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# Models for Detection of Genotoxicity *in vivo*: Present and Future

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Additional information is available at the end of the chapter

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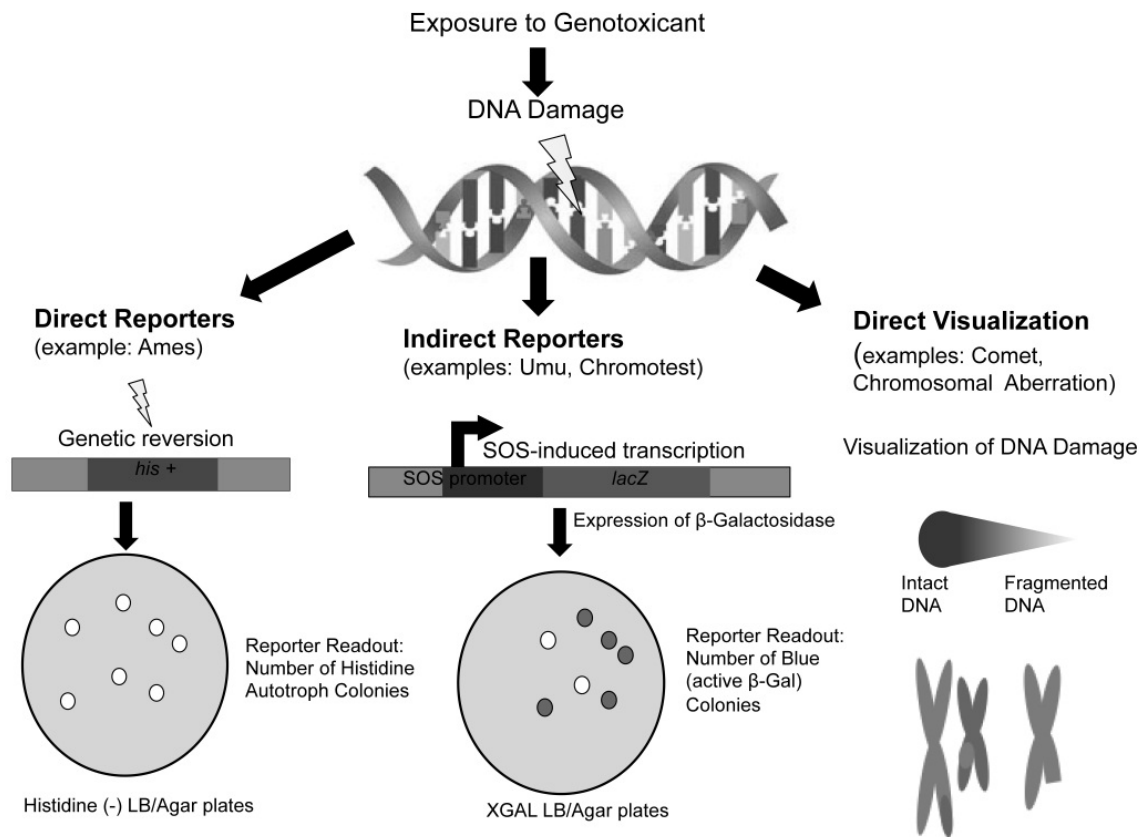
## 1. Introduction

DNA damage is toxic to the cell, both acutely (perturbing the cell cycle and inducing apoptosis), and in the longer term (accelerating senescence and causing cancer and genetic disease) (1,2). Therefore it is of great interest for public health to determine the potential of anthropogenic chemicals and other compounds found in the environment to cause DNA damage as likely toxicants, carcinogens and teratogens (3).

This chapter will review methods that use *in vivo* models (*i.e.* living organisms and cell lines) for detection of genotoxic damage caused by exposure to chemicals. The reason that living models play such a prominent role in mutagenesis detection is two-fold:

1. **Extremely low frequency of mutation:** the mutation frequency induced by exogenous agents is extremely low (in the range of 1 mutation in  $10^6$  to  $10^7$  nucleotides). The ability of living organisms to amplify these rare events through positive selection is the basis for a number of these model systems.
2. **Modulation by metabolism:** metabolism has a dual role for activation (bioactivation) and for detoxification of genotoxic compounds. Therefore, metabolism needs to be taken into account by models of genotoxic exposure. Living organisms incorporate metabolic activity into the equation, although they only approximate human metabolism to various degrees.

*In vivo* models fall into two broad categories according to how they detect genotoxicity: direct or indirect genotoxicity detection methods. Direct measurement detects alterations in DNA either by sequencing, by the generation of a phenotype linked to specific mutations, or by visualization of the DNA damage such as micronucleus formation, detection of aberrant chromosomes, or an increase in the number of DNA breaks (4-6) (Fig. 1).



**Figure 1.**

Phenotypic detection of DNA damage is based on loss-of-function (*forward*), or gain-of-function (*reverse*) reporters. Forward mutation reporters are based on the loss of a phenotypically-detectable trait such as color or sensitivity to a metabolic poison. Therefore they can detect a range of mutations (miss-sense, transcriptional termination, frameshift, indels, etc.) along a sizeable target sequence, which increases the overall frequency of detectable events, allowing in many cases for direct screening. Forward mutation reporters also provide a representation of the range of genotoxic effects induced by the relevant compound, although biased for changes that lead to functional inactivation. Reversion reporters, by contrast, are based on reversion of a specific mutation inactivating a selectable marker. Therefore, reversion markers report very specific mutations at pre-determined sites, which may not be representative of the range of lesions introduced into DNA. Also reversion events are exceedingly rare due to the small size of the target, and can only be identified by positive selection.

Phenotypic detection methods generally produce binary readouts, with the presence of growth on limiting solid or liquid media, or changes in color as primary readouts. This means that the generation of a single data point requires fine-tuning the dose and of the dilution to obtain countable colonies (on solid plates) or a number positive wells that follows a Poisson distribution (in liquid culture) (7).

Genotoxic potential can also be detected by indirect measurement methods, usually based on transcriptional fusion of a reporter gene to a promoter responsive to DNA damage

(specific examples are discussed below). Indirect detection methods provide an indication that the cell has sensed genotoxic stress, but the accuracy of each indirect reporting system depends on the range of lesions inducing the relevant transcriptional response and on the specificity of the relevant promoter for DNA damage relative to other types of stress. The reporter can be colorimetric, fluorescent or luminescent. Examples include: *lacZ* (beta-galactosidase), GFP (green fluorescent protein), luciferase, and *phoA* (alkaline phosphatase). Some of these markers (GFP and luciferase for example) have a wide dynamic range and are proportional to the amount of damage, greatly facilitating quantification (8,9). The use of GFP as a reporter, rather than reliance on an enzymatic reaction, produces a measurable response to DNA damage in a shorter time frame.

In general, indirect assays are better suited for high-throughput analysis because they can produce quantitative signals. Surface markers such as CD59 also provide forward mutation reporters that are quantitative, *i.e.* whose loss in a cell population following chemical exposure is directly proportional to the amount of genotoxic damage induced (10,11). Quantification of surface markers in large cell populations is made possible by the use of FACS (Fluorescence-Activated Cell Sorting) analysis, which is a high-throughput method that detects the presence or absence of the relevant marker in individual cells. The availability of quantitative reporters for mutagenesis substantially reduces the amount of test sample required. On the other hand, direct mutagenesis detection assays are more labor-intensive but more specific because they detect alterations in DNA.

DNA-damaging chemicals are frequently generated from precursors as reactive metabolic intermediates (12,13). The precursors are known as procarcinogens even though carcinogenicity has not in all cases been demonstrated. Procarcinogens include most genotoxic natural products and environmental agents since they would be expected to react with other molecules before reaching DNA. Chemotherapeutic drugs and other anthropogenic chemicals or contaminants, on the other hand, are frequently direct-acting. **Table 1** provides some examples of direct-acting and of bioactivation-dependent genotoxicants.

Xenobiotic metabolism, which is designed to solubilize lipophilic compounds as a way to facilitate their excretion, contributes prominently to bioactivation (12,13). The liver is the primary site of metabolic bioactivation, given its large metabolic capacity as well as its anatomical position as the gateway for compounds absorbed in the GI tract. Bioactivation by metabolism also occurs in other tissues, including skin, lung, bone marrow, and GI tract. Bioactivation in the GI tract can also result from the action of the intestinal flora, or due to the drastic pH changes that occur as food moves through the tract (12).

Xenobiotic metabolism often involves two reactions, an oxidative one and a conjugative one. Oxygenation is typically carried out by members of the cytochrome P450 family (CYPs). CYPs are membrane-bound heme-proteins that require an effective reductase system to provide electrons (14). These enzymes tend to exhibit low catalytic efficiency and broad substrate specificity. Humans bear over 50 different CYP genes, which have some overlapping substrate specificities. Conjugation on the other hand transfers N- or O- acetyl groups (acetyl transferases), sulfates (sulfotransferases) or glutathione (glutathione-S

transferases) to electrophilic substrates. Metabolism genes (particularly CYPs) are extraordinarily polymorphic, explaining the presence of wide interindividual differences in response to xenobiotics (13). Liver metabolism can be mimicked in testing paradigms by adding primary hepatocytes, liver slices, or various organ extract fractions to tester cell cultures, or by liver perfusion (15). The standard fraction is known as S9 fraction, which combines microsomes (containing CYPs) and cytosol (enriched for transferases) from the liver of rodents whose metabolism has been activated through xenobiotic pre-treatment (15,16).

Type of damage	Direct-acting Chemical class	Bioactivation-dependent Chemical class	
Alkylating	Diazo compounds	Triazenes	
	Nitrosamines	Azoxy compounds	
	Nitrosoureas	N-alkylnitrosamines	
	Aziridines	Aromatics	Polyaromatics
	Halogenated methanes, ethanes		Heteroaromatics
	Mustards		Nitroaromatic amines*
	Sulfates, sulfonates	Some proximate mustards	
Oxidizing	Simple epoxides	Quinones	
	Thiiranes, oxiranes		
	Simple peroxides		

**Table 1.** Examples of direct-acting and bioactivation-dependent genotoxic agents

In the context of drug discovery, *in vivo* methods are second-line assays performed to support the safety of a compound that is in the pipeline for clinical development (17,18).

Lead compounds are typically first prioritized by structure-activity relationship (SAR) analysis. This is a computational method that links specific chemical features of a given compound to individual biological activities, including genotoxicity. Due to its correlative nature, the predictive value of SAR analysis largely depends on how well represented the relevant class of compounds is in the database (19). Lead compounds that have made this first cut are then tested *in vivo* by direct mutagenesis detection methods for regulatory compliance. These include at least one prokaryotic phenotypic mutagenesis assay, one eukaryotic cell culture assay, and one animal test visualizing DNA damage (20,21). The standard test battery includes the Ames Test, the Mouse Lymphoma TK Assay, and the Micronucleus Test, respectively (see below). Transcriptional reporter-based assays can also be used for pre-screening prior to direct detection tests. Finally, the most reliable method to determine the potential carcinogenicity of a compound is testing it in a mammalian animal model (rat or mouse). This is done last, given the high cost of rodent carcinogenicity assays.

Here we discuss different *in vivo* models reporting on the ability chemicals to induce DNA damage, flagging these compounds as potential hazards to public health. This includes a variety of detection methods in prokaryotic, eukaryotic, tissue culture, whole-animal, and transgenic animal models. We finish by highlighting active areas of technology development and briefly speculate on the impact that next generation sequencing will likely to have in the field.

## 2. Prokaryotic reporter systems

Prokaryotes are useful for assessing DNA damage because they are haploid, reproduce quickly, and are easily grown in culture. Their use as a model for testing genotoxicity in humans is based on the universal nature of chemical mechanisms of DNA modification, as well as on the strong conservation of mechanisms of DNA repair between bacteria and humans (with the important exception of nucleotide-excision repair) (1). Genetic alterations are frequently used to enhance the sensitivity of prokaryotic reporter systems to DNA damage. Examples include mutations that increase membrane permeability (*rfaE*, *tolC*) and deficiencies in DNA repair (*uvrB*, *uvrA*, *umuD*). Most B- and K-derived laboratory strains of *E. coli* already exhibit increased permeability to xenobiotics as a result of loss of LPS selected during the long passage of these strains in culture (D. Josephy, personal communication). *E. coli* tends to be more sensitive to chemical mutagens than *Salmonella*, particularly to oxidizing mutagens, cross-linking agents and hydrazines (22). On the other hand, *Salmonella* facilitates detection of aromatic amines and of nitroaromatic compounds because of substantial endogenous bacterial nitroreductase (NR) and O-acetyltransferase (O-AT) metabolic activity.

### 2.1. Phenotypic reporter systems

Phenotypic reporters in prokaryotes are based on reversion of an auxotrophic marker. The *Ames Test* was the first of these assays to be developed, revolutionizing the field of genetic toxicology for its low cost and simplicity. This assay is based on reversion of a mutation preventing the biosynthesis of histidine. Reversion is detected by growth of colonies on solid agar in the presence of trace amounts of histidine (23). Growth on solid agar requires a large amount of test sample (~1 mg) but allows testing of non-water soluble compounds. A set of six strains have been developed to detect a broad range of point mutations and frameshifts (24). The Ames Test is still by far the most widely-used prokaryotic testing method, in part because it is mandatory for regulatory compliance.

Two variations have been developed to facilitate high-throughput formatting and to reduce the amount of sample needed: *Mini-Ames* and the *Ames Fluctuation Test*. *Mini-Ames* (also known as *Mini-Mutagenicity Test*) follows the standard Ames Test protocol, except at 1/5 the size. This reduces the amount of sample required to 300 mg of compound for the whole set of 6 reporter strains (25). Despite these advantages, *Mini-Ames* is still not widely used. The Ames Fluctuation Test is a variation of the Ames Test that is performed in liquid culture, using a chromophore as a binary indicator of growth (26). This assay has been adapted to a



microtiter format (*Ames II Test*) (27). This format can incorporate microsomes, S9 fraction, or hepatocytes for bioactivation. Commercially available, The Ames II Test has comparable accuracy relative to the traditional Ames Test for most compounds (and even higher accuracy for low-potency liquid mixtures) (28), and its use is overtaking that of the traditional Ames Test.

The *AraD Test* is an alternative assay that detects forward mutations in the arabinose operon. The cells used in this assay have a mutation in the *araD* gene, which leads to accumulation of a toxic intermediate when arabinose is present. Mutations that inactivate the operon prevent the metabolism of arabinose, allowing cells to grow on arabinose (29,30). The *AraD Test* exhibits a different sensitivity profile than Ames, although being a forward mutation assay it has two advantages over Ames: more sensitive to point mutations (larger target for mutagenic action) and producing more accurate spectrum of mutation than the Ames test (since mutations are not limited to a single site). However, in practice this assay does not represent a significant alternative to the Ames Test or other cell-based mutagenesis assays (17).

## 2.2. Transcriptional reporter systems

Transcriptional reporter systems are based on the fusion of reporter genes to promoters of the SOS regulon, which includes a battery of ~40 genes involved in the response to DNA damage (31). This regulon is under the control of the *lexA* repressor, which upon genotoxic stress is cleaved by RecA, relieving repression (31). Two systems enjoy widespread use: the *UmuC Test* and the *SOS Chromotest*. Both systems are based on transcriptional fusions of DNA damage-inducible promoters (*umuC* and *sfiA*, respectively) to *lacZ* (32,33).

*SfiA* detects a broader range of genotoxic damage than *umuC*. On the other hand, the host for the UmuC Assay is a *Salmonella* strain (NM3009), making this assay particularly suited for detection of nitroarenes, such as those found in combustion products. The UmuC Test has been adapted to micro-titer plate format. High throughput, fully automated microtiter plate versions are also available for the SOS-Chromotest as well as numerous commercially available kits for testing specific sample types. Thus, the SOS-Chromotest provides easily quantifiable, reproducible and customizable ways to measure genotoxicity in a variety of samples, from wastewater to blood serum (33). An additional system, based on a fusion between the SOS-inducible gene *sulA* and the alkaline phosphatase-encoding gene *phoA* has also been recently described (34).

## 3. Eukaryotic reporter systems

Eukaryotic systems are also extensively used for detection of genotoxic activity. They have the advantage of having a DNA repair machinery that is even closer to that of humans (with homologous nucleotide excision repair machinery and more translesion synthesis polymerases for example) and of having comparable replication machinery, allowing detection of genotoxins that interfere with mitosis. Higher eukaryotes also have metabolic systems that are much closer to our own, although even rodents show marked

differences in metabolism relative to humans (12,13). Another advantage is the larger size of the cell nucleus and genome, which facilitates detection of rearrangements and other genomic abnormalities. Disadvantages include higher cost (particularly rodent systems), the presence of efflux pumps that can prevent accumulation of xenobiotics, and a diploid genome, which masks the phenotypic effects of heterozygous recessive mutations. Different strategies for improved detection of recessive mutations have been devised. These include: sporulation (in yeast), heterologous expression of single human chromosomes (in Chinese hamster cells), and selection of X-linked and heterozygous loci as markers (which become dominant or homozygous recessive with only one mutation).

As in prokaryotes, genetic modifications in yeast enhance sensitivity to DNA damage. Examples include deletion of efflux pumps (35), removing *mag1* (a N3mA DNA glycosylase), hindering base excision repair (36), and deletion of *mre11*, preventing both homologous recombination repair and non-homologous end joining pathways (36).

We group eukaryotic models into three sections: 1) phenotypic detection; 2) transcriptional detection; and 3) direct visualization of DNA damage.

### 3.1. Phenotypic reporter systems

Phenotypic detection of genotoxic damage in eukaryotes follows principles of forward and reverse mutation analogous to prokaryotic systems. While some of these systems are as old as the Ames' test, others are still being actively developed. Below we discuss two yeast mutagenesis reporter systems (the *DEL Assay* and the *Mitotic Gene-Conversion Assay*) and four mammalian cell-based ones (HPRT, TK, *The Human-Hamster Hybrid (A(L))* and the *PigA assay*).

#### 3.1.1. Yeast *DEL Assay*

This assay detects chemical induction of recombination events by reversion of a *his3* locus that has been interrupted by short repeats. Reversion to *his*<sup>+</sup> can be measured by plating (37), or more recently in microtiter plate format, using a colorimetric readout (38,39). This assay has thus far proven to be very accurate, discriminating between carcinogens and non-carcinogens of the same chemical class, and showing a 92% correlation with two prokaryotic genotoxicity assays (Ames and UmuC) (39).

#### 3.1.2. Yeast *Mitotic-Gene-Conversion Assay*

The Mitotic Gene-Conversion Assay uses a combination of heteroallelic (*ade2-40/ade2-119* and *trp5-12/trp5-27*) and homoallelic (*ilv1-92/ilv1-92*) gene loci to detect induction of mitotic crossing over, mitotic gene conversion and reverse mutation (40). The original heteroallelic condition *ade2-40/ade2-119* forms white colonies. Mitotic crossing over can be detected visually as pink and red twin sector colonies due to the formation of homozygous cells of the genotype *ade240/ade240* (deep red) and *ade2-119/ade2-119* (pink). Mitotic gene conversion can be detected by the loss of auxotrophy for adenine (*ade2* locus) or tryptophan (*trp5* locus). Mutation induction can be followed by the appearance of isoleucine non-



requiring colonies on selective media. Detecting both reversions and repair-associated recombination events is a unique feature of this assay that increases the sensitivity to genotoxicity. This assay is widely used and included in the Code of Federal regulations of the United States of America. However, the need to assess mitotic cross-over by screening for changes of color makes full automation of this assay very difficult.

### 3.1.3. HPRT Assay

This assay measures inactivating mutations at the *hprt* locus, which encodes the salvage-pathway enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT). HPRT catalyzes the formation of inosine or guanosine monophosphate from hypoxanthine or guanine, respectively. Treatment with 6-thioguanine generates 6-thioguanine monophosphate (6-TGM), which is highly cytotoxic to wild-type cells (41). Inactivating mutations in the *hprt* gene are dominant because this gene is carried on the X chromosome and is subject to X-inactivation (42-44). The standard cells for use in this assay are CHO (Chinese hamster ovary) cells, V79 (Chinese hamster lung cells), G12 or G10 cells (V79-derived cells). A variation on this assay is the expression of bacterial *gpt* gene (the functional homolog of HPRT) in an HPRT- background (42).

### 3.1.4. Cell Line TK Assay (mouse or human lymphoma cells)

The cells used for the assay are mouse lymphoma cells L5178Y, which are heterozygous at the thymidine kinase locus (*tk1*) on chromosome 11. Inactivating the WT allele induces trifluorothymidine (TFT) resistance, and *tk*<sup>-/-</sup> mutants can be selected for in a background of *tk*<sup>+/-</sup> non-mutant cells (45-47). Colony size is an indicator of the type of mutation involved: large colonies typically correspond to small TK-inactivating mutations, while small colonies often indicate clastogenic damage. This test (which is mandatory for regulatory compliance) is the most favored of the cell-line based assays because of its sensitivity to mutagens (48,49). However, this assay is also very susceptible to false positives (48,49).

Two tests use surface proteins as forward mutation markers, the Human-Hamster Hybrid (A(L)) Cell Mutagenesis System and the PigA Assay. Surface markers offer several advantages over drug-dependent readouts. Results are quantitative, producing not only a binary (yes/no) result but an indication of the potency of the chemical tested. In addition, with these assays cells do not need to be lysed for analysis, which enables tracking of the phenotype over time, as well as testing multiple or constant low-level exposures.

### 3.1.5. The Human-Hamster Hybrid (A(L)) Cell Mutagenesis System

Human-hamster hybrid (A(L)) cells were generated containing a single human chromosome 11 in addition to a standard set of CHO chromosomes. This human chromosome expresses CD59, CD44 and CD 90 surface antigens. The presence of CD59 on the cells' surface makes them sensitive to binding by a polyclonal antibody known as E7. Upon binding of the E7 antibody, incubation with serum stimulates the complement cascade, which lyses the cells.

The yield of CD59- mutants can also be detected by immunofluorescence and quantified using flow cytometry, providing a quantitative readout for mutagenesis (50,51). Detection of CD59- mutants exhibited a linear correlation with clastogen (gamma-radiation) and point mutagen (MNNG) dose, confirming the quantitative nature of the assay (50).

### 3.1.6. *PigA* Assay

The phosphatidylinositol glycan complementation group A (Pig-A) gene encoded on the X-chromosome is essential for attaching GPI-anchored proteins to the cell surface. The PigA assay detects the loss of CD59 (incidentally the same marker used in the Human-hamster hybrid (A(L)) assay described above) in red blood cells as an indicator of loss-of-function mutations at the endogenous Pig-A locus. Anti-CD59-PE is used to stain blood cells, and individual cell fluorescence is monitored by FACS analysis (10,52). Thiazole orange is used to differentiate between mature erythrocytes, reticulocytes (RETs), and leukocytes; and anti-CD61 to resolve platelets (10). The assay has been adapted for monkeys, mice, rats and humans (11). In rats, phenotypes can be detected earlier in reticulocytes than in erythrocytes (2 weeks versus 2 months following exposure, respectively).

## 3.2. Transcription reporter systems

As in the case of prokaryotes, eukaryotic transcription reporter systems are transcriptional fusions to genes that are specifically induced in response to DNA damage.

In yeast, one of the promoters of choice is that of ribonucleotide reductase 3 (*rnr3*), which encodes a form of the large subunit of ribonucleotide reductase. This gene is transcribed in response to low levels of damage, discriminates between DNA damage and other forms of stress, and its expression reaches higher levels than other DNA damage-responsive genes (53). The *rnr3* promoter is therefore ideal as a reporter. An assay was developed with *rnr3* driving *lacZ* expression (*rnr3-lacZ*) (35), and has had a modest impact following its initial description (10 PubMed citations, and 260 Google Scholar hits). A promising variation was developed that uses secreted *Cypridina* luciferase as a reporter (*rnr3-luciferase*) in a DNA repair-deficient yeast strain (54,55). Secretion of luciferase into the culture medium facilitates sequential measurements of DNA damage because cells don't need to be collected. This allows the detection of chronic effects, *i.e.* accumulated damage due to chronic low-level exposure over an extended period of time. It is easy to envision a fully automated adaptation of this assay, which would be cheap, and would not require specialized technicians.

Another promoter that has been used in yeast is that of HUG1 (Hydroxyurea- UV- and Gamma radiation-induced). The HUG1 promoter is used to drive expression of GFP (56). While the specific function of HUG1 is unknown, it is a part of the Mec1p kinase pathway, a signal transduction cascade that has a pivotal role in DNA damage-sensing in yeast. The sensitivity of the initial strain was enhanced by two deletions: that of *mag1* and that of *mre11*. These changes increased the sensitivity of the assay to alkylating agents and to

inducers of strand-breaks, but did not change the sensitivity to other forms of DNA damage (36). However, in order for this system to have a clear advantage over other luminescence-based reporters, the sensitivity still needs to improve considerably.

In human cell lines, the promoter of choice is that of the Growth Arrest and DNA Damage 45 (GADD45) gene, which is a sensor for genotoxic stress in mammalian cells. GADD45 $\alpha$  is induced upon exposure to clastogens, aneugens, and mutagens. The *GreenScreen Assay*, which uses a transcriptional fusion with GFP transformed into human lymphoblastic TK6 primary cells as a reporter, showed high specificity for carcinogens that do not require metabolic activation (100% accuracy with 75 chemicals tested) (57), as well as for procarcinogens (91% accuracy with 23 chemicals tested) (58). This assay has since undergone extensive validation with more than 8,000 compounds. The overall specificity to genotoxins has remained quite high at 95% (59). The GreenScreen Assay has been commercialized by the company Gentronix, including a 96-well plate version, and is becoming increasingly popular.

### 3.3. Direct visualization of DNA damage

A sensitive way to visualize DNA damage in eukaryotic cells is the COMET assay. This assay detects DNA fragmentation, which can result from a wide range of lesions including double strand breaks (DSBs), single strand breaks (SSBs), alkali labile (abasic) sites, oxidative DNA base damage, and DNA-DNA/DNA-protein/DNA-drug crosslinking. Cells are embedded in a thin layer of agarose, which is mounted on a microscope slide. The slide is immersed in an ionic running buffer (usually TBE or TAE) and the cells are electrophoresed through the agarose. DNA fragments will travel faster than the intact parts of the nucleus, and will run in front of the nucleus. When the DNA is stained and observed with a microscope, the fragments form what looks like a comet's tail, and the nucleus forms the comet's head (17,18) (**Fig1. direct visualization**). The COMET assay does not test for a specific end-point and can therefore be used to monitor both the genotoxic effects of chemical exposure and the kinetics of DNA repair. The use of the COMET assay as a readout for genotoxicity is increasing. Full automation has recently been achieved (60,61), which will greatly facilitate standardization and use of the assay as a screening tool. In addition, the sensitivity of this assay is being improved through combinations with other visualization methods, such as FISH (6,62).

#### 3.3.1. Sister Chromatid Exchange Assay

The Sister Chromatid Exchange (SCE) Assay detects reciprocal exchanges between two sister chromatids of a replicating chromosome, apparently involving homologous loci (63). The DNA is labeled for two cell cycles (for example with bromodeoxyuridine) and visualized by fluorescence microscopy. It can be performed on a variety of cells, including cells from sentinel species like mussels and fish, which makes this assay extremely useful for environmental monitoring (64). This assay has also been used in humans as a marker for

genotoxic exposure (65). While this assay does not detect DNA damage *per se*, SCE is an indication of ongoing DNA repair and therefore a genuine indicator of genotoxicity.

Two other visualization methods (the *Micronucleus Test* and the *Chromosomal Aberration Test*) are largely aimed at the detection of clastogens (agents that produce alterations affecting more than a few contiguous bases) and of aneugens (agents that alter the number of chromosomes). Prokaryotic systems have poor sensitivity for clastogens and aneugens because prokaryotes do not have multiple chromosomes, and their replication shares little mechanistic homology with mitosis. In addition, the larger size of the genome and of the nucleus in eukaryotes greatly facilitates the direct visualization of large aberrations. Therefore, these two assays complement prokaryotic reporter systems and are required for regulatory compliance.

### 3.3.2. *Micronucleus Test (MN)*

Micronuclei (MN) are broken fragments of daughter chromosomes that did not make it into the nucleus during mitosis. MN formation is therefore diagnostic for chromosomal DNA damage. It is detected by staining for DNA, cell membrane and nuclear membranes, followed by observation of individual cells with microscopy. The approved method for scoring micronucleus induction is to image stained cells and count those with MN. Cells can be harvested from a live animal or from tissue culture. The presence of MN is best visualized in erythrocytes (because they are anucleate) but it can also be used with other cell types. The success of this test relies on proper cell harvesting and culturing techniques, as the integrity of the cell and nuclear membrane are vital. It also depends on careful scoring of cells, since the nucleus must be clearly defined in order to determine the occlusion of MN from the nucleus (5,66). Flow cytometry can be used to quantify cells with MN induction (67), although careful microscopy controls are recommended. Recently, micronuclei induction in TK6 cells by a battery of reference compounds was determined using both microscopy and flow cytometry (68). This study produced a good correlation between the two readouts, suggesting that MN assay by flow cytometry may become one of the methods of choice for routine genotoxicity testing in the near future, particularly in the pharmaceutical industry.

### 3.3.3. *Chromosomal Aberration (CA) Assay*

The Chromosomal Aberration Assay detects large-scale damage of chromosomes, including structural aberrations (fragmentation or intercalations) and numerical aberrations (aneuploidy and polyploidy). Numerical aberrations are most frequently the result of unequal segregation of homologous chromosomes during cell division, which can be caused by interference with cohesion during mitosis (69). The test is most commonly carried out *in vitro* by exposing cell cultures to the test substance, and then treating the cells with a compound that stops mitosis in metaphase (colcemid). Following staining, the chromosomes are analyzed microscopically for aberrations. FISH-staining techniques have been used to increase the sensitivity of CA, allowing each chromosome to be differentially stained to reveal chromosomal rearrangements not detectable with conventional staining techniques (70,71).

### 3.4. Transgenic animal models

Transgenic animals represent one of the pillars of toxicological analysis, because they combine exposure in a whole organism with efficient detection in microbial systems. Every cell of the transgenic animal carries a chromosomally-integrated vector-reporter fusion gene that is not expressed and is therefore free to accumulate mutations. The vector is either a bacteriophage or a bacterial plasmid. Following exposure of live animals to a test chemical, transgenes are recovered from the genomic DNA and placed in the appropriate bacteria for readout of mutational frequency. Mutants are identified through the use of phenotypic reporters and their mutational spectrum can be determined by sequencing (72). Transgene models are ideal for study of the effects of chronic and repeated exposure, given the genetic neutrality of the transgenic reporter in the live animal. When the goal is to obtain mutation spectrum information, prolonged and/or repeated genotoxic exposure maximizes the number of independent mutational events obtained.

#### 3.4.1. *Muta<sup>TM</sup>Mouse*

The Muta<sup>TM</sup>Mouse was the first transgenic rodent system to be introduced (73). In this system, the transgenic mice have, on their third chromosome, the  $\lambda$  g10 bacteriophage vector linked to a single *lacZ*. Following treatment, the genomic DNA is extracted from the tissues of interest, packaged into a lambda vector and transfected into *lacZ*- *E. coli*. Mutations result in white plaques in the presence of X-gal. This system is not nearly as popular as the LacZ Plasmid Mouse and the Big<sup>®</sup> Blue Mouse because of poorer yield of transgenic DNA (72).

#### 3.4.2. *Big<sup>®</sup> Blue Assay*

Another bacteriophage-based assay is the Big<sup>®</sup> Blue Assay, which exists in both mouse and rat backgrounds (74,75), and is available from several different companies (72). The original mouse version uses *LacI*, the lac repressor, as the reporter that is linked to the  $\lambda$ LIZ $\alpha$  phage vector. Multiple copies of the transgene (40 for the mouse model and 30 for the rat) are integrated in the chromosome, arranged head-to-tail. Transformation of recovered vector into *E. coli* followed by plating on X-gal medium allows the identification of blue plaques (with inactive mutant *lacI*) on a background of white plaques. The Big Blue system was greatly improved by the use of the *cII* gene as a reporter. The *cII*  $\lambda$  gene is responsible for transition from the lytic to the lysogenic phase at low temperatures, inducing expression of the CI repressor. Inactivating mutations sustained at the *cII* locus confer phages with the ability to form plaques, making detection of mutations a simple positive selection (76,77).

#### 3.4.3. *LacZ Plasmid Mouse*

The LacZ Plasmid Mouse has 20 copies of the pUR288 plasmid per haploid genome (a total of 40 copies) integrated into multiple chromosomes (78). Genomic DNA is recovered, and digested by the *HindIII* restriction enzyme, releasing single copies of linearized plasmid. Magnetic beads coated in Lac repressor protein are used to isolate plasmid DNA from the digest, and the isolated vector is then recircularized into individual plasmids. These



plasmids are transformed in *E. coli* and mutant frequency can be determined by scoring white colonies in the presence of X-gal. Compared with bacteriophage-based transgenic systems, plasmids can be isolated more efficiently and are more tolerant of deletions (both internal and involving flanking sequence) (72). A significant improvement to the test was the introduction of P-gal (which generates a toxic product when broken down by  $\beta$ -gal) as a positive selection for  $\beta$ -galactosidase loss-of-function (79).

#### 4. Conclusions

Mutagenesis detection *in vivo* is key for testing the genotoxic potential of anthropogenic chemicals produced for industrial or medical applications as well as of products present in our environment. Both prokaryotic and eukaryotic models are useful, and complement each other. Prokaryotic models are simple, inexpensive, and frequently amenable to high-throughput formatting but detection is largely restricted to mutagens that induce point mutations and frameshifts. Eukaryotic models, by contrast, are more labor-intensive and time-consuming but are more sensitive to clastogenic and aneugenic activity and facilitate visualization of DNA damage (nicks, breaks, abasic sites, etc.). Indirect methods are cost-effective and easily amenable to automation, while visual or phenotypic detection is more specific because it reports DNA damage or genetic alterations caused by DNA damage but is generally more expensive and labor-intensive.

Accurate reproduction of human metabolism in model systems of genotoxicity remains one of the most urgent challenges in the field. As mentioned in the introduction, bacterial strains used for genotoxicity testing exhibit some metabolic activities. However, they lack cytochrome p450 activity completely, making them poor models for human bioactivation. Individual CYP proteins have been expressed in *E. coli* (22). However, expressing active CYP proteins in *E. coli* is not trivial, as it requires special media and co-expression of a reductase system as electron donor. More importantly, only a few CYP alleles can be expressed at a time, so it will be extremely difficult to reproduce the complex patterns of CYP expression occurring in liver cells. In the classic Ames Test, mammalian xenobiotic metabolism is mimicked through the addition of post-mitochondrial hepatic rodent extract (S9 fractions). While this *in vitro* metabolic model allows detection of a range of procarcinogens, it misses short-lived metabolites that fail to cross the bacterial cell wall and suffers from low reproducibility because of the variable composition of the extracts (15% inter-laboratory variability) (48).

Whole-animal models are still the most sensitive systems available for detection of procarcinogens. Fish were proposed as a model organism early on due to their enhanced liver metabolism relative to humans and to the easy exposure to xenobiotics in the water or in the trophic chain (80). Transgenic reporters analogous to the ones created in mice were developed in fish (80,81), although their use is not yet widespread, possibly due to the need for specialized labor and facilities. *Drosophila melanogaster* is also likely to become more prominent in the future as a model for genotoxicity because it complements in many ways bacteria or yeast-based models. It is a whole organism, but extremely cheap and easy to maintain. Like fish, *Drosophila* produce large numbers of testable offspring (high *n*), and



have metabolic and DNA repair systems that are highly homologous to human systems. Assays for genetic damage in germ cells, mostly in males (*Sex-Linked Recessive Lethal Test (SLRLT)*, and *Reciprocal Recombination Test*) were the initially developed (82). Recombination assays were later devised in somatic cells for improved sensitivity (82). These assays rely on endogenous forward mutation markers, with visible developmental abnormalities in wings, eye morphology, or bristle shape as readouts. Flies can be exposed to test chemicals in early stages of development (larvae), further increasing the sensitivity of the assay. Larvae are very actively metabolically and have been shown to be sensitive to teratogenic effects of pro-carcinogens (83). The large number of endogenous targets, the suitability for early exposure, and its active metabolism make *Drosophila* possibly the most sensitive phenotypic detection model available and a very promising model for detection of genotoxic and teratogenic effects (83).

New molecular technologies are likely to enhance our ability to detect the presence of mutations at very low frequencies, as illustrated by the *Random Mutation Capture Assay* (84). This technique detects the loss of a specific restriction site in chromosomal or mitochondrial genomes using multiplex PCR amplification (65,84) and has enabled establishing spontaneous mutation rates in tumors (85), and in a mouse model of aging (86). Importantly, by limiting dilution of the template, this technique has the ability to detect mutations from single DNA molecules templates, identifying non-clonal mutations in a heterogeneous population (85).

High-throughput sequencing technology will also likely allow the determination of genotoxic effects in the near future with an unprecedented level of resolution. Next-generation sequencing is based on massive, parallel amplification of templates (87). While DNA amplification is PCR-based, and therefore susceptible to the error-rate of the polymerase, mutations present in the template can still be detected through redundant coverage (typically in the 30-fold range). The accuracy of coverage information can be ensured through adequate design of bar-coded primers for amplification. Because, given the structure of the human genome, most random mutations in a cell are expected to be neutral, they should occur randomly and increase the genetic diversity in exposed the population over a period of time. In the absence of positive selection, sequencing of clonal mutations (*i.e.* mutations that are present in a significant fraction of the population) would miss this underlying genetic diversity (88). Therefore, obtaining an adequate representation of chemically-induced mutations would require sequencing DNA from individual cells.

As these new models and molecular tools become established in the field of genetic toxicity, they will need to be incorporated into the regulatory process for approval of new chemicals or for reassessment of chemicals currently in use.

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