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Review

Current Status of Short-Term Tests for Evaluation of Genotoxicity, Mutagenicity, and Carcinogenicity of Environmental Chemicals and NCEs

Mahima Bajpayee, Alok K. Pandey, Devendra Parmar, and Alok Dhawan
Developmental Toxicology Division, Industrial Toxicology Research Center, M.G. Marg, Lucknow, India

The advent of the industrial revolution has seen a significant increase in the number of new chemical entities (NCEs) released in the environment. It becomes imperative to check the toxic potential of NCEs to nontarget species before they are released for commercial purposes because some of these may exert genotoxicity, mutagenicity, or carcinogenicity. Exposure to such compounds produces chemical changes in DNA, which are generally repaired by the DNA repair enzymes. However, DNA damage and its fixation may occur in the form of gene mutations, chromosomal damage, and numerical chromosomal changes and recombination. This may affect the incidence of heritable mutations in man and may be transferred to the progeny or lead to the development of cancer. Hence, adequate tests on NCEs have to be undertaken for the risk assessment and hazard prediction. Compounds that are positive in tests that detect such damages have the potential to be human mutagens/carcinogens.

Only long-term animal bioassays, involving lifetime studies on animals, were used earlier to classify substances as mutagens/carcinogens. These tests were cumbersome and time consuming and required a lot of facilities and personnel. Short-term tests, therefore, were brought into practice. A "battery" of three to four of these short-term tests has been proposed now by a number of regulatory authorities for the classification of compounds as mutagenic or carcinogenic.

This review deals with the current status of these short-term tests.

Keywords Short-Term Tests, Genotoxicity, Mutagenicity, Carcinogenicity, NCEs

INTRODUCTION

The advent of the industrial revolution has seen a significant increase in the number of chemicals released in the environment. These include pesticides, industrial chemicals, solvents, drugs, etc. Most of these chemicals are metabolized and excreted by humans; however, continued low-level exposure to environmental toxins/pollutants from cigarette smoke to factory emissions pose serious health risks. A number of disorders, including cancer, have now been attributed to the exposure of environmental chemicals. It becomes imperative to check the toxic potential of new chemical entities (NCEs) to nontarget species before they are released for commercial purposes. Since DNA is the carrier of inherited information and any change in its structure may potentiate serious biological changes, understanding the mutagenic/carcinogenic effects of chemicals is very important.

Mutagens are chemicals or compounds causing chemical or physical alterations in DNA structure, resulting in an inaccurate replication of that region of the genome. They can be mutagenic per se or after metabolic activation. Moreover, DNA or chromosomal instability and DNA repair deficiencies have also been implicated in cancer risks. Most cancers are shown to be the result of genetic alterations such as chromosomal loss and rearrangements (Klug and Cummings 2001). Mutations play a central role in cancer development, as evident from molecular studies of oncogenes and tumor suppression genes. The shift in equilibrium between fidelity of replication (in normal or damaged DNA) and DNA repair processes may lead to the induction of mutations (Sarasin 2003). Mutational alteration in the proto-oncogenes and/or suppressor genes can lead to development of cancers (Wijnhoven et al. 2000). Activation of cellular oncogenes by chromosomal translocation, which brings an oncogene under the influence of a highly active chromosome region, appears to play a pivotal role in the genesis of certain hematopoietic and lymphoid tumors (Cahill et al. 1998). Hence, an understanding of the molecular mechanisms of chemical carcinogenesis is central to the prevention of many environmentally induced cancers.

A carcinogen is an agent that causes or induces neoplasia (i.e., heritably altered relatively autonomous growth of tissue). Most chemical carcinogens must be metabolized in a cell before they exert their carcinogenic activity. The process of carcinogenesis involves a variety of biological changes, which to a great extent reflect the structural and functional alteration in the genome of

Received 14 February 2004; accepted 5 April 2004.
Address correspondence to Dr. Alok Dhawan, Developmental Toxicology Division, Industrial Toxicology Research Center, P.O. Box 80, M.G. Marg, Lucknow 226 001. E-mail: dhawanalok@hotmail.com, alokdhawan@yahoo.com
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the cell. It involves initiation (simple mutation in one or more cellular genes controlling key regulatory pathways of the cell), selective functional enhancement (promotion), and progression of the unstable karyotype. Hence, during cancer development, a normal somatic cell is transformed into a malignant linear cell via a complex multistage process ranging from point mutations to chromosomal aberrations affecting the function or expression of both oncogenes and tumor suppressing genes (DHEW 1977).

The primary objective of mutagen testing is to determine whether a chemical has the potential to cause genetic alterations in man. The fundamental concern in mutagenesis is the risk to future generations. The association of mutagenesis with other endpoints such as carcinogenesis, teratogenesis, and aging has been noted. Hence, it is necessary to obtain quantitative data from relevant animal model systems from which extrapolation to humans can be made to predict virtually safe or tolerable levels of exposure. Clinical observations, epidemiological studies, and experimental tests have led to identification of chemicals that are mutagenic and/or carcinogenic. These tests are divided into long-term, medium-term, and short-term assays.

The U.S. guidelines for testing environmental chemicals and food additives were delineated during the 1970s and 1980s (U.S. FDA 1982).

The first battery of tests had:

Salmonella test for gene mutation.
In vitro test for CA or a mammalian cell mutagenesis test.
A general test for DNA damage.

Then, in 1993, the U.S. FDA guidance for testing food and color additives recommended a "core" testing battery (U.S. FDA 1993).

Gene mutation in bacteria (*Salmonella*).
A test for gene mutation in mammalian cells in vitro.
An assay for cytogenetic damage in vivo in which rodent bone marrow assay was preferred.

Though similar recommendations were made from various countries, there were differences in the test system requirements in the in vivo/in vitro testing (MacGregor et al. 2000).

The guidelines by the Committee on Mutagenicity of Chemicals in Food Consumer Product and Environment (COM), U.K., in 2000, put forward three stages for testing of chemicals for mutagenic potential:

Stage 1—*In vitro assays*

1. Bacterial tests for gene mutation
2. Test for clastogenicity
   i. In vitro metaphase analysis
   ii. In vitro micronucleus test
3. Mammalian cell mutation assay
   Mouse lymphoma assay

Follow-up:

Positive result in any of the tests: the substance is an in vitro mutagen and in vivo testing is required.

Negative result in all the above tests: the substance is not mutagenic.

Stage 2—*In vivo somatic cell testing*

1. Rodent micronucleus assay or
2. Bone marrow or peripheral blood (mouse) assay for clastogenicity with kinetochore and centromeric staining.
3. Other assays include:
   i Liver UDS for thymidine incorporation outside S phase.
   ii Comet assay or other DNA strand breakage assay for DNA Single Strand Break/Double Strand Break.
   iii ^{32}P-post labeling for DNA adducts.
   iv Covalent DNA for DNA adducts.

Positive result: the compound is considered as an in vivo somatic cell mutagen and assessment for germ cell mutagenicity is required.

Negative results: the compound is not an in vivo mutagen.

Stage 3–*Germ cell assays*

Tests to show interaction with germ cell DNA: dominant lethal assay is the most useful [COM] guideline as an assay for clastogenicity and aneugenicity in germ cells. Assays for investigation of clastogenicity in mammalian spermatogonial cells and spermatocytes have also been recognized. All established germ cell mutagens have shown positive results in bone marrow assays, and there is no current evidence for germ cell-specific mutagens. Metaphase analysis of spermatogonia or micronuclei induction in the spermatocytes and transgenic mice models can also be used to investigate mutations in germ cells. Genotoxicity in germ cells can also be seen using various approaches as described in the Stage 2 studies. The mouse-specific locus test and the mouse heritable translocation test are also used to estimate risk of heritable effects under exceptional cases.

Various strategies (Fig. 1) have been employed for the identification of a chemical as genotoxic, mutagenic, and/or carcinogenic using different test systems (Table 1). The tests are broadly classified into three categories based on the timeframe required for the completion of the tests.

1) LONG-TERM BIOASSAYS

The essence of long-term bioassays is to observe the test animal for a major portion of its lifespan for the development of neoplastic lesions after or during the exposure to various doses of a test substance by an appropriate route (Bannasch et al. 1986).

The main stages of these assays for carcinogenicity of chemicals include design, conduct, analysis, and reporting, requiring careful planning, good animal care leading to long survival, high standard of pathology, and unbiased statistical analysis.

Undertaking long-term bioassays for carcinogenicity is time consuming and costly and requires adequate physical facilities and qualified personnel. Care is taken to include ideal animal species for the study, which would show identical biological responses to that of humans. The route of administration of the

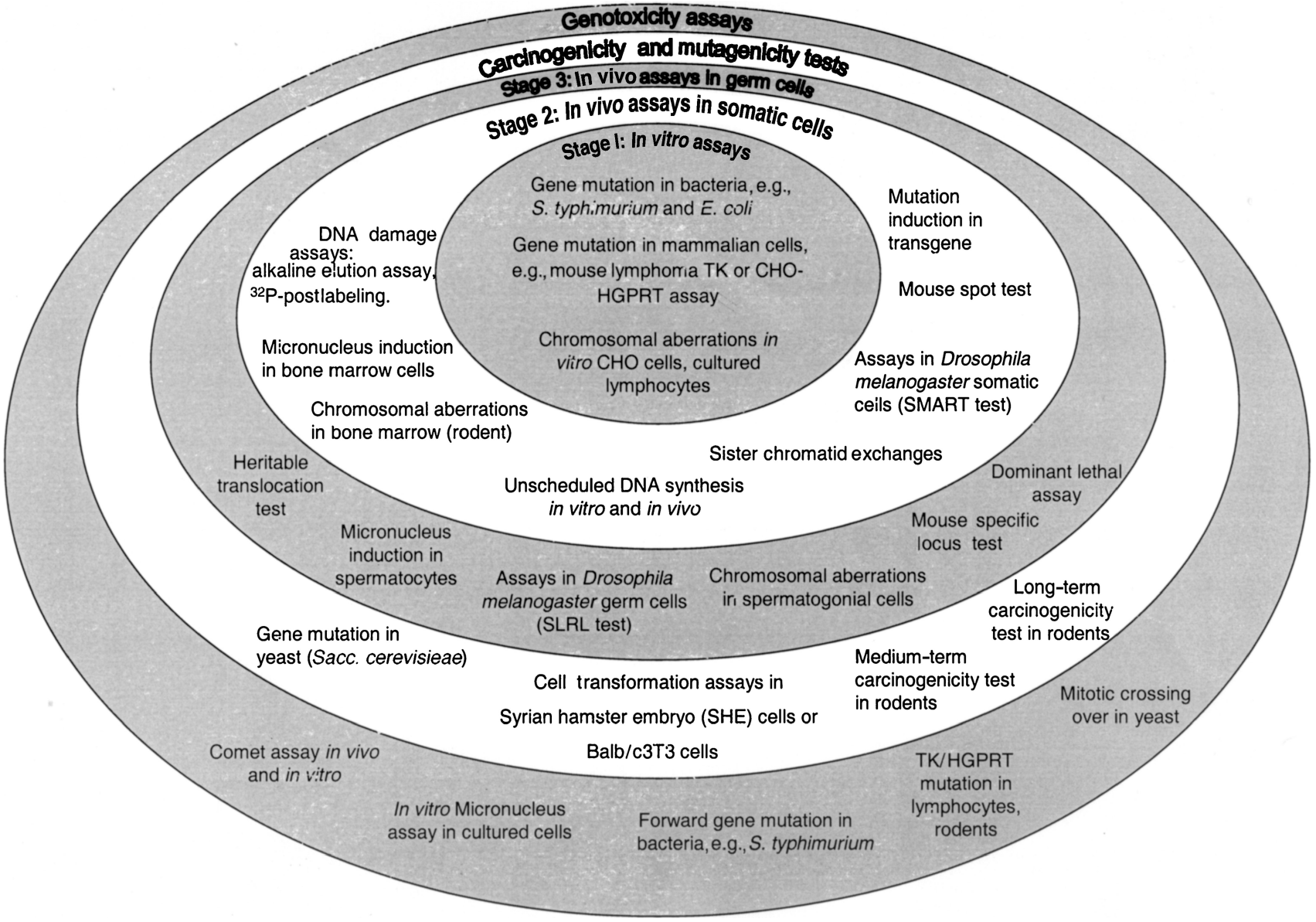

FIG. 1. Strategy for testing of genotoxicity, mutagenicity, and carcinogenicity.

TABLE 1
Tests for genotoxicity, mutagenicity, and/or carcinogenicity

Endpoint		Regulatory Agency Recommendation*
Short-Term Tests (Timeframe—Several Weeks, 1–3 Months)		
1. Gene Mutation Assays		
In vitro		
Prokaryotic mutagenesis in vivo; e.g., *Salmonella typhimurium* (Ames test)	Reverse or forward mutation in specific bacterial strains	OECD (471), ICH, COM
Mouse lymphoma thymidine kinase (TK)	Mutation in TK	OECD (476), ICH, COM
Chinese hamster ovary (CHO) and V79 hypoxanthine guanine phosphoribosyl transferase (HGPRT)	Mutation in HGPRT	OECD (476), COM
In vivo		
Mouse spot test	Somatic cell mutation, detects coat color variation	OECD (484), COM
Mouse specific locus test	Germ cell mutation, coat color, and ear size change	COM
Dominant lethal assay	Death of fertilized egg in mammalian implanted species	OECD (478), COM
Mutation induction in transgenes	Mutation in LacZ gene, LacI gene in mouse or rat	ICH, COM
2. Chromosomal Abnormalities		
Mitotic recombination, mitotic crossing over or mitotic gene conversion in yeast (in vitro)	Conversion of heterozygous alleles to homozygous state	OECD (481)
Chromosomal aberrations	Visible alteration in karyotype	
(in vitro)	In cell lines (CHO, V79 etc) and cultured lymphocytes	OECD (473), ICH, COM
(in vivo)	In rat bone marrow cells and spermatogonial cells	OECD (475,483), ICH, COM
Sister chromatid exchanges	Visible exchanges of differentially labeled sister chromatids	OECD (479)
Heritable translocation test (mice) (in vivo)	Translocation-induced in germ cells	OECD (485), ICH, COM
Micronucleus test		
in vivo	Appearance of micronuclei in bone marrow	OECD (474), ICH, COM
in vitro	Appearance of micronuclei in cell lines	OECD (487)
3. Primary DNA damage		
DNA repair in vitro	Unscheduled DNA synthesis (UDS)	OECD (482), ICH, COM
Rodent liver unscheduled DNA synthesis induction in vivo	UDS in primary rat hepatocytes	OECD (486), ICH, COM
DNA damage in vivo or in vitro	Comet assay, alkaline elution assay, ^{32}P-postlabeling, for DNA damage in lymphocytes or cell lines (CHO, V79, etc.)	ICH, COM
4. Cell transformation		
Induced neoplastic transformation of cells	Syrian hamster embryo (SHE) cells, Balb/c3T3	OECD (draft)
5. Assays in *Drosophila melanogaster*	Somatic mutations and Germ cells	OECD (477)
6. Flow cytogenetics	Using flow cytometry to study DNA damage	
Medium-Term Tests (Timeframe—2–8 Months)		
Ito model in rats	Hepatic adenomas and carcinomas staining for GST (pi)	
Newborn mouse model	Neoplasm of liver, lung, and lymphoid organs in mouse	
Long-Term Tests (Timeframe—18–24 Months)		
Chronic bioassay in animals	Tumors in all organs	OECD (451), ICH
Tissue specific bioassays; liver and lung (mouse), brain (rat), mammary gland (rat/mouse)	Hepatomas, pulmonary adenomas, gliomas, and carcinomas in rat and mice	

*OECD, Organization of Economic Co-operation and Development; number in parenthesis denotes the OECD guideline number of the test. ICH, International Committee on Harmonization. COM, Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, UK.

test chemical should be through the predominant route of human exposure (i.e., oral, cutaneous, inhalation, or intramuscular).

The "gold standard" for determining potential carcinogenic activity of a chemical is through the use of chronic two-year bioassays for carcinogenicity in rodents. This model has been used to detect compounds labeled as human carcinogens by the International Agency for Research in Cancer (IARC) (Vaino et al. 1991). The bioassay is used to detect complete carcinogens, tumor promoters, and co-carcinogens.

In addition to the long-term bioassay for carcinogenicity in animals, limited animal bioassays or tissue specific bioassays (Pilot III and Dragan 2001) have been developed that are useful for screening chemicals for carcinogenic activity. These assays have the advantage of getting results relatively quickly (12–18 months) as compared to long-term bioassay; the endpoints evaluated are:

1. high background rate of cancer and thus high sensitivity to induction of cancer
2. early detection of cancer.

The positive results in these assays are strongly suggestive of carcinogenicity but not conclusive in themselves, and negative results do not abrogate need for full long-term bioassay. Examples of these tests are:

1. Strain-A mouse lung tumor bioassay, developed by Stoner and Shimkin (1982), in which high incidence of lung tumor is monitored after carcinogen exposure.
2. Breast cancer development in female Sprague Dawley rats after treatment with carcinogen is observed.
3. Development of hepatomas, adenomas, and gliomas in the liver, lungs, and brain cells of rats or mice is observed in 12 to 18 months.

2) MEDIUM-TERM BIOASSAYS

These assay systems are conducted in rats with either the liver as the target organ or the whole body as the target organ (Shirai et al. 1999). Two assays have been developed that have reduced the time for development of an endpoint. The more intensively used is the Ito model developed by Dr. Nobuyuki Ito and colleagues (Ito et al. 1989, 1998) in Japan. Nodules and focal lesions in the liver of rats, staining for the glutathione S-transferase pi (GST-pi), are the endpoints in this assay and it takes eight weeks to complete. The results have been correlated with-long and medium-term results, indicating usefulness of this assay as a potential surrogate for the chronic bioassay.

The **newborn mouse model** of chemical carcinogenesis, initially described by Shubik and colleagues, has been used extensively in studies of mouse hepatocarcinogenesis and recently (Fujii 1991) used to evaluate the carcinogenic potential of 45 compounds. This assay determines endpoints of formation of neoplasms in various tissues in mice (e.g., liver, lung, and lymphoid organs), within a one-year period. The assay is relatively inexpensive, utilizing small amounts of test material, but needs longer periods to give results.

3) SHORT-TERM MUTAGENICITY TESTING ASSAYS

Short-term assays have been developed since the long-term methods are time consuming and require substantial resources and expensive laboratory animal upkeep. These tests are inexpensive and rapid as screening tools for mutagens and carcinogens. No single short-term test can, however, predict mutagenicity/carcinogenicity of a compound. Batteries of these short-term tests have to therefore, be performed before a compound is labeled as a carcinogen and/or mutagen. The result from a battery of short-term tests is considered equivalent to the predictive value of a long-term animal bioassay.

More than 200 short-term assays have been developed to test for the mutagenicity in bacteria, cultured mammalian cells, insects, and mammals. These assays identify carcinogenic agents by inducing mutations in DNA in vitro or in vivo. The primary use of short-term tests is for mechanistic evaluation, and results have impacted risk assessment.

The most frequently used tests include:

1. Reverse mutation assay (Ames test) using *Salmonella typhimurium*.
2. Forward mutation using lymphoma cells, mammalian cells, and Chinese hamster ovary (CHO) or lung fibroblast cells (V79).
3. In vivo cytogenetic assessment

   Chromosomal aberrations.
   Micronucleus assay.

4. Knockout transgenic mice models for in vivo assays to identify carcinogens.

3.1) Gene Mutational Assays

3.1.1) Bacterial Assays

Bacteria are widely used, both in fundamental studies of the mechanisms involved in the biological responses to DNA damage (mutagenicity) and in the short term-screening tests for potential carcinogens (Venitt et al. 1986). They possess elaborate mechanisms for responding to DNA damage. The damage may be measured by assaying for mutations, for increased killing of DNA repair deficient strains of bacteria, or for induction of lysogenic bacteria.

The bacterial short-term tests consist of two components:

1. The target cell (bacterial strain)
2. The metabolizing system (converts certain compounds to DNA-reactive species) and is supplied exogenously

The bacterial assays are easiest to conduct, rapid, and inexpensive. Effects are assayed by methods that detect forward or reverse mutations.

Forward mutation (from drug sensitivity to drug resistance) has the theoretical advantage of presenting a large genetic target

and should indicate many types of mutagenic effects (including deletions). They, however, fail to detect the increase in the induced frequency of a rare event against a background of common events.

3.1.1.1) Reverse mutation assays. These assays use bacteria that are already mutated at an easily detectable locus (e.g., amino acid autotrophy). The frequency with which the test chemical produces a second mutation, which abolishes the existence of the first mutation, is then determined. Several indicator strains or a single strain with multiple markers may be necessary to overcome mutagen specificity.

Ames Test. It is the most widely used in vitro primary screen for gene mutation, using *Salmonella typhimurium* bacterium, and detects most of the human mutagens and carcinogens (Ames 1971; Ames et al. 1973a, 1973b; Maron and Ames 1983). Testing of chemicals for mutagenicity in this assay is based on the premise that a substance mutagenic to the bacterium is likely to be a carcinogen in laboratory animals, and hence, by extension, presents a risk of cancer.

The test takes about three days and involves the use of several specially constructed strains of *Salmonella typhimurium* (e.g., TA1535, TA1537, TA97/TA97a, TA98, and TA100, TA102, and TA104) (Levin et al. 1982; Venitt et al. 1984; Bonneau et al. 1991; Ohe et al. 1999; Table 2). *Escherichia coli* strains WP2 and WP2*uvr*A have also been used (Barrueco and de la Pena 1988; Wilcox et al. 1990; Ohta et al. 2002; Table 2).

In this assay, bacterial tester strains that are deficient in DNA repair and lack the ability to grow in the absence of histidine are treated with different concentrations of the test compound. The test is conducted both in the presence and absence of the exogenous metabolic activation system. A drug-metabolizing system or the S9 fraction (obtained from the liver of rats after treatment with Arochlor 1254) is added to compounds requiring metabolic activation. Relevant positive and negative controls are also included in the study. These bacteria die when plated on an agar medium lacking histidine. Sometimes, a mutation occurs in a few of these cells that corrects the original mutation, which allows the reverse-mutated cells (revertants) and their progeny to grow on a histidine-deficient agar plate and form a visible colony after 48 h of incubation.

A chemical is positive if it causes a reproducible dose-dependant response in at least one tester strain. If no increase in mutant colonies is seen after testing various strains under different culture conditions, the test chemical is considered to be nonmutagenic in the *Salmonella* test. A positive result in this assay indicates that the substance is a mutagen and could be a carcinogen as well.

Strain engineering has enhanced the sensitivity of Ames assay to mutagens and toward specific classes of chemicals (Josephy et al. 1997; McCann et al. 1975). Plasmids encoding both mutagenesis and metabolic function (e.g., specific enzymes) on a single plasmid provide flexibility to mechanistic studies and allow for identification of mutagenic principles in complex mixtures and metabolic activation mechanisms (Josephy et al. 1997, Mortelmans and Zieger 2000; Suzuki et al. 1998; Carroll et al. 2002), thus providing broad substrate specificity and high mutagenic sensitivity toward chemicals.

3.1.1.2) Forward mutation assay. These assays use development of resistance in otherwise sensitive strains (e.g., *Arabinose resistance in Salmonella typhimurium*), in which forward mutation to L-arabinose resistance is detected (Ruiz Vazquez et al. 1978). Resistance to L-arabinose is mainly a result of forward mutation at any of at least three different genes (*ara* A, *ara* B, or *ara* D) for preceding production of a toxic intermediate L-ribulose-5-phosphate. The test uses a mutated strain of bacteria and detects changes from L-arabinose resistance in a medium containing L-arabinose and glycerol. Mutations in the structural gene for the enzyme L-ribulose5phosphate-4epimerase block the utilization of L-arabinose as a carbon source and also lead to the accumulation of toxic intermediate (i.e., L-ribulose-5-phosphate). Bacterial growth is thus inhibited in the presence of L-arabinose and a carbon source is unable to repress the *araBAD* operon. The assay has been used to study the genotoxic potential of river and tap water (Vahl et al. 1997; Shen et al. 2003).

The *Salmonella typhimurium* BA strain carries the *ara*D531 allele required for the Ara assay and a his auxotrophy (hisD3052 or hisG46) required for the His assay. The induction of forward mutations to L-arabinose resistance (AraR) and of reversions to histidine prototrophy (His+) can be quantitatively compared in these bacteria for mutagenicity of nitro-containing compounds (Jurado et al. 1994).

3.1.2) Mammalian Assays

3.1.2.1) In vitro assays: Two of the in vitro mammalian cell mutation assays that offer attractions over the microbial systems include:

(i) Thymidine kinase (TK) +/− Mouse lymphoma L5178Y assay (Clive et al. 1979)
(ii) Chinese hamster ovary − hypoxanthine guanine phosphoribosyl-transferase (CHO–HGPRT) assay (O'Neill et al. 1977; Li et al. 1987)

Cultures of established cell lines or cell strains can be used in the in vitro mammalian cell gene mutation test. The cells are selected on the basis of their ability to grow in culture and stability of the spontaneous mutation frequency (Aaron et al. 1994). This test is used to screen for possible mammalian mutagens and carcinogens.

These assays use forward mutations at specific loci in the TK or HGPRT gene (Fig. 2) as the endpoint that confers resistance to the toxic chemical (DeMarini et al. 1989). Here, too, phenotypic expression of a mutation in a gene is compared in treated and untreated cells. The damage to a gene results in loss of a phenotype (e.g., growth in presence of toxic compound). Forward mutation to such drugs as 8-azaguanine, 6-thioguanine, 5-bromo-2 deoxyuridine, and ouabain have been well characterized and widely used as genetic markers.

TABLE 2
Various bacterial strains used in the Ames test and their characteristics

Bacterial strain	Target DNA sequence	Target allele	Plasmid and other characteristics	Mutation detected	Primary mutations
S. typhimurium TA 97a	CCCCCC GGGGGG	*his*D6610	pKM101 (Ap^r), *rfa,* Δ*uvr B*	Frameshift	C or G deletion
S. typhimurium TA 98	CGCGCGCG GCGCGCGC	*his*D3052	pKM101 (Ap^r), *rfa,* Δ*uvr B*	Frame shift	GC or CG deletions and Complex frameshift
S. typhimurium TA 100	CCC GGG	*his*G46	pKM101 (Ap^r), *rfa,* Δ*uvr B*	Base pair substitution	GC→AT (ts) AT→ GC (ts) TA →GC (tv)
S. typhimurium TA 102	CAAGTAAGAGC GTTCAT TCTCG	*his*G428	pKM101 (Ap^r) and pAQ1 (Tc^r), *rfa*	Base pair substitution, oxidative and cross-linking mutagens	AT→ CG (tv) AT →TA (tv)
S. typhimurium TA 104	TAA GC ATT and CG	*his*G428	pKM101 (Ap^r), *rfa,* Δ*uvr B*	Base pair substitution oxidative and cross-linking mutagens	GC→ AT (ts), GC→ TA (tv) AT→GC (ts), AT→ CG(tv) AT→TA (tv)
S. typhimurium TA 1535	CC GG	*his*G46	None, *rfa,* Δ*uvrB*	Base pair substitution	GC→AT (ts) AT→GC (ts) TA →GC (tv)
S. typhimurium TA 1537	CCCCC GGGGG	*his*C3076	None, *rfa,* Δ*uvrB*	Frameshift	C or G deletion
E. coli. WP2 uvrA	AT TA	*trp*E	None*, uvr A*	Base pair substitution and cross-linking	GC→ AT (ts), GC→ TA (tv) AT →GC (ts), AT→ CG(tv) AT→TA (tv)
E. coli. WP2 uvrA (pKM101)	AT TA	*trpE*	pKM101, *uvr A*	Base pair substitution and cross-linking	GC→AT (ts), GC→TA (tv) AT→GC (ts), AT→ CG(tv) AT→TA (tv)

ts, transition; tv, transversion; *rfa* increases the permeability of cell wall to large molecules; *uvrB* deletes excision repair; plasmid pKM101 encodes *mucAB* gene that participates in SOS repair system; (Ap^r) ampicillin resistance selection of plasmid; (Tc^r) tetracyclin resistance selection of plasmid.

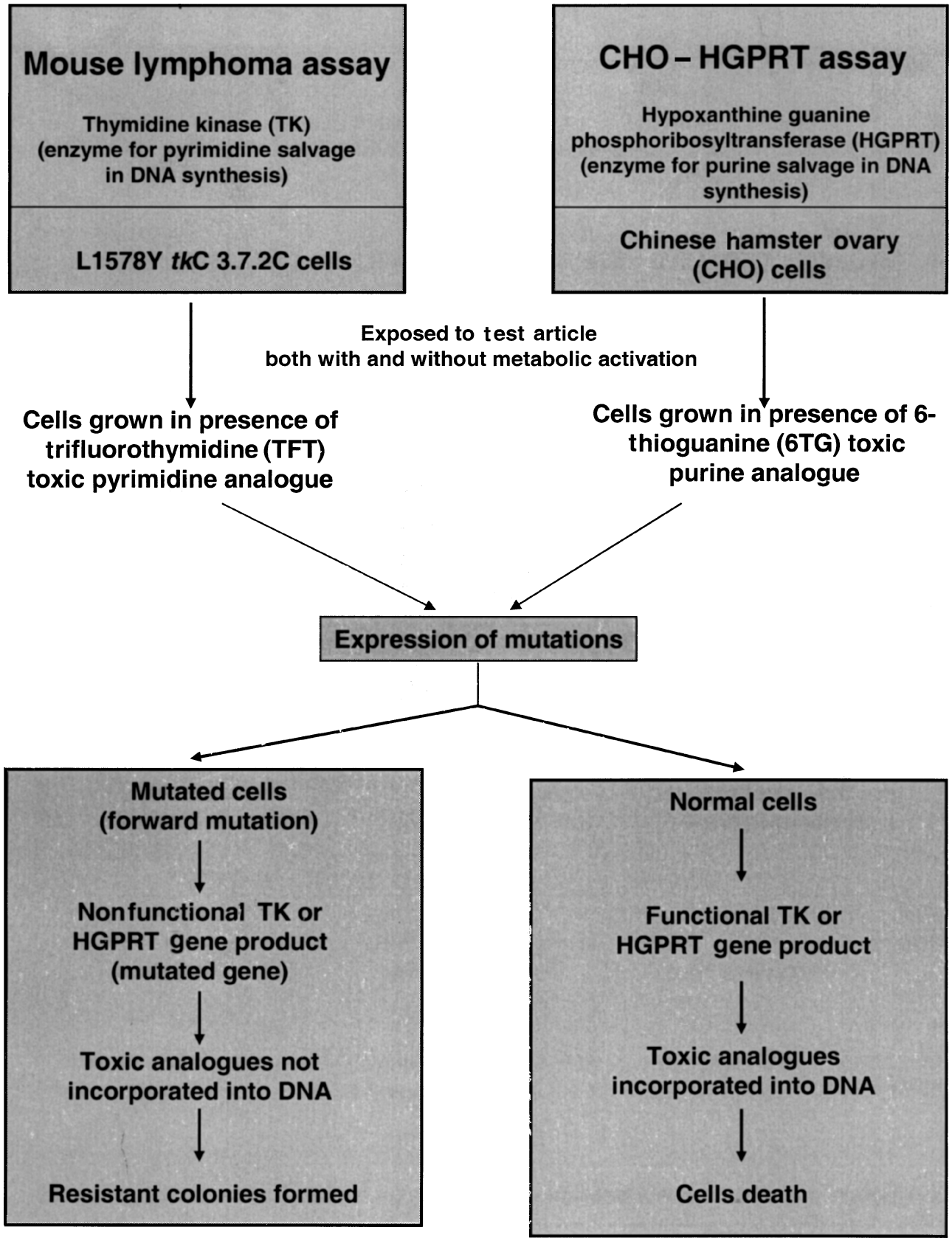

FIG. 2. Schematic representation of the in vitro mammalian cell mutation assays.

Chinese hamster ovary cells and L5178Y mouse lymphoma cells appear to be most suitable because of the relative ease with which mutants can be isolated. These assays detect point mutations involving deletions, base substitutions, frame shift, and rearrangement within the locus.

Cells are propagated from stock cultures, seeded in culture medium, and incubated at 37°C. The proliferating cells are exposed to the test substance, both with and without metabolic activation with the mammalian liver postmitochondrial fraction (S9) usually used at concentrations ranging from 1% to 10% in the final test medium. Concurrent positive and negative (solvent or vehicle) controls, both with and without metabolic activation, are included in each experiment. When metabolic activation is used, the positive control chemical should be one that requires activation to give a mutagenic response. The minimum time requirement to allow near optimal phenotypic expression of newly induced mutants for each locus is different; for example, while TK requires at least two days, HGPRT requires at least 6 to 8 days.

The TK enzyme is required for salvaging pyrimidines. Toxic pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU), and triflurothymidine (TFT), in the presence of TK enzyme, are incorporated into nucleotides, resulting in the inhibition of cellular metabolism and cytotoxicity. Mutant cells deficient in TK enzyme due to forward mutation ($TK^+ \rightarrow TK^-$) are resistant to these analogues and are able to grow, but normal cells are unable to proliferate in the presence of these antimetabolites. Presence of mutant cells shows that test compound is mutagenic (Clements 2000).

The cells (L5178Y *tk*+/−3.7.2C) used for the mouse lymphoma assay (MLA) are heterozygous at the thymidine kinase locus (*Tk1*) on chromosome 11. Inactivating the *tk*+ allele induces trifluorothymidine (TFT) resistance (Moore et al. 2000; Clements 2000), and *tk*−/− mutants can be selected in a background of *tk*+/− nonmutant cells. The test is capable of detecting gene mutation, chromosomal damage, and recombination induced by a wide range of chemicals. It also helps in detecting clastogenic lesions ranging from point mutations to multilocus mutations for agents indicating a mutagenic response in this system. MLA detects a wider range of genetic damage as compared to chromosome aberration analysis and also detects damage in viable cells capable of forming colonies. This is an advantage of MLA, particularly when using it as part of a test battery to provide information on hazard identification (Putman 1984). There are two versions of the assay—soft agar and microwell methods (Honma et al. 1999a; Omori et al. 2002; Moore et al. 2003), both of which have been adopted by ICH guidelines (Mitchell et al. 1997). The standard MLA, and other mammalian cell gene mutation assays have a short treatment period (3 to 6 h). However, short treatments may be insufficient for detecting some clastogens and spindle poisons, because the mutagenicity of these non-DNA targeting chemicals may be cell cycle dependent, and treatment extending over more than one cell cycle may be required to see their effect and for this 24 hour treatment should be used (Honma et al. 1999b, Moore et al. 2002).

The hypoxanthine guanine phosphoribosyltransferase (*hgprt*) locus on X-chromosome is used as the target gene in the CHO-HGPRT assay. HGPRT enzyme is important for purine salvaging and incorporates toxic analogues (e.g., 6-thioguanine or 8-azaguanine) into DNA, resulting in cell death or inhibition of cell growth. A mutation in this gene results in mutated cells with inactive gene product and phenotype loss that permits the survival (colony formation) in the presence of toxic analogues. The Chinese hamster ovary (CHO) assay, which measures newly induced mutations at the *hgprt* locus, has been widely used for mutagenicity testing (Li et al. 1987). The use of suspension cultures and soft agar cloning in the CHO assay provides a sensitive test for the identification of mutagens (Oberly et al. 1990) and is a viable alternative to the traditional monolayer procedure (O'Neill et al. 1977). A modification of this assay permits treatment in suspension and soft agar cloning comparable to the MLA. This methodology eliminates the risk of metabolic cooperation and the trauma of trypsinization. In addition, a larger population of cells can be treated and cloned for mutant selection (Oberly et al. 1993).

Many compounds that are positive in this test are mammalian carcinogens; however, correlation with carcinogenicity is not foolproof. There is increasing evidence that carcinogens acting through other, nongenotoxic mechanisms or mechanisms absent in bacterial cells are not detected by this test.

3.1.2.2) In vivo mutation assays. The analysis of mutations in somatic or germ cells in suitable tissues is carried out after in vivo treatment in rodents.

3.1.2.2.1) Dominant lethal assay. The assay is used to investigate clastogenicity or aneuploidy in germ cells in vivo. The term "dominant lethal" describes embryonic death due to chromosomal breakage in parent germ cells. A dominant lethal mutation is one occurring in a germ cell that does not cause dysfunction of the gamete but that is lethal to the fertilized egg or developing embryo and causes fetal death.

Male rats, treated with test substance, are mated with females, who are then sacrificed at 14 to 16 days of pregnancy, and the live/dead embryos from the implants are counted. The live implants in tested/control groups are compared. The postimplantation loss is calculated by determining the ratio of dead to total implants from the treated and control groups. Preimplantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups (Brewen et al. 1975). These lethal mutations are believed to be primarily due to structural or numerical chromosomal aberrations. Induction of a dominant lethal event after exposure to the test substance indicates that the substance has affected germinal tissues of the test species (Ehling et al. 1978). The working group on mammalian germ tests decided the protocol for the performance of the test and postimplantation loss, preimplantation loss, and fertility rate were the main parameters to be assessed (Adler et al. 1994). Spermatids appeared to be

the germ-cell stage most sensitive to dominant lethal induction (Barnett and Lewis 2003). A schematic method of data presentation was proposed, which enables the most pertinent assay data and parameters to be viewed and considered simultaneously (Ashby and Clapp 1995). N-Hydroxymethylacrylamide (NHMA), a mouse carcinogen, was found to be unique in inducing genetic damage in germ cells but not somatic cells of male mice in this test (Witt et al. 2003).

3.1.2.2.2) Mouse specific locus test. This test is an efficient method for studying the germ cell mutations induced by chemicals (Russell et al. 1981). Parental mice homozygous for wild-type genes are treated with test compounds and mated with a tester stock, which is homozygous recessive at that marker locus. The resultant offspring are expected to be heterozygous for the genotype while expressing the wild-type phenotype. If mutation occurs, the offspring expresses the recessive phenotype. The tester stock is homozygous for seven loci controlling coat color and the size of the external ear, and the recessive phenotypes are easily recognizable, allowing for the simple and rapid screening of F1 offsprings. Spontaneous mutations can be manifested both as whole-body mutants and mosaics (mottled coat color). The mosaics may either be visible in the F1 offsprings or masked, among the wild-type parental generation (Russell and Russell 1996). The test is an efficient germ cell mutation assay in mice due to simple methodology. It detects a wide range of mutations ranging from single base pair substitutions to insertions or deletions (Favor 1999) and intralocus changes to multilocus deletions (Ehling 1989).

3.1.2.2.3) Mouse spot test. The mouse spot test for the coat color variation is widely used for the detection of a number of genetic alterations, ranging from chromosomal damage to gene mutations (Styles and Penman 1985), which result in the loss of the heterozygosity of the loci controlling hair color. The procedure is inexpensive and the test is rapid for screening somatic cell mutations in mice in vivo. Mouse embryos heterozygous for recessive coat pigmentation are treated with the compound *in utero*. Treatment is given at a time when pigment precursor cells migrate to the dermis. Any alteration or loss of allele at one of the heterozygous coat color loci in one of the melanocyte precursor cells leads to the expression of the recessive allele. Mutant clone is observed when the offspring are two weeks old and is recognized by the colored patches (brown, grey, or yellow) of hair. Mouse assays detecting genetic instability at endogenous loci predict hazardous effects of an environmental agent and/or genetic predisposition to cancer (Reliene and Schiestl 2003). The disadvantage of breeding tests is that they cannot confirm the altered clone.

3.1.2.2.4) TK+/− and HGPRT in mice, rat, and human cells. The development of the hypoxanthine guanine phosphoribosyltransferase (HGPRT) assay employing human peripheral blood lymphocytes has contributed significantly to the understanding of in vivo somatic cell mutagenesis (Albertini 1985). HGPRT is finding use in in vivo studies for mutations arising in either somatic or germinal cells (Albertini 2001). Studies of thioguanine-resistant colonies show that the mutants are deficient in HGPRT and have structural alterations in the *hgprt* gene (Nicklas et al. 1988). These cells are either T-helper or T-suppressor cells (Albertini 2000). The mutant frequencies (MFs) of *hgprt* are increased in peripheral blood T-cells from melanoma patients when compared to normal controls (Albertini et al. 2001).

The rat HGPRT assay was developed with lymphocytes derived from the spleen, providing the opportunity for studying the in vivo mutations in endogenous genes, and it serves as a model for widely used human lymphocyte HGPRT assay (Aidoo et al. 1997; Casciano et al. 1999). The assay using rodents has the advantage that they are commonly used in toxicological studies and are readily available as inbred and hybrid strains, making the use of the rodent HGPRT assay desirable. Effects in the *hgprt* lymphocyte mutations due to caloric restriction has shown reduction in accumulation of spontaneous somatic cell mutation in aging rats, especially those caused by base substitutions and frameshifts hence leading to decreased tumor incidence and retardation in aging (Aidoo et al. 2003). In mice, an age-related study showed variation in the patterns of appearance and frequency of *hgprt* mutant T lymphocytes in the thymus and spleen at various periods of growth (i.e., 12-days, 22-days, and 8-weeks) (Walker et al. 1999).

3.1.2.3) Transgenic systems for in vivo mutagenesis. In vivo mutation system has been developed in transgenic mice (Provost et al. 1993). The advent of transgenic animal model with recoverable system genes has provided the opportunity to directly and efficiently measure spontaneous and chemically induced mutation in different tissue in vivo (Mirsalis et al. 1995). The transgenic mouse mutation assays began with two transgenic mice named Muta Mouse and Big Blue Mouse (Kohler et al. 1990; Nohmi et al. 2000). Both Muta Mouse and Big Blue Mouse systems are lambda phage-based and employ bacterial *lacZ* gene or *lacI* gene, respectively, as a reporter gene for mutations. In the *lacZ* system (Muta Mouse), colorless or pale blue plaques are judged as mutants in the blue plaques' background, whereas blue plaques are judged as mutants in the colorless background in the *lacI* system (Big Blue Mouse; Gossen et al. 1994; Fig. 3).

In the *LacI* system two transgenic C57BL/6 and B6C3F1 mice have been constructed, with each cell containing multiple copies of a bacteriophage lambda shuttle vector at an identical chromosomal integrating site. These vectors contain a *lacI* gene as a target for mutagenesis. The mice are exposed to the test compound and then DNA is extracted from the tissue of interest. The shuttle vector is recovered from genomic DNA by in vitro packaging and infected into *E. coli* cells, which are then plated onto dishes to form plaques or colonies. The mutated plaques are selected on the basis of their ability to cleave a chromogenic substrate. A mutation in the *lacI* gene inactivates the *Lac* repressor protein and allows functional expression of β-galactosidase enzyme. X Gal, a chromogenic substrate, is cleaved by this β-galactosidase from mutant plaques, giving blue color, while normal plaques appear colorless. The principle

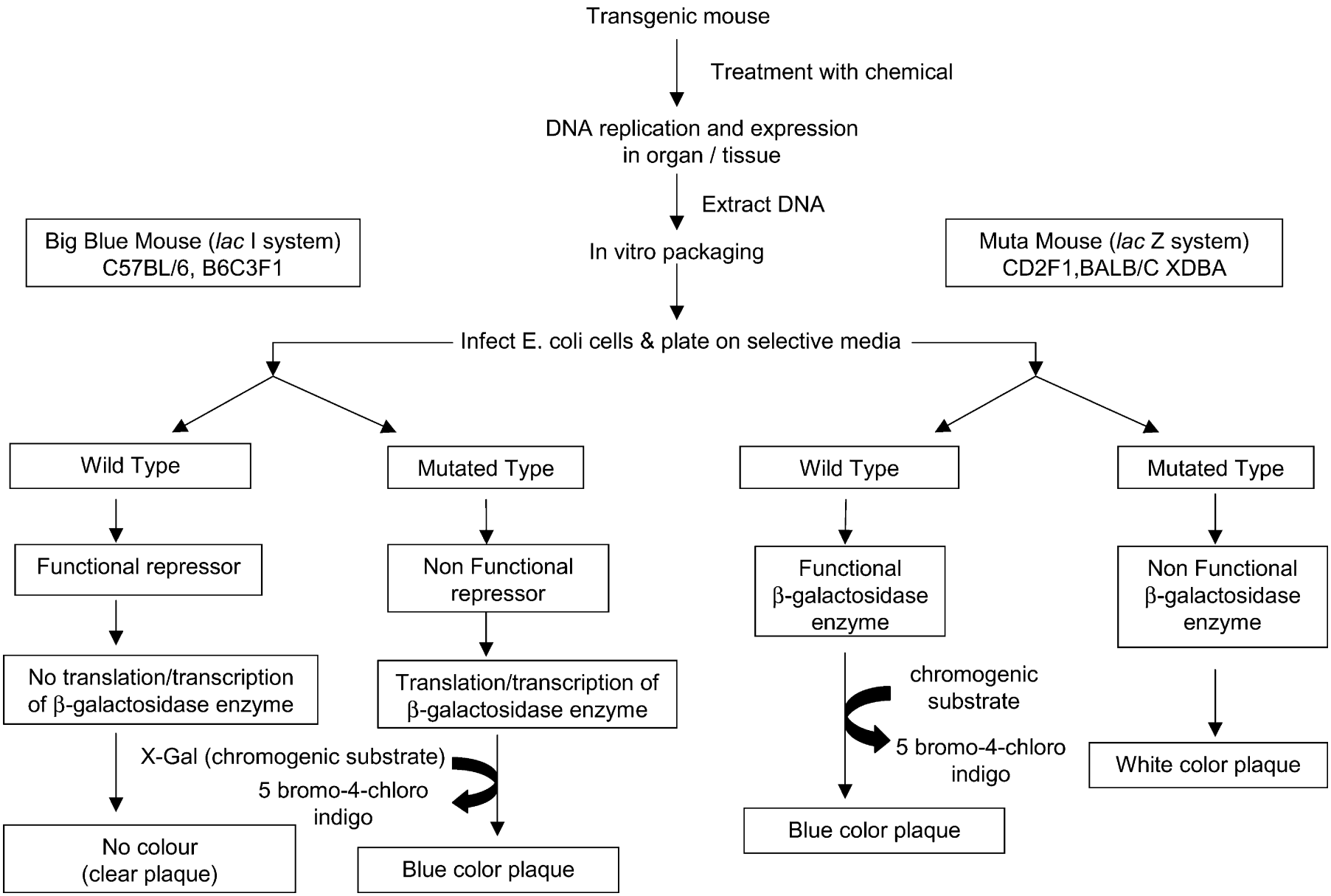

FIG. 3. Sequence of steps in the assessment of mutagenicity of chemicals in trasnsgenic rodent assay in vivo.

of colorimetric detection of mutants is also followed in the *lacZ* system; however, in this system, β-galactosidase enzymes are active in the wild type.

The transgenic mouse mutation system is highly adaptable to screening for mutagenic potential and provides a relatively quick and statistically reliable assay for gene mutations in the DNA from any tissue or organ (Thybaud et al. 2003). A significant increase in the mutant frequencies gives a positive response for the test article. Also, mutations induced in the parent germ cells are transmissible to the F1 offspring (Barnett et al. 2002). Other systems, which have been developed for specific genetic alterations to make them more suitable to carcinogenesis by external agents, include p53 $+/-$ deficient model, TG-AC model, and *Tg-Hras*2 model (Fig. 4).

The TG.AC mouse carries a v-Ha-ras oncogene fused to the zeta-globin gene promoter. The model was developed by Leder et al. (1990). The v-Ha-ras transgene has point mutations at codons 12 and 59, and the site of integration of the transgene allows these mice to show genetically initiated skin as a target for tumorigenesis. The untreated skin of these transgenic mice does not differ from the wild type, but repeated application of the skin tumor-promoting agent leads to the development of benign papillomas, which may become malignant. This model has been used for the study and differentiation of initiating agents, tumor-promoting agents, and complete carcinogens. Specific activation of the expression of the transgene is the critical event in the responses of the Tg.AC model (Cannon et al. 1997).

Heterozygous p53 $+/-$ deficient mice are used in the short-term (26 weeks) assay for the identification of mutagenic carcinogens. These mice have a single functional allele and an inactivated null allele of the p53 gene. The inactivation of the wild-type functional allele due to mutation/loss on carcinogen exposure leads to development of neoplasias. Hence, these heterozygous mice are viable, having low background incidence of tumors (Tennant 1995), but on becoming nullizygous, they develop high incidence of spontaneous tumors in 3 to 6 months. Studies in these mice have shown that potent carcinogens can increase loss of heterozygosity in heterozygous p53-deficient mice.

The *TgHras2* mice carry a human proto-oncogene c-H-*ras* and have an endogenous promoter for the c-H-*ras* gene (Yamamoto et al. 1997). The transgene is overexpressed in normal tissues and tumors (e.g., papillomas and lymphomas) that develop in the animal. It has been used to study the effect of carcinogens in Japan (Yamamoto et al. 1998).

3.2) Assays for Chromosomal Abnormalities

The simplest and most sensitive assays for investigating clastogenic effects of chemicals are those using cytogenetics in both in vivo and in vitro test systems. These assays use microscopy for direct visualization of the deleterious effects at the genetic level. Clastogenicity in bone marrow cells of rodents dosed with the suspect chemical can be studied either by analyzing chromosomal aberrations in metaphase cells or micronucleus formation. Both of these can also be used as tests for aneuploidy (numerical changes in chromosomes). Cultured mammalian cells are the most often used for in vitro studies.

The tests for chromosomal abnormalities include:

3.2.1) Mitotic Recombination Assay

This assay is used to measure mitotic recombination, that is, gene conversion or crossing over in yeast *Saccharomyces cerevisiae*, a eukaryotic unicellular fungus. There are two types of recombination; a) the classical mitotic crossing over where exchanges of DNA segments between genes occur and b) the mitotic gene conversion where unilateral DNA transfer within a gene occurs (Zimmermann 1984). It has been shown that both double-strand breaks and transcription leads to mitotic recombination in *Saccharomyces cerevisiae* (Gonzalez-Barrera et al. 2002) and recombination only by crossover or only by gene conversion has been studied (Freedman and Jinks-Robertson 2002). Yeast cells carry out recombinational repair of the single-strand breaks and a systematic study of mitotic recombination in *Saccharomyces cerevisiae* has been done (Aylon et al. 2003).

Strains of *Saccharomyces cerevisiae* have been developed, which detect such events by production of homozygous alleles from heterozygous alleles. These recombinations are essentially DNA exchanges between segments of homologous chromatids. This assay is indicative of nonspecific DNA damage. Mutagens are given in solvents with or without metabolic activation. The colonies are then counted on nonselective or selective medium after 4 to 6 days. Two statistical analyses for calculating the recombination rate have been described and their application has been studied using an intron-based inverted repeat recombination reporter system for the regulation of homologous recombination (Spell and Jinks-Robertson 2004).

3.2.2) Chromosomal Aberration

Cytogenetic assay for clastogenicity using metaphase analysis is one of the currently recommended in vitro tests for mutagenic properties of a substance. Chemical compounds that induce structural chromosomal aberrations are designated as "clastogens." These compounds give rise to primary DNA lesions, some of which are further processed during the replication and/or DNA repair into secondary lesions, which then develop into the various types of chromosomal aberrations visible in the metaphase cells.

The classification of chromosomal aberrations (CAs) is based on the structural unit involved, that is, whole chromosome or the single chromatid and the type of morphological alteration, that is, break (deletion/fragments) or rearrangements (inversion/translocation and exchanges between chromatids or chromosomes). Aberrations in the unreplicated G1 chromosome are duplicated in S phase and appear as chromosome type aberrations, while those formed after DNA replication (i.e., during or after S phase) the involve only one chromatid and appear as chromatid type aberration (Fig. 5). The majority of the chemical clastogens are S phase dependant and induce aberrations only

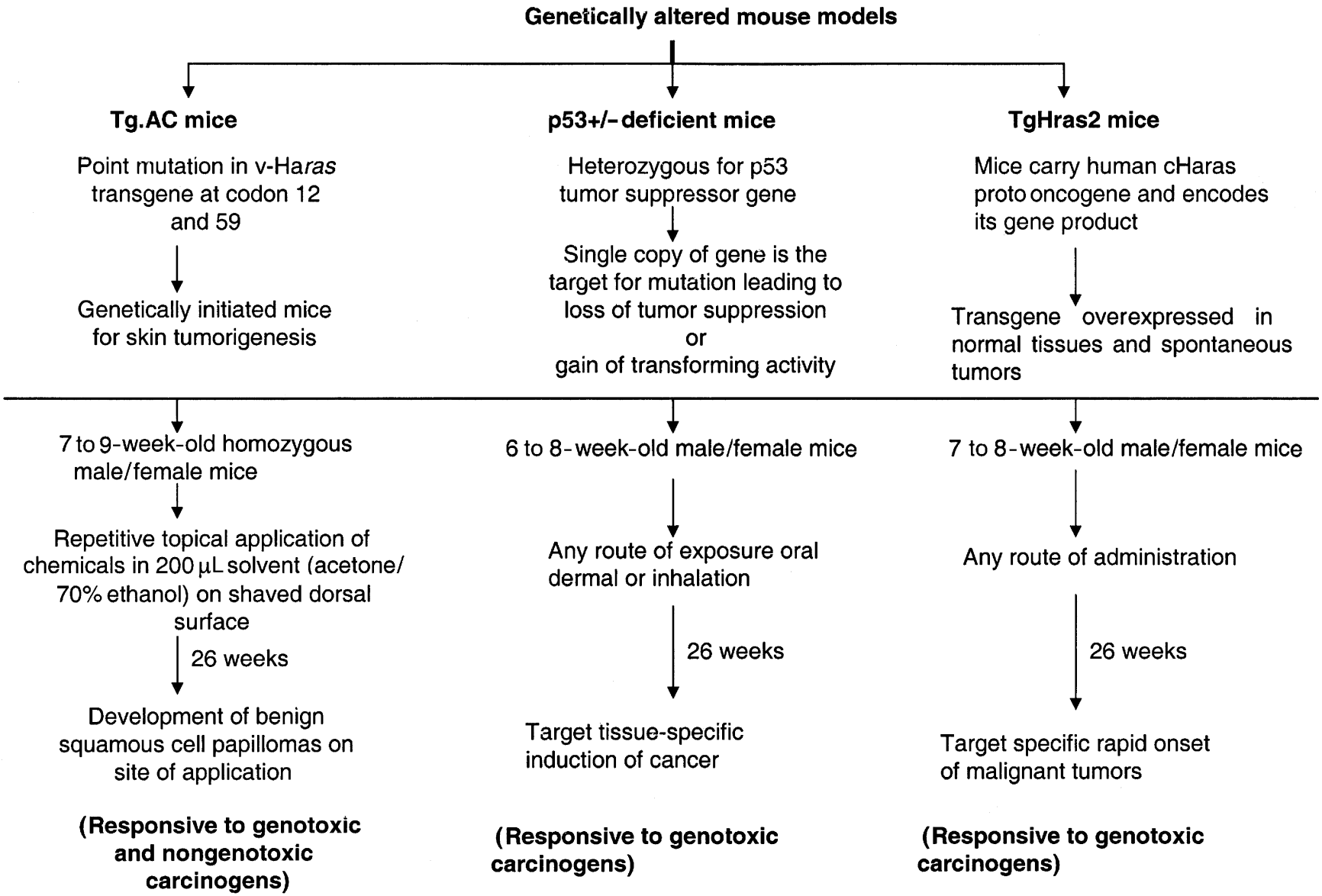

FIG. 4. Transgenic animals for development of short term assays in vivo.

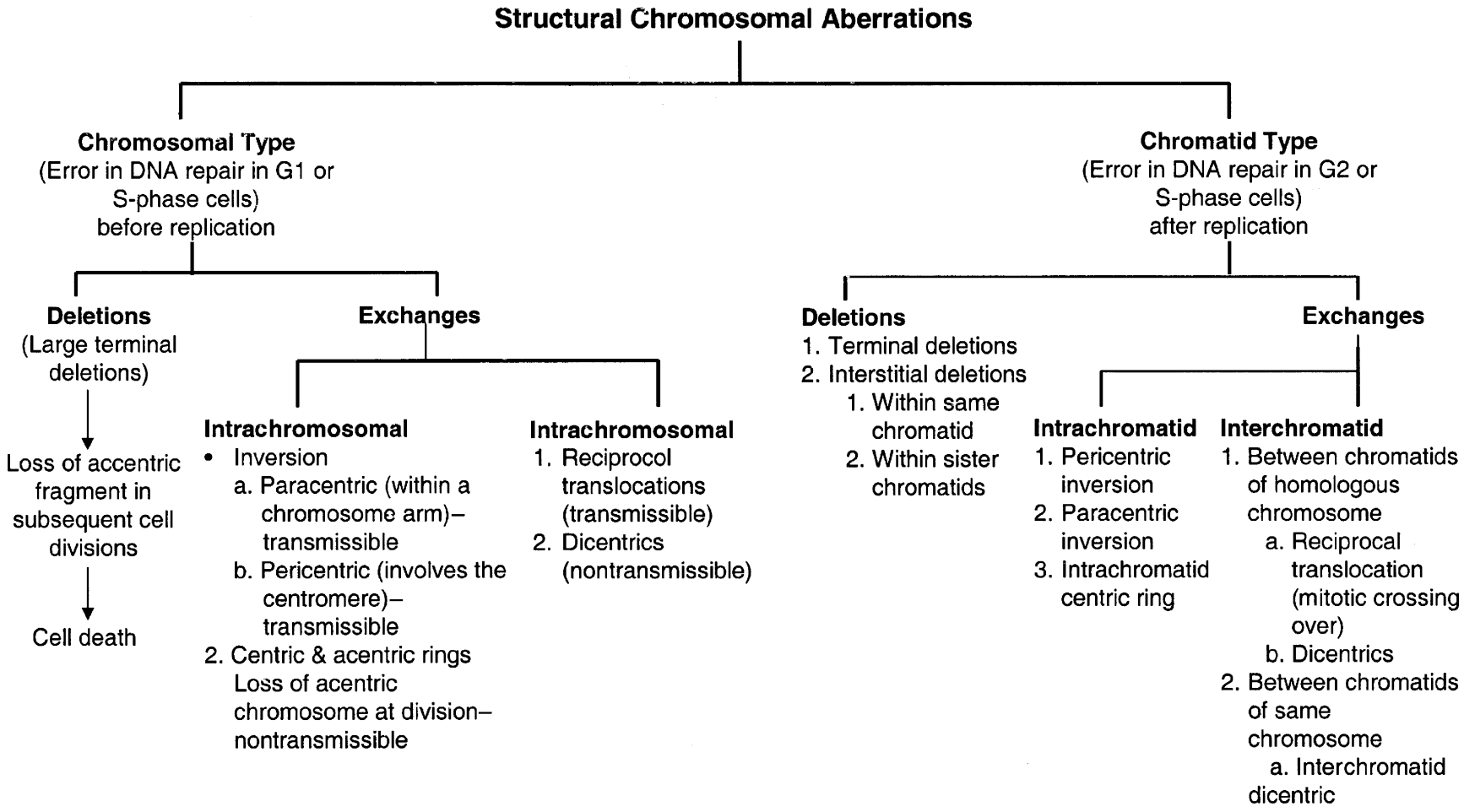

FIG. 5. Types of structural chromosomal aberrations.

in cells that pass S phase after exposure. Thus, it is not the type of primary DNA lesions, but the processing of the lesion in S phase that leads to aberration. Yield of CA is often higher when cells are exposed to a clastogen in early S phase. This is probably due to the limited time available for the error-free repair in the primary DNA lesion in the cell. The majority of cells with aberrations in metaphase may not survive cell division. Unbalanced chromatid type aberrations are frequently lethal because both chromatids are affected and both daughter cells will suffer from structural chromosome damage.

Cytogenetic alterations are involved in apoptosis and cell proliferation of normal and cancer cells. These include shortening of telomeres, formation of dicentric chromosomes, transfer of telomeric DNA to the homologous chromosomes, malfunction (inactivation) of the centromeres, endoreduplication of chromosomes, and other structural and numerical chromosome abnormalities (Pathak et al. 1994).

A key factor in the design for a cytogenetic assay is obtaining appropriate cell population for treatment and analysis. Cells with stable, well-defined karyotype, a short generation time, low chromosome number, and large chromosomes are ideal for cytogenetic analysis. CHO cells and human cells, especially peripheral lymphocytes, have been used extensively for in vitro testing (Evans 1984; Preston et al. 1987a; Galloway et al. 1994). These tests require cells to be treated during a sensitive period of cell cycle (e.g., 'S' phase and aberrations scored in the first mitotic division) to prevent loss of any unstable aberration. Interpretation of the assay for CAs in cell cultures may be affected by artifacts obtained due to extreme culture conditions (Scott et al. 1991). Assays at highly cytotoxic doses and extreme pH and metabolic activation system also may be genotoxic and give false-positive results. The study should be, therefore, extended until at least some cytotoxicity is observed (Galloway 2000).

In vivo CA involves treating of the animals and then collecting cells for cytogenetic analysis (Tice et al. 1994). A large number of dividing cells from tissues (e.g., bone marrow from mice and hamsters) can be collected. Animals are treated with a metaphase arresting compound two hours before sacrifice (e.g., colchicine) (Preston et al. 1987b). CA has also been used in human biomonitoring studies (Au et al. 2001; Au 2003).

This analysis is time consuming and hence, a new cytogenetic assay has been developed using fluorescent in situ hybridization (FISH) (Johnson et al. 1998) or chromosome painting to highlight the alterations in the number of copies of selected chromosomes (Natarajan 2001). FISH has found applications in cancer research. It gives increased resolution of the detection of aberrations and leads to a better understanding on the mechanisms of formation of chromosome aberration (Natarajan and Boei 2003). These methods are very useful for analysis of the development and spread of certain tumors and are also efficient tools for tumor diagnosis. Specific chromosomal translocations by FISH painting in mouse plamacytoma (MPC), human Burkitt lymphoma (BL), other B-cell derived tumors have been shown (Szeles 2002). Spectral karyotyping and multicolor or multiplex-fluorescence in situ hybridization allow for visualization of all chromosomes of a metaphase in a single hybridization step, which enables the screening for the aberrations and comprehensive analysis of complex karyotypes (Tchinda et al. 2003; Brizard et al. 2004).

3.2.3) Sister Chromatid Exchange

Sister chromatid exchange (SCE) represents a symmetrical exchange in chromatid segments within a single chromosome. SCEs are the end-products of homologous recombination (Helleday 2003). Such an exchange does not produce altered chromosome morphology, and SCE is not detectable unless the sister chromatids are differentially stained or labeled (Tucker et al. 1993). The points of exchange are cytotaxically at the same locus, but precision of exchange at molecular levels is not known. SCE *per se* is not lethal or cytotoxic but has been extensively used as an endpoint in cytogenetic/mutagenicity testing.

The detection of SCE involves pretreatment of cells with agents that are incorporated into the DNA and induce SCE, that is, bromodeoxyuridine (BrdU) and H^3-thymidine. Treatment is given in presence or absence of S9 metabolic activation systems.

A large number of chemicals have been shown to increase SCE frequency in animal/human cells in vivo (Rodriguez-Reyes and Morales-Ramirez 2003) as well as in vitro. A great majority of these compounds also give rise to DNA and chromosomal damage and gene mutation. Alkylating agents and other electrophilic compounds are inducers of SCE (Latt 1974). It is an S-phase dependant event; hence, agents increase SCE only if cells are allowed to pass through S phase after exposure. Treatment of cells in early S phase causes greater increase in SCE frequency than treatment in G1 or late S. The observations suggest that SCE-inducing damage can be removed before S phase and that formation of SCE is closely linked to the replication process. The removal of SCE-inducing damage is seen by a reduction in SCE frequency with time between exposure and sampling of cells for analysis in vivo. Thus, these times are important determinants in final yield of SCE.

In vivo, in vitro, and ex vivo SCEs can be performed (Perry and Evans 1975). Thus, lymphocytes from animals or humans can be removed from exposed population and cultured in presence of BrdU to analyze the cells in exposed population (in vivo-in vitro SCE). Cell lines can also be used for in vitro SCE (Sayed et al. 2002), while in vivo SCE is performed on animals implanted with BrdU tablets or given continuous infusion of BrdU.

Many SCE-inducing agents are mutagens. The majority of induced SCEs do not represent recombinational/mutational events, but some fraction of exchanges occur at nonhomologous loci, and these unequal SCEs could represent mutational events or chromosomal deletion or involvement in DNA amplification.

3.2.4) Micronucleus Formation

Micronucleus (MN) is the chromatin-containing body that represents chromosomal fragments or whole chromosomes not

incorporated in the nucleus during mitosis (Schmid 1975). The MN assay can be conducted both in vitro and in vivo for detection of both aneuploidy and clastogenicity (Fig. 6). The formation of micronuclei in peripheral blood lymphocytes is a valuable cytogenetic biomarker in human populations occupationally exposed to genotoxic compounds (Bolognesi et al. 2004). It is widely used in human biomonitoring studies (Majer et al. 2001; Neri et al. 2003).

In vivo micronucleus (MacGregor et al. 1987) assay is most often done in the erythrocytes from bone marrow of rodents, and a large number of chemicals have been studied using this method for the assessment of genotoxicity of chemicals, including clastogens and spindle poisons (Sato and Tomita 2001). The in vivo micronucleus assay using other tissues include liver (Igarashi and Shimada 1997; Müller-Tegethoff et al. 1997) and colon epithelium (Ohyama and Tokomitsu 1996). The single dose rat bone marrow micronucleus assay has enhanced sensitivity, and the rodent bone marrow MN assay is used as a predictor of possible germ cell mutagenicity of chemicals (Tinwell et al. 2001). Germ cell MN in rodent spermatids permits the analysis of chromosomal damage during meiosis. The in vivo MN test has been combined with FISH to discriminate MN of clastogenic and aneugenic origin (Attia et al. 2003).

The cytokinesis-blocked MN assay (CBMN) is the most improved and widely used assay for genotoxicity assessment (Fenech and Morley 1985) and human biomonitoring (Fenech 2002). In this assay, Cytochalasin-B is used to arrest the cell cycle at the cytoplasm division (cytokinesis). Hence, bi- and multinucleate cells are obtained and the MN counted in binucleate cells. The CBMN assay also allows nucleoplasmic bridges (NPBs) to be measured, which originate from dicentric chromosomes and centric ring chromosomes during anaphase (Thomas et al. 2003). These are structural rearrangements of DNA involving only one or two homologous chromosomes. NPB measurement can be achieved in CBMN assay, because inhibition of cytokinesis prevents the loss of NPB that would occur if cells were allowed to divide. Inclusion of NPB in the CBMN assay provides a valuable measure of chromosome breakage/rearrangement that would not be available in the micronucleus assay. The MN can also be used along with Kinetochore and centromeric staining to identify the nature of any micronuclei formed (Natarajan 1993; Lynch and Parry 1993; Dhawan et al. 2003). This enables one to categorize the test compound as a clastogen or aneugen (Parry et al. 2002). Induction of micronuclei is related either to dysfunction of spindle apparatus or formation of acentric fragments and is greatly influenced by the time of treatment and sampling in relation to the cell cycle. As micronuclei are detected in the interphase cells, it is essential that the cells in the analyzed population be allowed to divide between treatment and analysis. The majority of clastogens give rise to lesions during G1 and S phases, leading to formation of micronuclei in subsequent cell cycles. Thus, one cell cycle is allowed between treatments and harvesting of cells.

At shorter sample intervals micronuclei may escape detection, while longer sampling time may lead to loss or dilution of micronucleated cells. Micronuclei induced by dysfunction of spindle or S phase independent clastogen can be seen at shorter sampling intervals than S phase dependant chemicals.

3.2.5) Mouse Heritable Translocation Test

It is an in vivo test, that detects structural and numerical chromosome changes in mammalian germ cells as recovered in first-generation progeny (Kirsch-Volders et al. 2000, 2003). This test along with the dominant lethality test, is used to predict an environmental mutagen to mammalian germ cells. It detects reciprocal translocation in chromosomes (Generoso et al. 1980). Reciprocal translocation results from breaks in two nonhomologous chromosomes and the mutual exchange through reunion of the fragments. This does not alter the polarity of the exchanged material or the number of chromosomes. A mechanism by which dominant-lethal mutations and heritable translocations are produced following chemical treatment of male postmeiotic germ cells was proposed, which showed that induction of dominant-lethal mutations and heritable translocations is dependent on the stage at which the germ cell is treated (Generoso et al. 1982; Adler 2000). Several mutagenesis protocols can be used to generate mouse mutations with high efficiency and highlight the unique value of reciprocal translocations as tools for gaining access to the biological functions of mammalian genes (Stubbs et al. 1997).

Carriers of the translocation show reduced fertility, which is used to select F1 progeny for cytogenetic analyses. The translocations are cytogenetically observed in meiotic cells at diakinesis-metaphase I stage of male individuals. There may also be a loss of X chromosomes in females; hence, XO of females are cytogenetically identified by presence of 39 chromosomes in bone marrow.

3.3) Tests for Primary DNA Damage

The measurement of DNA damage and repair induced by exogenous chemicals has been used in short-term tests for potential carcinogenicity. These tests utilize nonreplicative DNA synthesis and involve the measurement of DNA strand breakage by alkaline elution method, detection of DNA adducts through ^{32}P-postlabeling, and the comet assay.

3.3.1) Unscheduled DNA Synthesis (UDS)

DNA lesions can be detected by means of repair mechanisms such as excision repair. DNA repair synthesis after excision of a damaged segment of the DNA in mammalian cells can be detected by autoradiography, after incorporation of H^3-thimidine, as unscheduled DNA synthesis or UDS, that is, DNA synthesis outside the S-phase of the cell cycle, using the opposite strand as template (Madle et al. 1994). There is a distinction between the heavy nuclear labeling of replicative DNA strands and light nuclear labeling of the H3-thymidine incorporation during repair. Tests employing UDS are conducted in primary rat hepatocytes

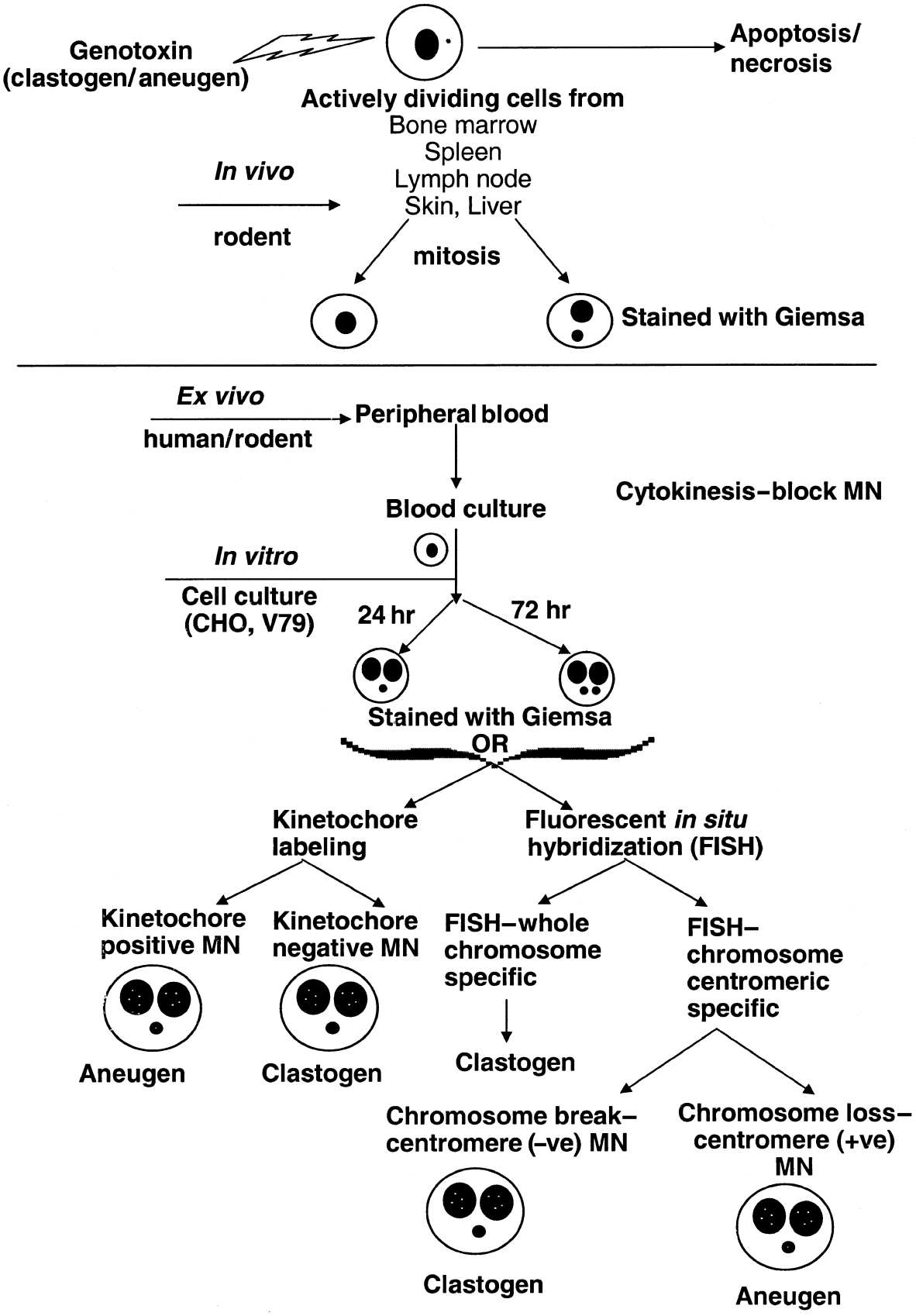

FIG. 6. Schematic representation of the micronucleus (MN) assay.

and human diploid fibroblasts. The in vitro UDS is carried out on the primary rat hepatocytes, which are sensitive to the effect of DNA-damaging chemicals. These cells do not require metabolic activation and are capable of metabolizing procarcinogens to the active carcinogen (McGregor and Anderson 1999). Since these cells are epithelial in origin, monitoring of genetic damage is relevant to the in vivo situation. The UDS has also been done in male germ cells (spermatogonia, meiotic spermatocytes, and early spermatid stages), and its correlation with other heritable mutation tests indicates that the in vivo/in vitro spermatocyte DNA repair assay is useful in predicting the mutagenic potential of chemicals in male germ cells (Bentley and Working 1988; Inoue et al. 1993; Sotomayor et al. 1996, 1999; Sotomayor and Sega 2000).

Ex vivo UDS assay is designed for the complex patterns of metabolic activation, detoxification uptake distribution, and excretion of chemicals (Mirsalis and Butterworth 1980). The hepatocytes from rats are isolated at two points, that is, 2 to 4 h and 12 to 16 h after dosage of test article given in vivo. These isolated hepatocytes are then made to undergo in vitro UDS. The detailed procedure has been reviewed (Casciano 2000). The positive results from UDS performed in vivo or in vitro are highly predictive of rodent carcinogenicity. The epidemiology of DNA repair capacity and its effect on cancer susceptibility in humans is an important area of investigation (Berwick and Vineis 2000). UDS in human leukocytes has become a popular tool for estimating an individual's DNA-repair capacity and sensitivity to mutagens (Pero and Ostlund 1980). Nucleotide excision repair (NER) is differentially expressed in human tissues. A unique culture system to measure the NER capacity in primary cultures of lymphocytes and foreskin fibroblasts using the UDS functional assay has been carried out to study the baseline DNA repair in normal human cell types, which forms a basis for evaluation of the effects of "mutator" genes as etiological factors in the development of cancer (Latimer et al. 2003).

3.3.2) Single Cell Gel Electrophoresis/Comet Assay

Conventional cytogenetics techniques are time consuming and cumbersome, and require pooled cell population. Ostling and Johanson (1984) developed a microgel electrophoretic technique to assess quantitatively and qualitatively the DNA damage in individual cells, which after a few modifications, (Olive et al. 1990; Singh et al. 1991) has now become the state-of-the-art technique known as single cell gel electrophoresis (SCGE)/comet assay. The SCGE/comet assay has gained popularity during the last decade as a short-term test to measure the level of DNA damage in terms of strand breaks and alkaline labile sites due to environmental chemicals. It is a rapid, sensitive, reliable, inexpensive, noninvasive, and visual technique for the detection of DNA damage in individual cells, requiring very low amounts of both test material and target cell population. Its ability to evaluate DNA damage in nonproliferating cells makes it a useful tool to work on any eukaryotic cell.

The principle of the assay is to lyse cells embedded in agarose on a microscope slide and subject the DNA to unwinding and electrophoresis under neutral or highly alkaline conditions (pH $>$ 13). The migrated DNA after neutralization is stained with appropriate dye (e.g., Ethidium Bromide, Propidium Iodide, YOYO-1, Hoescht 33258, DAPI, and Acridine Orange) and visualized under a fluorescent microscope. Using a software, both qualitative and quantitative assessment of DNA damage is carried out by counting 50 to 100 cells per dose/individual. There are several parameters monitored by the software; however, the most frequently used are tail DNA (%), tail length (μm), and tail moment (arbitrary units).

The assay can be performed both in vivo and in vitro (Tice et al. 2000). A variety of samples, obtained as a single cell suspension can be used for Comet assay (Anderson and Plewa 1998). Peripheral blood lymphocytes, nasal and buccal epithelial cells have extensively been used to assess human genotoxicity in clinically or occupationally exposed population (Valverde et al. 1997). In vivo assay using different tissues and organs from mice has also been used (Sasaki et al. 2000). In vitro studies have also been conducted in cell lines (e.g., CHO, V79) and primary cell cultures. Both DNA damage and repair studies can be conducted. Also, a variety of information related to genetic toxicology (Dhawan et al. 2002), human epidemiology (Dhawan et al. 2001; Bajpayee et al. 2002), monitoring of human genotoxicity (Kassie et al. 2000; Palus et al. 2003; Basaran et al. 2003; Piprakis et al. 2003), patients undergoing radio/chemotherapy (Vaghef et al. 1997), and aging (Piperakis et al. 1998; Singh et al. 2003) can be obtained. Also, monitoring dietary factors in various diseases such as diabetes (Raslova et al. 2000; Pitozzi et al. 2003), and thalassemia (Anderson et al. 2001; Ruf et al. 2003) have been carried out using this assay. The assay has been used for environmental biomonitoring and has utilized earthworms, fish, and molluscs exposed to polluted environments (Tice 1995; Akcha et al. 2003).

Besides giving information about the single- and double-strand breaks, it has been used to study oxidative damage (Giovannelli et al. 2003), incomplete excision repair sites (Green et al. 1992), and repair in the extract of lymphocytes (Collins and Harrington 2002). Discrimination of necrosis and apoptosis has also been carried out (Fairbairn et al. 1995). The comet assay has also been coupled with fluorescent in situ hybridization (Comet-FISH) for evaluating DNA-specific damage (Santos et al. 1997; Rapp et al. 2000; McKenna et al. 2003).

Although the comet assay is a useful technique, the single cell data (rate limiting), small cell sample (sample bias), sensitivity (technical variability), and interpretation are some of its disadvantages.

3.4) Cell Transformation Assays

Cell transformation can be defined as the induction in cultured cells of certain phenotypic alterations that are related to neoplasia (Barret et al. 1986; LeBoeuf et al. 1999). The assay is based on the assumption that neoplastic transformation in the cultured

cells occurs by the same mechanism as neoplastic alterations of cells in vivo. These assay systems are used to study the multistage process of neoplastic transformation and the mechanism of action of carcinogens. Both genotoxic and nongenotoxic compounds can be assessed in these systems (Yamasaki et al. 1996).

Mammalian cell culture (in vitro) transformation assays are important short-term tests for potential carcinogens. The endpoint scored is morphological transformation. With fibroblast cultures, the cells start showing loss of growth inhibition and cell-to-cell orientation, and subsequent passages of these cells result in other characteristics (e.g., growth in semisolid medium and capacity to produce tumors in immunosuppressed animals). Two cell systems frequently used in this assay are:

3.4.1) SHE Cell System

The first cell transformation system, using Syrian hamster embryo (SHE) cells for detecting carcinogens, was developed by Berwald and Sachs (1965).

Primary cultures of SHE cells from hamster fetus at 12 to 14 days of gestation are used to test the carcinogenic potential of chemicals. The embryos, after trypsinisation and washing under sterile condition, are plated into cultured flask and grown for 24 hours. These target cells are plated onto culture dishes containing X-irradiated feeder cells (viable cells unable to replicate), which support the growth of the cells. The cells are again grown for 24 hours so as to obtain 25 to 45 colonies per dish. SHE cells are treated with test chemicals for 24 hours or for the entire period of the study. The cells are refed the culture medium after removing the test chemical and left for 6 to 7 days. The colonies formed are fixed with methanol and stained with Giemsa (Barrett et al. 1986).

The advantage of this assay is that it is the only transformation system that employs normal diploid cells as target cells, and these cells have a wider range of metabolic capabilities than other cell lines. The assay is rapid, and scoring for transformed cells is possible within 10 days after plating. Also, SHE cells appear to contain activation systems for the metabolism of a wide spectrum of carcinogens to their active form, and thus it does not require any exogenous metabolic activation.

The early passages of cells are used for the study, but to reduce culture variations and cell variations from different embryos, cryopreserved SHE cells are preferred. For this, primary cultures are screened for susceptibility to transformation by standard compounds, and then highly susceptible batches of the cells are cryopreserved in liquid nitrogen to be used later. It has been used for assessment of carcinogenic potential of chemical (Yamaguchi and Tsutsui 2003), physical, and biological agents (Isfort 2000; Engelhardt 2004). Alkylating agents, polyaromatic hydrocarbons, nitrosamines and amides, aromatic amines, etc., have been tested for their ability to produce morphological transformation. This system has also been extended to include methods for metabolic activation of indirect carcinogens.

Normal clones have cells growing in a monolayer in an organized pattern and minimal cell stacking at confluency. Morphologically transformed colonies contain cells in an extensive random orientation stacked growth, with criss-crossing at the periphery and center. SHE cell assay has been developed at low pH for reducing/eliminating the problems associated with the standard assay (Kerckaert et al. 1996).

3.4.2) BALB/c3T3 Mice Cell System

Transformation of BALB/c3T3 clone has been utilized for screening assays for potential carcinogens and is based on a spontaneously immortalized cell line derived initially from a mouse embryo by repeated passages. This established cell line was originally developed by Aaronson and Todaro (1968) and has high sensitivity to chemical transformation. Chemicals with short half lines can be screened on this cell line, and a dose-response curve can be obtained with relatively small number of plates having large number of cells per plate. Improvement in the in vitro transformation assay of BALB/3T3 cells has been made with the use of T medium (modified DME/F-12) supplemented with insulin, transferrin, ethanolamine and sodium selenite and 2% FCS, which resulted in a five times higher transformation frequency and earlier appearance of transformation foci (Tsuchiya and Umeda 1995). A new assay method was developed in which the cells were replated in a medium containing a low concentration of serum after treatment improved the sensitivity and reproducibility of the transformation assay (Kajiwara et al. 1997). The replating method and time between initiator and promoter treatments for optimal transformation of Balb/3T3 cells has been shown to be efficient for screening of carcinogens and studying multiple stage carcinogenesis. (Fang et al. 2001). An ITES-medium-improved BALB/c 3T3 cell transformation assay was developed recently, which showed a concordance of 73.5% with rodent bioassay and can be said to be a reliable and useful short-term test procedure of screening for potential carcinogens (Kajiwara and Ajimi 2003).

The disadvantage of this cell system is that this subclone BALB/c3T3 is relatively unstable with regard to karyotype and other properties in culture. Thus, maintenance of cell stock with original properties is important. Actively growing cells are treated with chemicals with or without activation and then plated onto dishes (20 dishes each for each concentration of the chemical) at an appropriate cell density and then transformation of cells is seen. Positive transformed foci of the BALB/c3T3 cells show basophilia, multilayering of the cells, random orientation, and invasion into monolayer (Fitzgerald et al. 1989).

3.5) Assays in *Drosophila melanogaster*

Drosophila has been widely used in genetic toxicology for the identification of potential carcinogens and also for the studies of mechanisms of mutagenesis of chemicals (Vogel et al. 1999).

Due the short generation time (10 to 12days) and a broad range of suitable reporter genes, detection of a variety of genetic damages in germ cells of *Drosophila* is possible. Earlier, the sex-linked recessive lethal (SLRL) mutation at multiple loci served for screening genotoxins to mammals. SLRL mutation is an in vivo forward mutation assay, detecting both point mutation and small deletion in the germ line of the insect. These

mutations, occurring in the X chromosome of *Drosophila*, are phenotypically expressed in the mutant carrying males. Most of the database of chemicals is based on this forward assay. The SLRL assay has been used to study adaptive response of germ cells to alkylating agents, where the response was demonstrated in larvae but not in adult flies (Savina et al. 2003).

Assays of mitotic recombination and mutations in somatic tissues have replaced the costly SLRL germ line tests (Wurgler et al. 1984). These assays cost 5% to 10% of the SLRL test and evaluation of a large number of chemicals is possible. The somatic mutations in eye or wing disc cells have been validated and are well established. These detect mutagenic and recombogenic activity of chemical compounds. The wing spot test or the wing somatic mutation and recombination test (SMART) is a short and simple in vivo test in which larvae can be exposed to the chemical compound through feeding or inhalation. The recessive wing markers (multiple wing hair, *mwh* and flare, *flr*) are heterozygous, and the loss of heterozygosity leads to clones. This system also allows for the detection of various endpoints as mitotic recombination, point mutations, deletions, nondisjunction, and chromosomal loss, which gives rise to a single wing spot phenotype comprising of either *mwh* or *flr*. Twin spot phenotypes, with adjacent *mwh* and flr areas, give a direct proof for the recombogenic property of the test compound (Zimmering et al. 1997). This test is highly sensitive and has been validated for distinct types of genotoxic compounds that damage the DNA. The wing spot test in *Drosophila* is suited to the detection of recombinogenic activity of genotoxic chemicals (Spano et al. 2001) and has been used to assess the mutagenic activity of herbicides (Kaya et al. 2004) and fungicides (Osaba et al. 2002) and the genotoxicity of vitamin A (Klamt et al. 2003).

Another test is the white/white$^+$ eye mosaic system for homologous recombination, which is also based on the loss of heterozygosity. In this method the larvae are exposed to the chemical, and the light spots in the adult females are monitored (Vogel 1992). Point mutations and deletions, which inactivate the white$^+$ allele, chromosomal aberrations, and recombination give rise to the expression of white in the female genotypes. This assay has been used to detect both mitotic recombination and X chromosome loss induced by antineoplastic drugs (Rodriguez-Arnaiz et al. 2004).

Drosophila melanogaster has been used as an in vivo model for assessment of genotoxicity using the alkaline comet assay (Bilbao et al. 2002). The midgut and brain ganglia cells have been used in the modified comet assay for the genotoxicity assessment of a pesticide, cypermethrin (Mukhopadhyay et al. 2004).

3.6) Recent Advancements

Flow cytogenetics is an emerging field, which has immense potential in assessment of genotoxicity of compounds at very low concentrations due to its high sensitivity and has rapidly evolved into a technique for rapid analysis of DNA content, cellular marker expression and electronic sorting of cells of interest for further investigations. It is less time consuming due to its fast acquisition. The technique involves the use of flow cytometry to assess cytogenetic parameters. Flow cytometers are being extensively used for monitoring of cellular DNA content, phenotype expression, drug transport, calcium flux, proliferation and apoptosis. Phenotypic analysis of marker expression in leukemic cells has become an important tool for diagnostic and therapeutic monitoring of patients. Recent studies have explored the use of flow cytometry for monitoring hormone receptor expression in human solid tumors and for studies in human genomics.

Flow cytometry is a laser-based technology that is used to measure characteristics of cells and their constituents such as nuclei and other organelles. Flow cytometer scans single particles or cells as they flow in liquid medium past an excitation source. The fluorescence emitted is then captured through an optical system and transferred to software. These data are used to examine a variety of biochemical and molecular aspects of cells (Jaroszeski and Radcliff 1999). This is a powerful tool for many scientific disciplines, as it allows characterization of cells or particles within a sample (Cram et al. 1998). Flow cytometry is particularly important for biological investigations, because it allows qualitative and quantitative examination of whole cells and cellular constituents that have been labeled with a wide range of commercially available reagents (fluorochromes), such as dyes and monoclonal antibodies (MAbs).

For new chemical entities (NCEs), flow cytogenetics is increasingly being used for:

1. aneuploidy studies by estimating DNA contents in cells (Pajor et al. 1998)
2. flow karyotyping of cells, in which quantitative estimation of fluorochrome-stained chromosomes of mitotic cells is made. It finds application in patients with aberrant chromosomes (Fiegler et al. 2003).
3. in vitro analyses of micronucleus in CHO cells (Wagner et al. 2003), cultured lymphocytes (Nusse and Kramer 1984), reticulocytes in human blood (Abramsson-Zetterberg et al. 2000), or in vivo mouse erythrocyte micronucleus assay for clastogenicity (Adler 1993)
4. chromosomal abnormalities and DNA fragmentation
5. studying the HGPRT variants in human population (Kotova and Grawe 2002), which is a rapid and sensitive alternative to the autoradiography technique

CONCLUSIONS

Short-term tests have been used for more than two decades to identify chemicals as mutagenic and/or carcinogenic. Tests for mutagenicity have a well-established role in identifying a potential human mutagen and its probability as a carcinogen. These procedures have been applied to pharmaceuticals, food additives, pesticides, and environmental chemicals. Most substances causing mutations in somatic/germ cells can be detected by in vitro tests. A combination of two tests, one for bacterial

gene mutation (Ames test) and one for chromosomal aberrations in cultured mammalian cells, can adequately detect all compounds mutagenic in vivo. Another test for gene mutation in mammalian cells can complement the above two tests. Induction of micronuclei and chromosomal aberrations in rodent bone marrow can be performed, as these assays have been observed to detect most known germ cell mutagens. Short-term tests to detect gene or chromosomal mutations/aneuploidy (in mammalian in vivo) continue to provide vital information for evaluating the carcinogenic risks to humans.

The short-term tests provide better understanding of mechanisms involved in carcinogenesis and valuable information to identify mutagens and/or carcinogens. Working groups can then use the results of these tests to define mechanisms of carcinogenesis. Efforts toward harmonization of tests have suggested testing strategies that include bacterial reverse mutation assay, in vitro gene mutation and chromosomal aberration, in vivo rodent bone marrow micronucleus, and chromosomal aberration assays. Sensitivity of conventional assays have been enhanced using molecular probes, painting techniques such as fluorescent in situ hybridization in chromosomal aberration, and DNA probes to assess micronuclei. Newer assays including the comet assay, mutation in transgenic models, and cell transformations assays, which provide additional information about chemicals having direct effects on the genome in low levels.

Current short-term tests cannot mimic all the stages in carcinogenesis and are assumed to detect only the events leading to the initiation phase (i.e., ability to induce a mutagenic or clastogenic DNA lesion). The main value of these tests, thus, lies in their ability to identify chemicals that may, under certain exposure conditions, either cause cancer by a predominantly genotoxic mechanism or induce the initial phase of the carcinogenic process. The predictivity of the tests for detection of potential carcinogens is usually derived from their performance in validation studies.

Short-term tests for genotoxicity, mutagenicity, and carcinogenicity not only save time but also cut down animal experimentation. Several tests for the assessment of the genotoxic, mutagenic, and carcinogenic potential of compounds are available; however, a battery of these tests should be employed before labeling the compound.

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