

Table 4 Chemical analysis of soil samples

Chemicals	Ecological standard (mg/kg)	Pollutants in soil samples (mg/kg)						
		Clean regions samples			Polluted regions samples			
		S1	S3	S5	S2	S4	S6	S8
Pb	50	31	24	29	364	386	348	287
Cu	150	19	36	32	98	105	80	199
As	25	15	9	12	113	93	82	31
Zn	200	178	108	98	452	395	274	355
Cd	2	0.4	0.3	0.5	2.2	3.2	1.5	1.8
Naphthalene	0.05	0.05	0.05	0.05	0.08	0.05	0.10	0.07
Acenophthene	0.03	0.0	0.00	0.00	0.01	0.02	0.01	0.08
Acenaphthalene	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.03
Flourene	0.03	0.00	0.00	0.02	0.00	0.00	0.03	0.04
Phenanthrene	0.04	0.04	0.05	0.04	0.05	0.02	0.05	0.08
Anthracene	0.05	0.00	0.01	0.02	0.04	0.06	0.02	0.09
Flouranthene	0.02	0.28	0.19	0.03	0.24	0.06	0.13	0.68
Pyrene	0.02	0.02	0.02	0.02	0.28	0.05	0.18	0.49
B(a)A	0.02	0.06	0.01	0.00	0.10	0.02	0.05	0.47
Chrysene	0.01	0.01	0.00	0.00	0.12	0.07	0.06	0.24
B(e)P	0.02	0.01	0.01	0.00	0.09	0.18	0.00	0.18
B(a)P	0.02	0.00	0.00	0.00	0.06	0.18	0.00	0.21
Indeno(1,2,3cd)pyrene	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.01
Dibenzo(a,h)anthracene	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Benzo(ghi)pyrene	0.02	0.00	0.00	0.00	0.09	0.00	0.00	0.01
Petrol products	50	0.00	0.00	0.00	2	8	25	195

gens in samples from polluted regions. Special attention should be given to samples S1 and S3 containing increased amounts of fluoranthene (see Table 4) and showing negative responses in concentration-dependent and kinetics experiments (Tables 2, 3). It has consistently been shown that flouranthene proved to be a potent mutagen in bacterial and mammalian in vitro test systems; however, results from in vivo carcinogenicity studies in rodents indicated that it is not carcinogenic (Goldman et al. 2001; Verschueren 2001).

Chemical analysis of soil samples

Parts of the protocols for the quantitative analytical study of the soil samples are given in Table 4. For all chemicals that were analyzed but not included in the table (indicated in "Materials and methods"), concentrations values, which were below the accepted ecological standards for all studied samples, were found. The results obtained showed a significant pollution of samples collected from polluted regions. The amounts of some heavy metals (Pb, As, Zn) were 4- to 6-fold higher when compared to ecological norms. Most samples were also polluted with the carcinogenic genotoxins B(a)P, B(a)A, benzo(hgi)perylene, petrol products and different mutagenic compounds. The samples collected from the clean regions did not contain any carcinogenic substances or heavy metals above the ecological standards; however, some of them (S1, S3) contained significantly higher amounts of flouranthene.

The results obtained confirmed previous analytical data of NEEA for a significant pollution of some regions (named "polluted" in this study) with different pollutants, including carcinogenic genotoxins. Soil samples, for which the presence of carcinogens over the ecological norm was evidenced, gave positive results in the Ty1 test, while samples polluted with noncarcinogenic mutagens (S1, S3) or samples collected from the clean regions (S5) were negative in this test.

Discussion

The study in Ty1 test of several laboratory genotoxins, free and conjugated bile acids and soil samples polluted or not polluted with carcinogenic substances showed a dose-dependent positive response of the test to carcinogenic genotoxins or environmental samples, for which the presence of carcinogens has been evidenced by chemical analysis. The Ty1 test gave negative results with laboratory

noncarcinogenic genotoxins and with soil samples not polluted with carcinogens. A special interest represents the negative responses to B(e)P, B(b)A and flouranthene, which induce mutations that are not necessarily carcinogenic. It is now evidenced that Ty1 elements are activated for transposition by certain type of injuries to the genome through the DNA-damage signaling pathways (reviewed in Garlfinkel 2005). Our observation suggests that DNA damages induced by carcinogenic genotoxins are strong activators of Ty1 transposition. It should be noted however that some of our results were obtained by testing chemicals selected purposely for characterization of the Ty1 test, and the conclusion for a selective or nonselective test response to carcinogenic genotoxins can be made only after the study of large number randomly chosen genotoxins.

Several explanations can be proposed for the sensitive response of Ty1 test to genotoxins. First, Ty1 is a retrotransposon with structure and life cycle that are very similar to those of the known retrovirial oncoviruses (Garfinkel 1992). This may be considered as precondition for the similar responses to carcinogenic genotoxins of Ty1 retrotransposons and oncoviruses. It has been shown that Ty1 transposition induced by laboratory carcinogens 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 the DNA damage-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 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 transposition and neoplastic differentiation of cells, which can be interpreted in favor of a positive response of Ty1 test to carcinogens.

Second, the integration of Ty1 into new locations of genomic DNA creates genome instability and is accompanied by the appearance of DNA damages such as deletions, insertions, point mutations, gene-conversions, gene rearrangements (Garfinkel 1992). Most of these DNA damages were also found in different tumors induced by treatment with carcinogens (Bertram 2001). While each of the available short-term tests was developed to detect a specific genetic end-point, the Ty1 test will respond to the different DNA lesions induced by carcinogenic genotoxins, which increases its detection spectrum.

Third, results published recently evidence an increased production of reactive oxygen species (ROS) by the genotoxins that are positive in Ty1 test. B(a)P and B(a)A induced oxidative damages in human DNA with the formation of 8-oxo-7,8-dihydro-2-deoxyguanosine (Seike et al. 2004). At least 15 reports from 1980 to 2003 indicated that free but not conjugated bile acids cause induction of oxidative stress and enhanced production of ROS that damage DNA (Bernstein et al. 2005). These data suggests that the positive response of Ty1 test to genotoxins may depend on increased production of ROS by some carcinogens. Our preliminary data with mitochondrial rho$^-$ mutants of the tester strain indicated that the selectivity of Ty1 test to carcinogens is lost in cells with mitochondrial dysfunctions (Stoycheva et al. 2007). Since the oxidative phosphorylation in mitochondria, which is lacking in rho$^-$ mutants, is the main source of ROS production, this result also suggests the role of ROS in determining the response of Ty1 test to carcinogenic genotoxins. We favor this possibility for explaining the response of Ty1 test to genotoxins, and a detailed study on the role of elevated ROS production in the induction of Ty1 transposition is now in progress.

The advantages that the Ty1 test may have in environmental studies consist in its properties to detect sensitively the carcinogenic pollutants. It is a cheap, fast and sensitive method for detection of carcinogenic genotoxins, including those that are negative in other short-term tests. The usage of Ty1 test together with other tests for monitoring the environment might give a more complete picture of the pollution status in the studied regions.

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