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The Ty1 Retrotransposition Short-Term Test for Selective Detection of Carcinogenic Genotoxins

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1. Introduction

A number of breakdowns in human's health including cancer have been attributed to the exposure of environmental pollutants, and it became imperative to check the potential toxicity, mutagenicity or carcinogenicity of a number of chemicals. The tests designed for such studies are divided into long-term and short-term assays.

Long-term tests are bioassays for carcinogenicity using animals (mainly rats or mice) as testers. Targets of the carcinogenic action are either organs or the whole body of the tester animal studied by dissection and histochemical analysis. These bioassays are not suitable for the study of a large number of substances because they are time consuming, expensive, require special facilities and qualified personal. A full long-term assay is completed in an average period of two years which stimulated the development of medium-term bioassays (Ito et al. 1998). These assays determine formation of neoplasms in various tissues of rats or mice within a period of 12-18 months. However, the existence of thousands genotoxins with potential carcinogenic effect requires the application of much faster assays and short-term tests have been developed and widely used. According to the timeframe required to obtain results in an assay, the transgenic mouse systems are intermediate between long-term and short-term tests. Although designated as "short-term bioassays" (Tennant et al. 1995) results from these tests are obtained in 4-6 months. The modified genes of transgenic animals cause them to respond rapidly to carcinogens (Cannon et al. 1997). Transgenic mouse systems are statistically reliable *in vivo* assays and the positive results obtained in the tests are highly predictive of rodent carcinogenicity.

1.1 Advantages of short-term tests

Short-term tests identify genotoxic agents by detecting the three major end-points associated with human cancer diseases: gene mutation, clastogenicity and aneuploidy. The main value of the short-term tests lies in their ability to identify chemicals that may cause cancer under certain exposure conditions. More than 200 *in vitro* and *in vivo* short-term assays have been developed using bacteria, yeast, cultured mammalian cells and insects as testers. Most of the

testers are engineered to detect one genetic end-point, such as mutations, deletions, genome rearrangements, loss of chromosomal fragment or whole chromosome. Tester strains were also constructed for simultaneous detection of several end-points. Typical example is the *Saccharomyces cerevisiae* D7 tester strain (Zimmermann et al. 1975) for detection of mitotic crossingover, gene conversion and reverse mutation. The current status of short-term tests for evaluation of genotoxicity, mutagenicity and carcinogenicity of chemicals and environmental pollutants has been recently reviewed (Bajpayee et al. 2005).

Generally, the short-term tests are rapid, inexpensive and easy to perform: results from bacterial tests are obtained in 3-4 days, and mammalian cell based assays are accomplished in about 10 days. Some bacterial cell-based assays are commercially available with results obtained in few hours. However, the advantage of having a fast procedure is compensated by the high unspecificity of the positive response: these tests detect all kind of DNA-damaging agents. Together with the possibility to analyze several samples simultaneously, these mentioned characteristics seem to be the main advantage of short-term tests. The mouse lymphoma assay and Chinese hamster ovary assay (Clive et al. 1979; Li et al. 1987) can detects genetic damages in viable cells capable of forming colonies, which permits the automatization of the methods. Cultured mammalian cells (*in vitro*) or bone marrow cells of rodents (*in vivo*) have been developed to study clastogenic response by analyses of chromosomal aberrations in metaphase cells or micronucleus formation. The micronucleus test (Neri et al. 2003) can be used along with kinetochore and centromeric staining to categorize the test compound as a clastogen or aneugen (Parry et al. 2002). Recently, the single cell gel electrophoresis (comet) assay gained popularity due to its rapidity and low cost (Singh et al. 1991). This assay can be performed both *in vivo* and *in vitro* to detect substantial DNA damages leading to fragmentation of the target DNA.

A significant advance was the development of cell transformation assays for a specific detection of carcinogens or to study the process of neoplastic transformation. The Syrian hamster embryo cell system (Le Boeuf et al, 1999) detects carcinogens within 10 days and has the advantage to be the only transformation system that employs normal diploid cells capable of metabolizing a wide spectrum of pro-carcinogens to their active form. The BALB/c3T3 mice cell system is based on a spontaneously immortalized cell line showing high sensitivity to transformation. Improvements of the assay showed a concordance of 73.5% with long-term bioassay (Kajiwara et al., 1997).

In conclusion, results from several appropriately selected short-term tests is now considered equivalent to the predictive value of a long-term bioassay. Short-term tests not only save time and expenses but also cut down the animal experimentation.

1.2 Disadvantages of short-term tests

The results of collaborative studies and the data accumulated, evidence that at present no single short-term assay can detect all genotoxic substances and discriminate noncarcinogenic mutagens from carcinogens. It is now accepted that batteries of tests have to be used in the study of mutagenic and/or carcinogenic properties of each potential genotoxin. Recommendations were made for strategies in testing which include several stages (usually three), each one consisting of 2 to 4 different assays and some of the tests (like the *Salmonella* mutagenicity test) have to be performed with 3-5 tester strains (Bajpayee

et al. 2005; Eastmond et al. 2009). The necessity to perform a number of assays depreciates the main advantage of short-term tests – their rapidity and low cost. Short-term assays are used to study toxicity, mutagenicity and carcinogenicity of substances, however, the deep concern in such studies is mainly the presence of carcinogenic properties in pesticides, drugs, industrial wastages as well as pollutants of environment. Therefore, the need of having a short-term test for detection of carcinogenic activity in the start of testing for chemical risk assessment still exists. The cell transformation assays use the induction of certain phenotypic alterations that are related directly to neoplasia. The disadvantages of the assays seem to be the instability of the cell cultures used. Special precautions should be made to avoid culture and cell variations from different embryos and to maintain cells with the original karyotype. Therefore, the cell transformation assays can be successfully performed only in specialized laboratories by well trained personnel.

The *in vitro* mammalian cell assays detect a number of DNA damages in well characterized genes used as markers. However a correlation between the positive results and carcinogenicity is not always foolproof. There is increasing evidence that carcinogens acting through nongenotoxic mechanisms are not detected in these assays (Bajpayee et al. 2005). The interpretation of results from other assays may be also difficult. Thus, the chromosomal aberration *in vitro* assay is affected by artifacts due to cytotoxic doses of the studied compound, extreme pH values and metabolic activation that give false-positive results (Galloway 2000; Scott et al. 1991). The *in vivo* chromosomal aberration assay (Johnson et al. 1998) is used mainly for diagnosis, development and spread of tumors, rather than for screening studies (Szeles 2002). False-positive results can also be obtained with sister chromatide exchange measuring assays, since the majority of them do not represent genuine mutational events and are due to exchanges occurring in nonhomologous loci (Rodriguez-Reyes & Morales-Ramirez 2003).

The induction of micronuclei is related to dysfunction of spindle apparatus or formation of acentric fragments. Carcinogens give rise to these lesions during G1 to S phase transition of the cell cycle. The formation of micronuclei takes place in subsequent cell cycles and therefore one cell cycle is allowed between treatment and harvesting of cells. At shorter sample intervals micronuclei are not detected and at longer sampling time micronuclei are diluted and difficult for detection. Thus, results in the micronucleus test are greatly influenced by the time of treatment and sampling in relation to the cell cycle.

Some disadvantages of the comet assay have also been found. The main are: variations of results obtained because of the small cell sample, rate limiting of results due to the analysis of single cells, different interpretation of results due to variability in technical performance and cells utilized. Beside, the comet assay detects significant damages that result in DNA fragmentation and degradation usually associated with cell death (Split & Hartmann, 2005). Therefore this assay is less suitable for monitoring mutagenicity and clastogenicity of genotoxins.

In conclusion, most of the short-term tests have one genetic end-point associated with the activity of some, but not all carcinogens in the multistage process of differentiation the normal into malignant cells. Because of this, no one short-term test can detect all carcinogens and the usage of batteries of assays is recommended, which compromises the main advantage of the short-term assays – their rapidity and low cost. The few short-term tests

having the process of neoplastic differentiation as an end-point are high tech methods requiring well equipped and specialized laboratories.

2. The Ty1 retrotransposon of *saccharomyces cerevisiae* is very similar to oncogenic retroviruses

Transposons are mobile DNA elements that replicate independently of the cellular genome and transpose the new copy into different places of nuclear DNA by nonhomologous recombination. They are ubiquitous from bacteria to human cells with a clear tendency to increase their copy number in higher eukaryotes: 45% of human DNA represents transposons and their remnants. Mobile elements that transpose to new sites in the genome via RNA intermediate are called retrotransposons because in many aspects they are analogous to the retroviruses, such as equine anemia virus, human immunodeficiency virus type 1 (HIV-1), yeast Ty1 transposon ect. (Garfinkel et al. 2006). Retroelements have been extensively studied in the yeast *S. cerevisiae*. Five distinct families of retrotransposons exists in this organism, named Ty1 to Ty5. Ty1 elements are the most abundant (33 copies per haploid genome), most highly expressed and most transpositionally active.

The structures of Ty1 retrotransposons and oncogenic retroviruses are very similar and consist of long terminal repeats (LTR) flanking a central coding domain including *gag* and *pol* genes (Figure 1). In the case of infectious retroviruses, an *env* gene is also present. The *gag* gene encodes structural proteins that form the retrotransposon virus-like particles (VLP), or the retroviral core particles, while the *pol* gene encodes the enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). LTR retrotransposons and retroviruses are transcribed by RNA polymerase II from LTR to LTR to form a terminally redundant RNA molecule which is translated and packaged into the virion core particle or VLP.

Translation of Ty1 mRNA initiates close to its 5'end (Figure1). Like retroviruses, Ty1 elements employ translational frameshifting at the beginning of *pol* gene to regulate the expression of *gag* and *pol* gene products. The two open reading frames are overlapping and separated by a +1 frameshift occurring within a highly conserved sequence. The primary translation products are Gag-p49 and GagPol-p199 that are cleaved by PR to form mature Gag-p45, PR-p20, IN-p71 and RT-p63. The endogenous protease PR of Ty1 and retroviruses employs an aspartic residue in its catalytic centre. Finally, the processed subunits are assembled in VLPs where Ty1mRNA is used by RT as a template to generate double stranded Ty1 copyDNA that can be integrated by IN at new sites within the genome. The integration of the new Ty1 copy is often accompanied by the appearance of mutations, deletions, insertions, gene conversions, inversions or large genome rearrangements which labilize the structure of the host genome and may cause partial or complete loss of chromosomes (Garfinkel 2005). It should be noted that similar labilization of genome structure accompanied with chromosome loss occurred frequently during neoplastic differentiation of mammalian cells (Jeronimo et al. 2001).

The analysis of large variety of target sites has shown that 5' flanking regions of tRNA genes are the preferred target sites for Ty1 transposition (Mewes et al. 1997). Since the upstream regions of tRNA genes do not contain any special DNA sequences, the region-specific integration may be due to specific interactions of Ty1 integrase with the genome structure

formed over the promoter elements of the tRNA gene (Nyswaner et al. 2008). Thus, Ty1 integration does not involve specific nucleotide sequence but a particular chromatine structure has the dominant role.

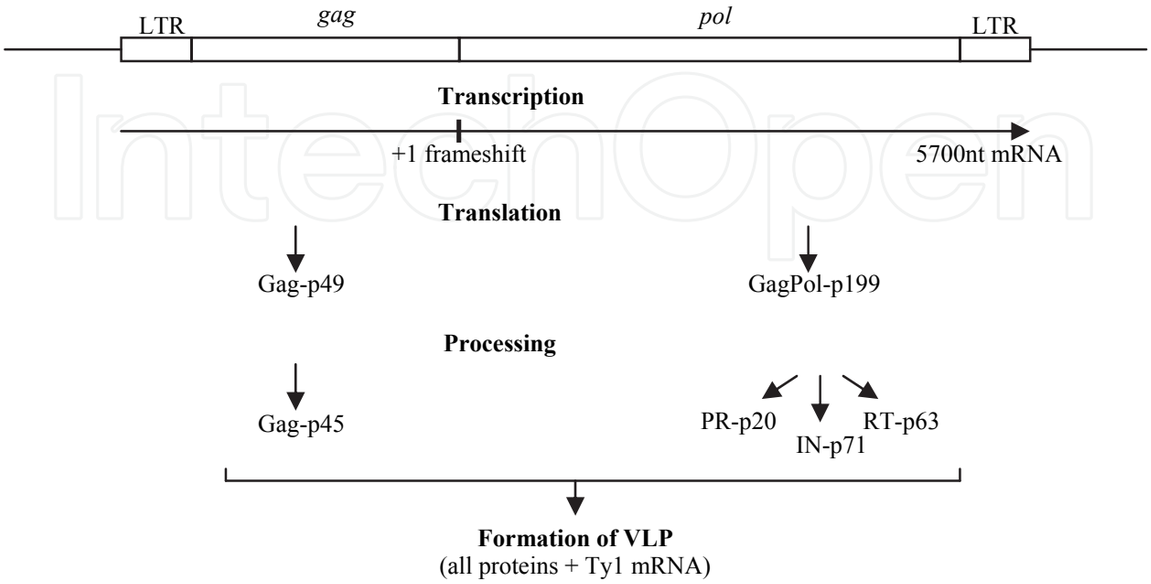


Fig. 1. Expression of Ty1 retrotransposon

Despite the high levels of Ty1 mRNA, transposition occurs at low level (10^{-5}-10^{-7}/cell/generation), cells contain low amount of mature Ty1 proteins and few VLPs are present. The balance needed between the level of *de novo* transposition and the Ty1 activity that can be tolerated by host yeast cell is reached by (1) a mechanism of transposition to specific target sites (upstream of tRNA genes), which are less hazardous for the cell because are devoid of protein coding function and, (2) regulatory mechanisms, that restrict Ty1 transposition to a low level. Both of these mechanisms lead to a decrease of successful Ty1 transpositions, thus saving the host from dangerous damages of DNA that may appear in the target site and result in labilizing the integrity of the genome.

The mobility of Ty1 retrotransposons is restricted by a large collection of proteins that preserve the integrity of the genome. Most of these repressors of transposition (Rtt) are ortologs of mammalian retroviral restriction factors (Curcio et al. 2007). The Rtt integrity factors inhibit transposition at posttranscriptional steps resulting in low level of Ty1 copyDNA. Two checkpoint pathways, the replication stress pathway and the DNA damage pathway, are involved in this type of Ty1 regulation in a cell-cycle dependent manner. Earlier studies (Staleva & Venkov 2001) also evidenced the dependence of Ty1 transposition upon transit through the cell cycle and *RAD9* gene product, which is a member of DNA damage signal transduction pathway. The protein product of *RAD9* checkpoint gene is the yeast functional counterpart of the human oncogene suppressor protein p53, which has among its functions monitoring the integrity of the genome and the delay of DNA replication until repair has been completed (Bertram 2001). These data suggest the existence of similarities in certain steps in regulation of Ty1 transposition and the neoplastic cells.

The analogy between Ty1 retrotransposons and retroviruses was recently confirmed in studies dealing with details in the mechanisms of their replication. The human APOBEC3G protein is similar to the APOBEC proteins of other eukaryotes and has been shown to inhibit the replication of Ty1 retrotransposon and retrooncoviruses. In all studied retroelements this inhibition represents a deamination of cytosines to uracils in copyDNA, which leads to degradation of the retroelement genome. An absolute requirement for a glutamate (E250Q) in the catalytic center of the different APOBEC proteins was found evidencing that DNA cytosine deamination is part of a mechanism, that restricts successful replication of Ty1 retrotransposons and retroviruses (Schumacher et al., 2008).

The surprising similarities between Ty1 retroelements and retroviruses become more understandable by the results obtained in phylogenetic studies (Llorens et al. 2009). The usage of combined graph and phylogenetic analysis based on genome sequencing data made possible the evolutionary history of LTR retroelements to be traced as a time-evolving network. The results obtained show that the Ty1 family represents the oldest pattern in this network and that diverse retroviruses evolve from LTR retrotransposons.

Mobile elements has a significant influence on evolution. Sequencing of diverse genomes reveals, that about half the spontaneous mutations in *Drosophila* result from insertions of mobile elements. In mammals mobile elements cause a smaller proportion of spontaneous mutations – 10% in mice and 0.2% in humans. It is presumed that mobile genetic elements have contributed to the evolution by promoting the creation of new genes with mutations favoring the adaptation (Garfinkel 2005). Thus, mobile genetic elements are probably one of the most powerful endogenous factors driving the evolution of all forms of life on earth.

In conclusion: according to genome structure, gene expression, life cycle, regulation of gene expression and evolutionary history, Ty1 retrotransposons are very similar to the oncogenic retroviruses, except for the absence of an infectious extracellular phase in their life cycle.

3. The Ty1 transposition test

Stress conditions, such as exposure to UV light (Rolfe & Banks 1986), low temperature treatment (Paquin & Williams 1984; Stamenova et al. 2008), severe adenine starvation (Ribero-dos-Santos et al. 1997) increase Ty1 transposition frequency. Chemical carcinogens can also increase transposition rate (Bradshow & McEntee 1989; Staleva & Venkov 2001). This, and the similarity in structure, gene expression and regulation reaching full analogy at certain points between Ty1 retrotransposons and oncogenic retroviruses suggested that the carcinogen-induced Ty1 transposition may be used for detection of carcinogenic genotoxins and substantiated the development of the Ty1 short-term test.

3.1 Construction of the tester strain

Two features have been considered important in the construction of the tester strain: (1) easy phenotypic detection of retrotransposition events and (2) increased permeability of tester yeast cells to carcinogens.

The indicator gene *HIS3AI* developed by Curcio & Garfinkel (1991) fulfills the first requirement. This reporter gene represents the *S. cerevisiae HIS3* gene interrupted by an artificial intron (AI) in the antisense orientation. The *HIS3AI* construction was inserted into a

Ty1 element with the intron on the sense strand of Ty1 and the marked Ty1 retrotransposon is integrated into the genome of a *S. cerevisiae* strain with deleted *HIS3* gene (Curcio & Garfinkel 1991). Thus, a successful transposition of the marked Ty1 element requires transcription, splicing the artificial intron, encapsulation in VLPs where Ty1 mRNA is reverse transcribed to give Ty1 copyDNA with functional *HIS3* gene. Every integration of this Ty1 copyDNA gives rise to one His^+ colony on selective medium lacking histidine. In contrast to other methods for determination of Ty1 transposition only in specific target loci (Ribeiro-dos-Santos et al., 1997), the usage of *HIS3AI* indicator gene permits tracing the Ty1 transposition in the genome as a whole. Another advantage of *HIS3AI* construction is that it allows direct selection of Ty1 transposition events in a single-step test. Since the number of His^+ transposants correlates directly with the number of transposition events for the marked Ty1 element, the usage of *HIS3AI* construction allows a *quantitative* determination of the transposition events. We used *HIS3AI* indicator gene for constructing the tester strain of Ty1 short-term test.

Yeast cells have been used to develop a variety of systems monitoring mutagens and carcinogens. Few of these tests, however, have an application in laboratory or environmental studies. The main reason seems to be the lower response of yeast cells as an indicator organism, due to limited uptake of genotoxins (Parry 1976), because of low permeability of *S. cerevisiae* cells (Morita et al. 1989). We overcome the natural inertness of yeast cells to mutagens and carcinogens by constructing a tester strain with increased cellular permeability. Previous studies revealed that a temperature-sensitive mutation, initially designated *ts1*, causes an increased cellular permeability of *S. cerevisiae* to different substances, including mutagens and carcinogens (Staleva et al. 1996). Cloning and sequencing showed that the *TS1* gene is the previously isolated *SEC53* gene and the *ts1/sec53* mutant allele represents a change of CCA to CTA (proline to leucine) at the 5'end of the gene (Staleva 1998). The essential *SEC53* gene codes for phosphomannomutase required in an early step in the pathway of O- and N-linked mannosylation (Kepes & Schekman 1988). Since the permeability barrier of *S. cerevisiae* is determined by the most superficial layer of the cell wall composed of highly glycosylated mannoproteins (Zlotnik et al. 1989), the impairment of protein glycosylation in *sec53* mutant cells results in formation of a permeability barrier with insufficient functions.

Strain	Cell wall porosity after cultivation at:	
	23°C	30°C
550 (*SEC53*)	25.58 ± 3.47	23.96 ± 4.86
551 (*sec53*)	29.25 ± 5.83	93.85 ± 7.22

	Transformation with plasmid DNA (transformants per 1µg DNA)			
	+LiAc		-LiAc	
	23°C	30°C	23°C	30°C
550 (*SEC53*)	1820 ± 43	2024 ± 18	0	0
551 (*sec53*)	1682 ± 67	2120 ± 31	2 ± 1	172 ± 44

LiAc –Lithium Acetate

Table 1. Increased permeability of *S. cerevisiae* 551 cells LiAc –Lithium Acetate.

Average values ± SD of 6 experiments. The porosity of cell wall was calculated as percentages following the recommendations of the authors (DeNobel & Barnett, 1991). The transformation procedure (Ito et al., 1983) with YCP50 plasmid DNA was used to obtain Ura$^+$ transformants.

The replacement of *SEC53* gene in the tester strain with the *sec53* mutant allele was performed by means of the two-step gene replacement method (Sherman et al. 2001). Briefly, the *sec53* allele was cloned in an integrative *URA3*-marked vector, restricted in the unique AlfII site of the *SEC53* gene and tester cells were transformed to give Ura$^+$ integrative transformants. Strains having the *sec53* allele instead of the *SEC53* wild type gene were selected (Pesheva et al. 2005), one of the selected transformants was named *S. cerevisiae* 551 and used as a tester strain in the Ty1 assay. Its genotype is: *MATa ura3 his3Δ200 TymHIS3AI sec53, rho*$^+$. The *S. cerevisiae* 551 strain is deposited with №8719 into the National Bank for Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria).

The temperature of 37°C is nonpermissive for both, Ty1 transposition (Paquin & Williams, 1984) and the growth of cells having the *sec53* temperature sensitive mutant allele. Cultivated at the semirestrictive temperature of 30°C, tester cells are growing exponentially with generation time of about 150 min in YEPD liquid medium and partial expression of the *sec53* phenotype. Significantly lower survival rates due to increased uptake of genotoxic substances were obtained for *S. cerevisiae* 551 cells grown at 30°C and treated with a variety of genotoxins compared to the isogenic *SEC53* harboring cells at equal concentrations of the chemicals. Direct evidence for increased permeability of yeast cells were obtained using the porosity test (DeNobel & Barnett 1991) and the transformation procedure with plasmid DNA (Ito et al., 1983). A fourfold increase in porosity was found only for *S. cerevisiae* 551 cells cultivated at 30°C (Table1). The porosity of the isogenic *S. cerevisiae* 550 cells (having the wild type *SEC53* gene) remained identical after cultivation at 23°C or 30°C. The increased permeability of *S. cerevisiae* 551 cells was also confirmed by their transformability with plasmid DNA. Wild type yeast cells are not permeable for plasmid DNA and in order to be transformed, the permeability of intact *S. cerevisiae* cells is increased by treatment with lithium acetate (Ito et al. 1983) or the cells are converted to spheroplasts. We obtained relatively high frequency of transformation (5-6%) for intact 551 cells grown at 30°C and not treated with lithium acetate (Pesheva et al. 2005). Transformants were not obtained from 550 cells without lithium acetate treatment either at 23°C or at 30°C, as expected. All these results evidence an increased cellular permeability of *S. cerevisiae* 551 cells at 30°C due to the *sec53* mutation and supported the usage of *S. cerevisiae* 551 as a tester strain in the Ty1 short-term assay.

3.2 The Ty1 transposition test: A laboratory protocol

1. *S. cerevisiae* 551 cells are cultivated at 30°C in water bath shaker in YEPD liquid medium (the recipes of used media are given in Sherman et al. 2001) to a density of 5-7x10^{7}cells/ml.
2. The culture is divided into samples of 4ml and appropriate concentrations of the studied genotoxins added. Water insoluble substances are dissolved in dimethylsulfoxide (Me_2SO) or ethanol and used in volumes not exceeding a final concentration of 5% of Me_2SO or ethanol. The control samples are treated with the same volume of the solvent.

3. Samples are cultivated at 30°C for 30 min in water bath shaker; cells are washed with YEPD liquid medium by centrifugation and suspended in 4ml fresh YEPD medium.
4. Cells are cultivated at 20°C in water bath shaker for 24h to complete initiated Ty1 transposition events. As it will be shown later on the cultivation at 20°C results in about 5 fold higher rate of successful transpositions relative to cultivation at 30°C.
5. Appropriate dilutions (usually 10^{-4}-10^{-5}) are made in sterile water and 0.1ml is plated on each YEPD plate. Use at least five YEPD plates to determine cell titer of survivals for control and for each concentration of the genotoxin.
6. The remaining part of each sample is centrifuged; cells are washed twice with sterile water and suspended in 3.9ml of water. Ten SC-His plates are plated with 0.2ml suspension per plate to determine the number of His^+ transformants for controls and for each concentration of the genotoxin.
7. Plates are incubated at 30°C for 3 days (YEPD plates) or 5 days (SC-His plates) and colonies are counted.
8. Median transposition rates are determined by the equation:

Fold Increase = Fts/Ftc, where

$$Fts = \frac{Number\ of\ His + transposans\ of\ treated\ culture}{Number\ of\ survivors\ devided\ by\ fold\ dilution\ of\ treated\ culture}$$

$$Ftc = \frac{Number\ of\ His + transposans\ of\ control\ culture}{Number\ of\ survivors\ devided\ by\ fold\ dilution\ of\ control\ culture}$$

Results can also be presented as "fold increase" of Ty1 transposition rates relative to the rate of transposition in the control sample taken as a fold increase of 1.0. A fold increase equal or higher than 2.0 is considered as a positive response of the Ty1 short-term test.

Note: When metabolic activation is needed to convert pre-mutagens and pre-carcinogens to their active forms, S9 mix is added to samples 60 min before the treatment with genotoxins. *S. cerevisiae* cells have cytochrom P_{450} and P_{488} required for the metabolic activation of pre-genotoxins. The activation of this intrinsic metabolic system can be achieved by switching cells growing in low dextrose (0.2%) to high dextrose (10%) YEPD liquid medium (Kelly & Perry 1983). After 3-4 generations in the high dextrose medium cells can be used in the Ty1 test without addition of an exogenous S9 mix and the presence of S9 mix does not change the results obtained. The change from low to high dextrose medium is made in a very early exponential growth phase (about $2x10^7$cells/ml) to allow for several generations before the treatment with pre-genotoxins.

4. Response of Ty1 test to laboratory genotoxins

Laboratory genotoxins with different structure and mode of action have been used to study the main characteristics of the Ty1 short-term test.

4.1 Increased sensitivity of Ty1 test

Benzo(a)pyrene [B(a)P] although being a very active agent in the *Salmonella* system, does not induce genetic damages in wild type *S. cerevisiae,* because little of this aromatic compound is

taken up by yeasts due to the limited permeability of cells. The results obtained in the Ty1 test (Table 2) show that this is true when *S. cerevisiae* 550 cells with wild type of *SEC53* gene are used as testers. However, if *S. cerevisiae* 551 (*sec53*) strain was used, the Ty1 test responds to increased concentrations of B(a)P with enhanced rate of Ty1 transposition. The positive answer of the test appears at low concentrations of B(a)P having negligible effect on cell - survival and the fold increase of Ty1 transposition rates enhanced in a concentration dependent manner.

Strain	B(a)P (mM)	S9 mix	Survival (%)	Ty1 test (fold increase)	$O_2^{-\cdot}$ (pM/cell)[a)]
551 (*sec53*)	0	-	100	1.00	0.048
	0	+	100	1.00	0.054
	0.08	-	105	0.71	0.045
	0.08	+	91	4.87	0.581
	0.16	-	85	1.41	0.080
	0.16	+	80	9.18	1.221
	0.32	-	63	2.00	0.080
	0.32	+	56	18.41	2.450
551+NaN_2[b)]	0.15	-	38	1.22	0.063
550 (*SEC53*)	0	-	100	1.00	0.040
	0	+	100	1.00	0.045
	0.08	-	98	1.27	0.061
	0.08	+	102	1.18	0.082
	0.16	-	105	1.85	0.062
	0.16	+	95	2.00	0.079
	0.32	-	88	1.67	0.055
	0.32	+	82	2.50	0.121
550+NaN_2[b)]	0.15	-	52	1.25	0.068

Typical results from one out of six identical experiments

[a)] Amount of superoxide anion ($O_2^{-\cdot}$) in pM per one cell determined by the method described in Stamenova et al. (2008)

[b)] Cells treated only with NaN_2

Table 2. Increased sensitivity of Ty1 test to metabolically activated B(a)P

Similar increase in the values of Ty1 transposition rates was also found in kinetic experiments where cells were treated for periods of 15 to 90 minutes. The positive answer of Ty1 test is not due to the stress conditions generated by the increased cellular toxicity at higher concentrations of B(a)P, since treatment with NaN_3, a powerful cell poison, had no effect on Ty1 transposition. Similar positive responses of the Ty1 test were found in concentration dependent and kinetic experiments with a number of carcinogenic genotoxins showing low response in tests based on wild type of yeast cells (Dimitrov et al., 2011; Pesheva et al., 2005; 2008; Staleva & Venkov 2001).

4.2 The Ty1 test responds positively only to active carcinogens

B(a)P is a pre-carcinogen which has to be metabolically activated in order to produce its effect in the cell. The Ty1 test used with the highly permeable 551 strain as tester cells did

not respond positively after treatment with B(a)P in absence of S9 mix (Table 2). The percentage of cells that survived this treatment decreases with increasing B(a)P concentration suggesting that ones inside cells the pre-carcinogen has a toxic effect which however is not an inducer of Ty1 transposition. The Ty1 test was positive with B(a)P and different other pre-carcinogens only in presence of S9 metabolizing mix (Dimitrov et al. 2011; Pesheva et al. 2005; 2008). These results evidence that the *sec53* mutation in *S. cerevisiae* 551 tester strain increases the sensitivity of Ty1 test due to increased permeability of cells. In addition, the positive response of the test to active carcinogens and the negative results obtained with pre-carcinogens suggest a specificity of the test response to the active forms of the genotoxins.

4.3 The Ty1 test is positive with carcinogens not detectable in the other short-term tests

Every short-term test has a range of carcinogen's detection and the usage of batteries of tests extends the limits of detection. Nevertheless, a fraction of genotoxins with carcinogenic potential proved in long-term experiments did not induce any short-term assay tested up to the maximum solubility and the reasons for this are not known (Ramel et al. 1996). We studied representatives of these genotoxins in the Ty1 test and results are summarized in Table 3.

Carcinogen	Concentration (mM)	Survival (%)	Ty1 transposants per/10^8cells	Ty1 test (fold increase)	$O_2^{\cdot-}$ (pM/cell)
Control	-	100	25 ± 5	1.0	0.045±0.001
Carcinogens negative in Salmonella tests					
Acetamide	34	60	172 ± 18	7.82	0.284±0.041
Thioacet-amide	13	71	182 ± 24	8.23	0.315±0.048
Dichloro-methane	10	58	193 ± 31	8.76	0.421±0.055
Carcinogens negative in all short-term tests					
Furfuryl alcohol	5	45	110 ± 8	5.0	0.470±0.028
Tetrahydro-furan	5	58	121 ± 12	5.5	0.520±0.055
tert-butyl-hydroquinone	0.05	53	134 ± 16	6.1	1.020±0.099
Noncarcinogenic mutagens					
5-bromuracil	52	50	33 ± 4	1.5	0.050±0.015
Benzo(e) pyrene[a)]	160	61	41 ± 6	1.9	0.068±0.005
Anthracene[a)]	34	72	26 ± 7	1.2	0.048±0.010

Average values ± SD of 5 experiments

[a)]Benzo(e)pyrene and anthracene were dissolved in Me_2SO and tested in presence of S9 mix of Ty1 test did not result from secondary effects of dying cells and the test responds positively to some carcinogens not detectable by the other short-term assays.

Table 3. Wider range of Ty1 test in carcinogen's detection

Furfuryl alcohol, tetrahydrofuran and *tert*-butylhydroquinone which are carcinogens not detectable in the other short-term tests showed a positive response in Ty1 test (Dimitrov et

al., 2011;Pesheva et al. 2005). With increasing the time of exposure (15 to 45 min) the frequency of Ty1 transposition steadily increased and reached a plateau suggesting saturation of transposition rates. After 30 min exposure to different concentrations, the tester cells exhibited a dose response with an increase at higher concentrations of the carcinogens. The induction of Ty1 transposition (fold increase >2.0) started before significant cell death become apparent. Based on these data, it is concluded that the carcinogen-stimulated positive response to some carcinogens not detectable by the other short term assays.

Negative results in Ty1 test were obtained with 5-bromuracil, anthracene and benzo(e)pyrene [B(e)P] which are mutagens without carcinogenic potential (Table 3). The positive results in Ty1 test found for a number of carcinogens, the specific test response to only metabolically activated carcinogens and the absence of response to noncarcinogenic mutagens suggested that Ty1 test may have the ability to differentiate carcinogens from mutagens without carcinogenic properties.

Genotoxin	Concentration [a)] (mM)	Survival (%)	Ty1 test (fold increase)	$O_2^{\cdot-}$ (pM/cell)
Controls				
H_2O	-	100	1.0	0.048±0.009
Me_2SO	5%	98	1.1	0.058±0.010
Carcinogens				
B(a)A	26	57	14.5	2.450±0.205
CDH	1000	48	16.0	2.851±0.221
LH	1000	53	22.0	3.446±0.255
CrVI	5	54	47.3	4.405±0.366
Noncarcinogenic mutagens				
B(b)A	26	70	1.8	0.068±0.005
TDH	1000	61	1.8	0.045±0.009
GDH	1000	80	1.5	0.058±0.010
CrIII	5	59	1.3	0.031±0.005

Average values of 6 experiments
Me_2SO – dimettylsulfoxide; B(a)A – benzo(a)anthracene; B(b)A– benzo(b)anthracene; CrVI – hexavalent chromium; CrIII – trivalent chromium; CDH – chenodeoxycholic bile acid; LH – lithocholic bile acid; TDH - taurodeoxycholic bile acid; GDH- glycodeoxycholic bile acid
[a)]CrVI and CrIII were dissolved in water; all the other genotoxins were dissolved in Me_2SO and tested in the presence of S9 mix

Table 4. Selective response of Ty1 test to carcinogens

4.4 Selective response of Ty1 test to carcinogens

The possibility that Ty1 test may have a selective positive response to carcinogens was studied using pairs of genotoxins with close to identical chemical structure: the one being strong carcinogen and the second having only mutagenic properties without being a carcinogen. One such pair is the well known carcinogen B(a)P and the B(e)P which is a noncarcinogenic mutagen. The carcinogenic status of all compounds used in our studies was according to YARC (1990) and recent publications in the field (Donkin et al. 2000). As it was already shown (Tables 2, 3) B(a)P induces strong positive answer in Ty1 test, while B(e)P was without effect on Ty1 transposition rate.

Benzo(a)anthracene [B(a)A] and benzo(b)anthracene [B(b)A] represent another pair of genotoxins studied in the Ty1 test (Dimitrov et al.,2011; Pesheva et al. 2008). B(a)A is assessed as carcinogenic for animals and humans while B(b)A is a non-carcinogenic mutagen (Salamone 1981). The study of these genotoxins in the Ty1 test (Table 4) showed a strong positive answer for the carcinogenic B(a)A and negative result (fold increase < 2.0) for the noncarcinogenic mutagen B(b)A. Table 4 shows results obtained at equimolar concentrations and the absence of positive test-response was also found for higher concentrations of B(b)A killing up to 70% of tester cells.

Considerable evidence support the view that free, but not conjugated, bile acids are carcinogenic in humans (reviewed in Bernstein et al. 2005). We took advantage from these observations to further characterize Ty1 test and studied the effect of the free chenodeoxycholic (CDH) and lithocholic (LH) bile acids and the noncarcinogenic conjugated taurodeoxycholic (TDH) and glycodeoxycholic (GDH) bile acids (Pesheva et al. 2008). The results obtained (Table 4) show that the carcinogenic CDH and LH bile acids induced positive responses in the Ty1 test, whereas the noncarcinogenic TDH and GDH bile acids give values close to the controls. While the two free bile acids increase the frequency of Ty1 transposition over a tenfold concentration range, the conjugated acids showed no such activity, even though toxicities of up to 60% were produced.

Although some heavy metals (the so-called trace elements) are essential for the survival of all life forms, others can be quite toxic and carcinogenic. In preliminary experiments we studied the response of Ty1 test to arsenic (As) classified as confirmed human carcinogen, lead (Pb) categorized as probable human carcinogen and zinc (Zn) which is not classifiable with regard to human carcinogenicity (Dimitrov et al. 2011). The results obtained were positive for As and Pb in concentration and time dependent experiments, while Zn was without effect on Ty1 transposition. A special interest represents the pair hexavalent chromium (CrVI) and trivalent chromium (CrIII). While CrVI is confirmed as strong human carcinogen, the data for CrIII are controversial. An extensive recent review (Eastmond et al. 2008) points to many instances of conflicting information. Thus, *in vitro* data suggest that CrIII has the potential to react with DNA and to cause DNA damages which however required experimental conditions incompatible with cell-life. *In vivo* evidence suggest that genotoxic effects did not occur in human cells or animals exposed to CrIII and the trivalent chromium complexes are widely used for decades as nutritional supplements and insulin enhancers in patients with type 2 diabetes. Although firm data for a carcinogenic effect of CrIII are lacking, there is a growing concern (Levina & Lay 2008) over a possible carcinogenicity of CrIII based on the assumption that once in the cell, part of CrIII can be oxidized to CrVI which is a confirmed human carcinogen. The study of CrVI and CrIII in the Ty1 test confirmed the strong carcinogenic effect of CrVI with a fold increase of 47 following treatment with 5mM CrVI for 30 min (Table 4). At the same conditions CrIII gave values close to the controls, as did all studied till now noncarcinogenic mutagens. Regardless of results that will be obtained in the future in favor of presence or absence of carcinogenicity for CrIII, it should be noted that the data obtained with CrVI and CrIII evidence that the Ty1 short-term test possesses the characteristic to discriminate opposite effects of a heavy metal, determined by changes in its valence.

5. Response of Ty1 test to environmental pollutants

Biomonitoring requires accurate, sensitive, easy and fast methods to assay environmental pollution. The specific and selective response of Ty1 short-term test to carcinogens found

with laboratory genotoxins suggested a verification of the characteristics of the assay in environmental studies monitoring pollution. The study was conducted during 2006-2007 and consisted of monitoring pollution in soil, water and air in regions in Bulgaria (Pesheva et al. 2008), using Ty1 test and quantitative chemical analysis of the samples. Processed samples were chemically analyzed for presence of mutagens, carcinogens, pesticides, polychlorinated biophenols, heavy metals, etc., at a total of 53 substances. In this review only short excerpts of the protocols will be presented, and, results obtained for air and water samples will be omitted.

Soil samples were collected from regions with low (odd number) or high (even number samples) pollution levels in June 2006 (S1, S2), November 2006 (S3, S4), May 2007 (S5, S6) and October 2007 (S8). Samples were processed according to standard procedures and each extract of sample was directly analyzed chemically and studied with Ty1 test in presence of S9 metabolic activating mix. Such experimental program gives the advantage to directly compare results obtained in the short-term test with the amount of genotoxins found in the chemically analysis of the samples. Samples collected from clean region showed low toxicity and gave negative responses in Ty1 test (Table 5). These results evidence that the Ty1 assay does not give false positives with extracts of samples. Samples collected from polluted regions showed different toxicities on yeast cells with a higher toxic effect of samples containing petrol products (S8). All samples from polluted regions gave positive results in Ty1 test with a fold increase ranging 7-12. The positive answers were confirmed in concentrations (= sample volume) dependent and kinetics experiments (Pesheva et al. 2008). The chemical analysis Table 5 shows that samples from the clean regions did not contain any carcinogenic genotoxins, while all samples from polluted regions contain mixtures of carcinogens. Only substances found in amounts above the accepted ecological standards are shown on (Table 5). Since each sample from polluted regions contained several carcinogenic substances, their positive results evidence that the Ty1 test responds positively to mixtures of carcinogens, as well.

Special attention should be given to samples S1 and S3 collected from clean regions which showed negative results in the Ty1 test. These samples were polluted with flouranthrene at concentrations 0.28 mg/kg and 0.19 mg/kg for S1 and S3, respectively. Given the accepted ecological maximum of 0.02 mg/kg, it is clear that although S1 and S3 samples are heavily polluted with flouranthrene they show negative results in Ty1 test. It has consistently been shown that fluoranthrene proved to be a potent mutagen in bacterial and mammalian *in vitro* test systems, however results from *in vivo* carcinogenicity studies in rodents evidenced it is not carcinogenic (Goldman et all. 2001; Verschueren 2001). Thus the selective response of Ty1 test to carcinogens found in studies with laboratory genotoxins is also characteristic for the assay used in environmental studies.

A short excerpt of the chemical analysis of soil samples is given in Table 6. For all mutagens and carcinogens that were analyzed but not included in the table, concentrations values below the accepted ecological standards were found. The results showed the significant pollution of samples collected from polluted regions. The amounts of some carcinogenic heavy metals (Pb, As, Cd) were 4-6 fold higher and the samples were polluted with mixtures of the carcinogenic genotoxins (B(a)P, B(a)A, benzo(hgi)pyrene, petrol products) and different mutagens.

Sample	Survival (%)	Ty1 transposants [a] (per 10^8 cells)	Ty1 test (fold increase)	Mutagens/carcinogens [b] found in chemical analysis
Control				
Me_2SO 5%	100	18 ± 5	1.0	
Clean Regions				
S1	92	22 ± 6	1.2	fluoranthrene
S3	90	27 ± 8	1.5	fluoranthrene
S5	97	31 ± 6	1.7	
Polluted regions				
S2	78	131 ± 16	7.3	**As**, **B(a)P**, **B(a)A**, **pyrene**, **crysene**, **benzo(ghi)perylene**, **Pb**, B(e)P, fluoranthrene
S4	67	127 ± 18	7.1	**As**, **B(a)P**, **pyrene**, **crysene**, **Pb**, B(e)P,
S6	79	99 ± 9	5.5	**As**, **B(a)P**, **pyrene**, **crysene**, **Pb**, fluoranthrene
S8	42	228 ± 21	12.7	**B(a)P**, **B(a)A**, **pyrene**, **crysene**, **petrol products**, **Pb**, B(e)P, B(b)A

[a] Average values ± SD from 10 experiments
[b] Carcinogenes are shown in bold

Table 5. Response of Ty1 test to soil samples

Carcinogens or mutagens	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
As	25	15	9	12	113	93	82	199
Cd	2	0.4	0.3	0.5	2.2	3.2	1.5	1.8
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	0.49
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
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
Pyrene	0.02	0.02	0.02	0.02	0.28	0.05	0.18	0.49
B(a)A	0.02	0.02	0.02	0.02	0.10	0.03	0.05	0.47
Chrysene	0.01	0.01	0.00	0.00	0.12	0.07	0.06	0.24

Table 6. Chemical analysis of soil samples

The samples collected from clean regions did not contain carcinogens above the ecological standards, although some of them (S1, S3) were polluted with flouranthene as already discussed. The analysis of environmental samples in Ty1 test permits an evaluation of the

Ty1 test sensitivity in environmental studies. An assay should be sensitive enough to detect levels of carcinogens over the accepted standards and to not give false positives with carcinogen's concentration below the ecological standards. The accepted standards for the different mutagenic or carcinogenic substances indicate the amounts of genotoxins that are not harmful for humans and animals. Table 6 (the three bottom lines) show that samples S1, S3, S5 contain pyrene at concentrations of 0.02 mg/kg which is the ecological standard. Samples S1 and S3 contain also B(a)A at concentrations close to the ecological standard and sample S1 contains 0.01 mg/kg of chrysene which is the accepted standard for this carcinogen. All these samples give negative results in Ty1 test (Table 5) evidencing that this assay remains negative with concentrations of carcinogens bellow the ecological standards. Together, the results obtained in environmental studies evidence that Ty1 test detects selectively carcinogenic pollutants only if they are in concentrations above the accepted ecological standards.

6. The molecular mechanism of the selective response to carcinogens

The positive and selective response to carcinogenic genotoxins requires explanation and two observations directed our efforts to clarify the molecular mechanism of the Ty1 test response.

First, we noted that the appearance of *rho*$^-$ mutants among His$^+$ transposants induced by carcinogens, is a very rare event. *S. cerevisiae rho*$^-$ cells are mitochondrial mutants representing large deletions of mitochondrial DNA (mtDNA), that appear spontaneously (Dujon, 1981), or can be induced by treatment with ethidium bromide (Sherman et al. 2001) or freezing (Stamenova et al. 2007). Nine out of ten studied carcinogens induced only *rho*$^+$ transposants (with wild type mtDNA) and the percentage of *rho*$^-$ transposants induced by *tert*-butylhydroquinoline was below the spontaneous frequency. This results and the observation that treatment with different carcinogens of *rho*$^-$ mutants was without effect on Ty1 transposition (Stoycheva et al. 2007; 2008), strongly suggested that the carcinogen induced positive response in Ty1 test depends on the function of mitochondria.

Although the deleted mtDNA fragment spans over different number of genes in *rho*$^-$ mutants, the genes for oxidative phosphorylation are deleted in all *rho*$^-$ isolates irrespective of the way they are induced (Dujon 1981). The nuclear gene *SCO1* codes for a protein that is transported to mitochondria where it participates in formation of oxidative phosphorylation complexes (Glerum et al. 1996). Mutations in, or disruption of the *SCO1* gene are associated with respiratory deficiency, however in contrast to *rho*$^-$ mutants, *sco1* mutants lack only mitochondrial oxidative phosphorylation in otherwise intact and functional mitochondria. We disrupted *SCO1* gene in *S. cerevisiae* 551 tester strain and the study of carcinogen-induced Ty1 transposition in 551 *sco1*Δ cells showed absence of Ty1 induction with almost equal Ty1 transposition rates in *sco1*Δ, *rho*$^-$ or control cells (Stoycheva et al. 2010). Therefore, the Ty1 transposition induced by carcinogens depends on oxidative phosphorylation and not on another mitochondrial function.

The second observation we made came from an extensive survey of the literature showing that all carcinogens with positive answer in Ty1 test are powerful generators of reactive oxygen species (ROS) in different cells (Bernstein et al. 2007; Toyooka & Ibuki 2007)

including *S. cerevisiae* (Brennan & Schiestl 1998; Herrero et al. 2008). The data for noncarcinogenic mutagens, although not abundant, showed absence of, or negligible effect on cellular level of ROS (Scandalios 2005; Toyooka & Ibuki 2007). ROS are generated in aerobic organisms through both endogenous and exogenous routes. The majority of endogenous ROS are produced through leakage of superoxide anions ($O_2^{\cdot-}$) from the mitochondrial respiratory chain.

ROS are generated by one electron reduction of O_2 or formed from enzymatic systems (Fridovich 1997). Dismutation of $O_2^{\cdot-}$ (spontaneously or by superoxide dismutases) produces hydrogen peroxide (H_2O_2) which in the presence of metal ions is partially reduced to hydroxyl radical ($^{\cdot}OH$). To minimize the damaging effects of ROS, aerobic cells evolved nonenzymatic and enzymatic antioxidant defenses. At increased levels, however ROS can not be detoxified sufficiently and may have deleterious effects on lipids, fatty acids, proteins and DNA. A variety of DNA lesions, block of replication and chromosomal loss were frequently detected in cells under oxidative stress.

We studied the possible link between carcinogen-induced Ty1 transposition and ROS level by simultaneous determination of Ty1 mobility and $O_2^{\cdot-}$ levels following treatment with carcinogens. As shown on Tables 2, 3 and 4 (last columns) the treatment with carcinogens increased both, Ty1 transposition rate and $O_2^{\cdot-}$ in tester cells, while noncarcinogenic mutagens (Table 4) have little effect on Ty1 mobility and $O_2^{\cdot-}$ level. We used a method for quantitative determination of $O_2^{\cdot-}$ in live cells only (Stamenova et al. 2008), so that the various toxicities of genotoxins can not account for the different amount of $O_2^{\cdot-}$ found in cells treated with carcinogens or noncarcinogenic mutagens.

Strain	Temperature of cultivation (°C)	MMS (3.5mM)	Ty1 transposants (per 10^8 live cells)
551 *rho*$^-$	15	-	72 ± 6
	30	-	16 ± 3
551 *rho*$^+$	15	-	87 ± 7
	30	-	19 ± 6
551 *rho*$^-$	15	+	78 ± 8
	30	+	17 ± 4
551 *rho*$^+$	15	+	641 ± 32
	30	+	155 ± 18

Average values ±SD from 6 experiments

Table 7. Ty1 transposition at different temperatures

Another possible reason to fall into error studying the interactions between carcinogen-induced Ty1 transposition and ROS level would be that the whole process of transposition in *rho*$^-$ cells is impeded from unknown yet reason and cells are unable to carry out Ty1 transposition at all. It has been shown that the rate of spontaneous transposition in cells cultivated at temperatures of 15°C increases relative to cells grown at 30°C (Paquin & Williamson 1984). We confirmed this result and as shown on Table 7 the number of *spontaneous* His$^+$ transposants in *rho*$^-$ cells at 15°C was significantly higher compared to cells grown at 30°C. However, treatment of *rho*$^-$ cells with the carcinogen

methylmethanesulfonate (MMS) did not enhance Ty1 transposition rates at any of the two temperatures. In *rho*$^+$ cells the increase of spontaneous and MMS-induced Ty1 transpositions was evident after cultivation at 15°C and 30°C. Since *rho*$^+$ and *rho*$^-$ cells increase spontaneous transposition rates at 15°C by an equal factor of 5 relative to cultivation at 30°C, the reason for absence of carcinogen-induced Ty1 transposition in *rho*$^-$ cells is not a general defect in the transposition process and most likely is due to loss of the start signal for the carcinogen induced transposition.

Several lines of evidence confirmed the role of increased ROS levels as inducers of Ty1 transposition in *S. cerevisiae* cells treated with carcinogens.

Although tightly coupled, the two processes in mitochondrial phosphorylation, electron transfer and ATP synthesis, can be selectively inhibited: antimycinA inhibits the transfer of electrons while carbonyl cyanide-3-chlorophenyl hydrazine (CCCP) blocks ATP synthesis (Epstein et al. 2001). Tester 551 cells cultivated in presence of antimycinA showed significant higher Ty1 transposition rates (fold increase 30-40) upon treatment with MMS compared to cells with not inhibited electron transfer and treated with the same concentration of MMS (fold increase 6-8). The block of ATP synthesis with CCCP had little or no effect on MMS-induced Ty1 mobility (Stoycheva et al. 2010). The inhibition of electron transfer along the respiratory chain is known to increase the leakage of electrons and to enhance in this way the production of ROS (Rasmussen et al., 2003). Higher ROS levels are also generated in exponential cells and cells growing in high dextrose (2%) media vs. stationary cells and cells cultivated in low dextrose (0.2%) media (Barros et al.,; 2004Lin et al., 2002). The study of MMS-induced Ty1 transposition showed increased mobility of Ty1 in exponential cells and cells from high dextrose medium compared to stationary cells and cells grown in low dextrose medium. The activation of Ty1 mobility by carcinogens seems to depend on accumulation of $O_2^{\cdot -}$ generated by the carcinogens since addition of N-acetylcysteine (NAC), a scavenger of $O_2^{\cdot -}$, resulted in absence of both, the increase in $O_2^{\cdot -}$ levels and the induction of Ty1 transposition rates (Table 8). The elevation of $O_2^{\cdot -}$ level with menadione, a superoxide generator, also increases Ty1 mobility without any additional treatment with carcinogen. Taken together, these results show that yeast cells with functioning oxidative phosphorylation accumulate $O_2^{\cdot -}$ upon treatment with carcinogens and indicate a key role of enhanced ROS levels in activating the initiation of the Ty1 transposition process.

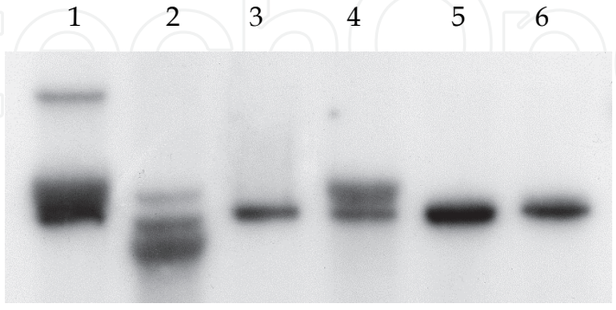
S. cerevisiae has been shown to have distinct protective responses to superoxides and peroxides (Jamieson et al. 1992). The *YAP1* gene encodes a transcription factor that activates target genes involved in response against H_2O_2 (Nguyen et al. 2001) and mutants deleted for *YAP1* gene accumulate H_2O_2 in the cells. We disrupted the *YAP1* gene in 551 tester strain and found 551*yap1*Δ cells to respond upon carcinogen treatment with significantly higher induction of Ty1 mobility compared to cells with functioning *YAP1* gene. A dose-dependent increase of ROS following exposure of cells to exogenously added H_2O_2 was found in numerous studies (Tample et al. 2005 and citations therein). We used this observation to supply *rho*$^-$ cells with the missing ROS and studied Ty1 transposition in the 551 *rho*$^-$ strain treated with increased concentrations of H_2O_2. Treatment with 0.5, 1.0 and 3.0mM H_2O_2 induced Ty1 transposition with a fold increase of 6, 10 and 25, respectively, relative to the nontreated control.

Carcinogen (mM)	NAC[a)] (60mM)	MD[b)] (mM)	Survival (%)	$O_2^{\cdot-}$ (pM/cell)[c)]	Ty1 test (fold increase)
Control (H_2O)	-	-	100	0.045 ± 0.015	1.0
MMS (3.0)	-	-	45	0.387 ± 0.115	8.5
MMS (3.0)	+	-	78	0.040 ± 0.010	0.9
As (2.0)	-	-	51	0.871 ± 0.250	33.5
As (2.0)	+	-	82	0.053 ± 0.009	0.8
CrVI(5.0)	-	-	54	1.440 ± 0.410	41.3
CrVI(5.0)	+	-	75	0.048 ± 0.018	1.7
-	-	2	81	0.788 ± 0.115	4.5
-	-	5	41	2.675 ± 0.550	8.1

a) N-acetylcysteine added 1h before treatment with carcinogens
b) Menadione added for 1h
c) Average of 4 experiments

Table 8. Response of *S. cerevisiae* 551 cells to carcinogens depends on ROS level

In addition to the transposition process, the movement and integration of Ty1 transposon to a new location in the genome can also occur by gene conversion (Roeder & Fink 1982). In this process a resident Ty1 element without being replicated undergoes homologous recombination with a second Ty1 element located on another place in the genome. There are data in the literature (Winn 2003) indicating an activation of homologous recombination by elevated ROS levels. Given the importance of having an activation of Ty1 mobility in *rho*$^-$ cells only after treatment with H_2O_2, Southern blot experiments were performed to differentiate *de novo* transposition from gene conversion (Figure 2). DNA samples isolated from *rho*$^+$ transposants induced with MMS or H_2O_2 have dispersed bands in addition to the 5kb band of the parental insertion. These bands indicated *de novo* transposition events in different locations of the genome. In *rho*$^-$ transposants such additional bands were found only after treatment with H_2O_2 and not with MMS. This result evidenced that the supply of ROS by addition of H_2O_2 to the cell culture activates a process of Ty1 transposition *de novo* and not by gene conversion. In addition these results evidence that the overall enhanced level of ROS and not the increased production of a particular oxygen species is involved in the induction of Ty1 mobility by carcinogens.



Lanes 1, 2, 3: DNA from *rho*$^+$ cells; lanes 4, 5, 6 – DNA from *rho*$^-$ cells. Lanes 1 and 4 – cells treated with 1mM H_2O_2 for 30 minutes. Lanes 2 and 5 – cells treated with 3.5mM MMS for 30 minutes. Lanes 3 and 6 – control cells. DNA was digested with *PvuII* and probed with *HIS3*. The 5kb fragment indicates the position of parental TymHis3AI insertion while dispersed bands represent *de novo* transposition events.

Fig. 2. Southern blot analysis of Ty1 retrotransposants

The accumulated data clarify to some extent the molecular mechanism of the selective Ty1 test response to carcinogens. The early work of Wilkie & Evans (1982) suggesting that in yeast cells carcinogens primarily attack the mitochondria, found nowadays its explanation and support in the observation that most, if not all, carcinogenic genotoxins generate high levels of ROS. The generated oxidative stress has different after-effects for the cells, including DNA damages. The oxidative DNA damages act in concert with the DNA damages caused directly by the carcinogens to labilize genome structure, which seems to be one of the first step towards neoplastic differentiation of the cell (Bertram 2001; Garfinkel 2005). Numerous studies (Lesage & Todeschini 2005; Nyswaner et al. 2008; Salmon et al. 2004; Tucker & Fields 2004) indicated the role of DNA damages, induced directly by the carcinogens, in the activation of the Ty1 mobility.

Some of our data, however, suggest an alternative explanation. When *S. cerevisiae* cells are deficient in production of ROS, the treatment with carcinogens does not activate Ty1 mobility, although there are no reasons to suppose that direct DNA damages are not generated by the genotoxin. Cells with compromised production of ROS (*rho*$^-$, *sco1*Δ) respond with a dose-dependent increase of Ty1 mobility only if the missing ROS are supplied to the culture. The use of N-acetylcysteine to scavenge the burst of $O_2^{\cdot-}$ following treatment with carcinogens also resulted in lack of activation the Ty1 transposition despite the appearance of direct DNA damages caused by the carcinogen. Opposite results were obtained with menadione, a generator of $O_2^{\cdot-}$ for which literature data (Cojocel et al. 2006) did not support a direct DNA damaging effect. Menadione increased Ty1 transposition without any additional treatment with DNA damaging agent. Finally, cultivation at temperatures at 15-20°C instead of 30°C increases Ty1 transposition (Paquin & Williamson 1986) because of increased ROS production (Zhang et al. 2003) at suboptimal temperatures and not because of the appearance of DNA damages. Together, these data evidence that the increased ROS level may have an independent key role on the induction of Ty1 transposition by carcinogens. We suppose that in cells with functioning mitochondrial oxidative phosphorylation, the carcinogen-induced burst in ROS production is the major initiator of Ty1 transposition. The effect of the direct carcinogen-induced DNA damages is later multiplied by the appearance of secondary oxidative DNA damages due to the already existing oxidative stress. Cells with compromised mitochondrial oxidative phosphorylation can not produce the major initiator in the Ty1 transposition process and the carcinogen activated induction of transposition can not take place in such cells.

Thus, the selective positive answer of Ty1 test to carcinogens is due to activation of Ty1 transposition by the burst of ROS generated by carcinogenic genotoxins. Noncarcinogenic mutagens are not strong ROS generators in *S. cerevisiae* cells and therefore give negative results in the Ty1 test. Both, carcinogens and noncarcinogenic mutagens are DNA damaging agents and the discrimination between the two kinds of genotoxins in Ty1 test is based on the ROS generating capacity of carcinogens which is not an intrinsic property of noncarcinogenic mutagens. These conclusions have been made on the basis of results obtained with 48 carcinogenic and 12 noncarcinogenic but mutagenic genotoxins studied till now in the Ty1 test. Given a number of genotoxins with carcinogenic and/or mutagenic potential of several hundreds, we can well believe that our current interpretation of the molecular mechanism underlying the selective positive response of Ty1 test to carcinogens may undergo some changes depending on the results obtained in future studies.

7. Applicability of Ty1 test: Advantages and shortcomings

7.1 Advantages

The Ty1 test is a short-term test based on fast growing yeast cells and results with this assay are obtained in 5 days. The test is easy to perform and requires expertise at the level of technicians. The usage of only one strain as tester cells makes possible the study of a number of samples per day by one person. The characterization of the test required repetitions to obtain statistically reliable results, but the presence or absence of carcinogenic activities in the studied sample is obvious after the first implementation of the Ty1 test. The accomplishment of the test does not require special equipment and can be performed in every microbiological laboratory. *S. cerevisiae* cells have an intrinsic P_{450}/P_{488} based metabolic system which can be activated by a change in cultivation from low to high dextrose medium. Although the results in Tables are presented with usage of exogenously added S9 mix, very similar values were obtained in almost all experiments using the endogenous metabolic system of the tester 551 cells. The utilization of the intrinsic metabolic activation system in the tester strain further lowers the anyway low cost of the assay.

A significant advantage of the Ty1 test is its ability to respond positively to carcinogens and negatively to noncarcinogenic mutagens. The advantage of having a selective answer to carcinogenic genotoxins becomes evident when the Ty1 test is used at the beginning of a battery of tests in mutagenicity/carcinogenicity studies. Most short-term tests in bacteria or mammalian cells have been designed primarily for hazard identification of genotoxicity and thus represent the starting point in the process of risk assessment. The answer of the important question about the carcinogenic or not carcinogenic nature of a substance is obtained at the end of the battery and is given by studies of the genotoxin in long-term tests. The implementation of Ty1 assay at the beginning of the study will give in 5 days the information about presence or absence of carcinogenic potential of the genotoxin and will spare time, efforts and costs. Therefore, the Ty1 assays is an indicator test that provides evidence of carcinogenicity and is thus an indicator of carcinogenic potential.

Another important advantage of Ty1 test is its wide range in detection of carcinogens. Several carcinogenic genotoxins not detectable by the other short-term tests were tested and found to give positive results in Ty1 assay. The following reasons can be considered in explanation the wider range of Ty1 test in carcinogen detection.

First, the tester cells have mutationally increased cellular permeability to genotoxins which facilitates the uptake of carcinogens in otherwise low permeable, and therefore low sensitive *S. cerevisiae* cells. Second, the Ty1 test is based on activation the transposition of a Ty1 transposon which is a typical genetic retroelement with structure, function and cell-cycle analogous to the known oncogenic retroviruses. Similarities in regulation of certain steps of Ty1 transposition and neoplastic differentiation of cells were detected, which might explain to some extent the selective character of Ty1 test response to wider range of carcinogens. Finally and probably most importantly, the molecular mechanism of the positive Ty1 test response is principally different compared to other assays. Most short-term tests were constructed to detect one genetic *end-point* raised by one, or several (but not all) carcinogens. On the opposite, the positive response of Ty1 test is due to induction the *initiation step* of the transposition process. Instead of detecting a DNA-damage as end-product of the effect of some carcinogens, the Ty1 test is based on activation the start of a process. This activation is

due to increased ROS levels generated by the carcinogens, which seems to be a property common to most, if not all, carcinogens. We consider such molecular mechanism of carcinogen detection as important in explanation the selective Ty1 test response to wider range of carcinogens.

7.2 Shortcomings

The advantage of Ty1 test to selectively detect carcinogens may be assessed as a shortcoming, if the test is used alone in environmental studies. Results obtained with laboratory genotoxins and in field studies evidenced a negative response of the test to noncarcinogenic mutagens. Thus, environmental samples taken from regions polluted with mutagens that are not carcinogenic will remain silent in the test. A way to overcome this shortcoming is to use the Ty1 assay for environmental studies together with a short-term test detecting mutations, if information about the general pollution of the region is required.

Another disadvantage of Ty1 test would be that the assay will give positive results with substances that are ROS generators in *S. cerevisiae*, irrespective of their carcinogenic or noncarcinogenic nature. The ROS generators represent a small group of chemicals used for research only and not found as environmental pollutants. Their carcinogenic potential is not well known and remains to be studied in the future.

A positive result of environmental sample in the Ty1 test means that the region this sample was collected from is polluted with carcinogen(s). The test does not show if one or a mixture of carcinogens are present in the sample. This feature of the Ty1 test is shared by the other short-term tests, as well.

The Ty1 test is a quantitative assay in the sense that every transposition event of the marked Ty1 element activated by the studied genotoxin gives rise to one His^+ colony. A concentration dependence of the positive answer was found for all studied carcinogens in the test, suggesting that higher fold increase of Ty1 transposition means higher concentration of the genotoxin in the sample. However, the activation of the Ty1 transposition process depends on increased ROS generated by the genotoxins. Studies, now in progress, indicate that different carcinogens produce various levels of ROS in *S. cerevisiae* and that the fold increase of Ty1 transposition depends on ROS level and not on the concentration of the genotoxins. Therefore, the fold increase of transposition reflects the ROS generating capacity of carcinogen(s) and not the amount of the genotoxin in the studied sample. The property of Ty1 test to give a quantitative measure of carcinogen's effect has to be taken into consideration only when different concentrations of one particular genotoxin are studied.

7.3 Fields of applications

The Ty1 test can be successfully used in research to study carcinogenicity of chemicals, including such applied in agriculture, nutrition and pharmacy. A positive answer of the test would be a strong indicator for carcinogenic potential of the studied substance or drug. The test can also be applied to study ROS generators and scavengers. Since the increase of Ty1 transposition depends on the ROS level, a decrease in the rate of transposition caused by an added substance indicates scavenging properties of this substance.

Another application of the Ty1 test is its usage in control studies of food products. Some foods, such as smoked meat, fish, cheese, etc., have to be checked according to the requirements of the European Commission for Nutrition, for presence of carcinogens that accumulate on the surface during product's processing. The usage of liquid additives instead of wooden smoke does not guarantee a full absence of carcinogens in food products.

The usage of Ty1 test in environmental studies has been described in order to better characterize the assay. In this research environmental samples were studied in Ty1 test and chemically analyzed for presence of genotoxins. The results obtained showed a positive response of Ty1 test to samples from regions polluted with carcinogens in amounts exceeding the accepted ecological standards and negative results with samples from region not polluted with carcinogens. The high sensitivity of the test, its selectivity towards carcinogenic pollutants and the absence of false positive responses substantiated the inclusion of Ty1 assay in the battery of tests used by the National Executive Environmental Agency (Sofia, Bulgaria) to control pollution of environment.

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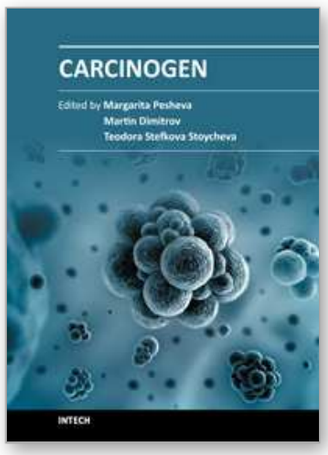

Carcinogen

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During the last decades, cancer diseases have increased all over the world. The low quality of food and strong pollution of environment are the main prerequisites for carcinogenesis. The main problem for scientists is to find strategy for prevention of cancer diseases. Therefore, the information about the models for studying carcinogenesis and mutagens which appear during cooking, environmental pollutants, and tests for specific detection of carcinogens is particularly important. The book "Carcinogen" is intended for biologists, researchers, students in medical sciences and professionals interested in associated areas.

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