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# The detection of genotoxic activity and the quantitative and qualitative assessment of the consequences of exposures

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## Abstract

A wide range of assays are now available which enable the effective detection of the mutagenic (the induction of gene and chromosomal changes) and more generally genotoxic (cellular interactions such as DNA lesion formation) activity of individual chemicals and mixtures. However, when genotoxic activity has been detected and human exposure occurs the critical questions relate to the qualitative and quantitative activity of the agent and the parameters such as routes of exposure, target organs and metabolism. Of major importance in hazard and risk estimation is the nature of the dose response relationship of each chemical and their potential interactions in mixtures. In this paper, we illustrate the methods available to produce quantitative and qualitative data in vitro using the micronucleus assay (as a measure of chromosomal structural and numerical mutations) and the HPRT assay (as a measure of induced gene and point mutations) and the current limitations (such as the large numbers of animals required) for obtaining such information in vivo. We recommend that in vivo studies should primarily focus upon confirmatory mechanistic analysis. For individual chemicals, profiles of the base changes induced can be obtained using the HPRT gene mutation assay and comparisons produced both in vitro and in vivo and thus allow identification of mechanistic differences between different modes of exposure.


***Keywords:*** Gene and chromosomal mutations; Quantitative and qualitative assessment; Mutation profiles

## Introduction: exposures to genotoxins

Irrespective of the route of exposure to a single chemical or complex mixtures the aims of an assessment of potential genotoxic consequences depends upon the provision of information which measures interaction with targets such as the DNA and the consequences of interaction. Measures of exposure to genotoxins can be provided by DNA adduct/lesion quantification by techniques such as $^{32}$P post-labelling (reviewed in Phillips et al., 1993) and damage to the chromatin by techniques such as the single cell electrophoresis or Comet assay (reviewed in McGregor and Anderson, 1999).

To determine the consequences of exposure it is necessary to quantitate the induction of both gene/point mutation and the induction of chromosome damage. In the case of both endpoints the quantitative effects of genotoxin exposure will be based upon three factors:

1. The proportion of lesions that are processed into mutations.

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2. The relationships between toxicity and mutation induction.
3. The identification of specific sites of exposure.

When chemicals have been demonstrated to be genotoxic in any one or all of the basic in vitro screening methods which involve the use of bacterial and mammalian cell cultures, all of which have been extensively reviewed elsewhere (for example see COM, 2000), the critical question is then "what will be the quantitative and qualitative effects of exposure to the agent in vivo?". Assessment of potential in vivo effects will involve consideration of both somatic (e.g. cancer) and germ cell (inherited defects) consequences. However, in most cases when positive results have been obtained in somatic cells it is assumed that germ cell effects will also be produced and further studies are rarely performed (COM, 2000).

## The quantitative analysis of genotoxic chemicals and mixtures

In situations where exposure may occur to genotoxic chemicals it is essential that information is available which demonstrates the nature of the dose–response relationships and whether thresholds of activity may be detected in the low dose range which would allow the estimation of no observable effect levels (NOEL). Ideally, such data would be available measuring genotoxic responses in vivo. However, the generation of such data in intact animals would involve the use of extremely large numbers in order to achieve appropriate statistical power. We have thus taken the decision that the most practical approach to generating quantitative data is to undertake detailed exposure studies using appropriate in vitro models, to demonstrate the operation of the same mechanism of mutation induction in vivo and to evaluate potential exposure scenarios and factors such as activation and inactivation. It is thus critical that the genetic endpoints which provide quantitative data can be used both in vitro and in vivo.

In our studies we have made use of two methods capable of detecting chromosome and gene mutation induction, namely the in vitro micronucleus assay and the HPRT mutation assay. Both methodologies were used in the human lymphoblastoid cell lines AHH-1 and MCL-5 which have the capacity to metabolise a wide range of potentially genotoxic compounds (for example see Doherty et al., 1997). As a model test compound we have used the alkylating agent methyl methane sulphonate (MMS). This compound primarily alkylates DNA at the N7 position of guanine as well as low levels of alkylation at the $O^6$ position of guanine.

## The quantification of induced chromosome mutations in mammalian cells

Genotoxicity can be assessed by cytogenetic methods either by measuring structural and numerical chromosome aberrations directly by classical metaphase chromosome analysis or by the micronucleus assay in interphase cells. Both methods can also be developed to provide additional information by using methods such as fluorescence in situ hybridisation (FISH).

Acentric chromosome fragments and lagging whole chromosomes may fail to segregate during mitotic anaphase to form micronuclei. These alternatives can be identified by the use of kinetochore antibodies or centromeric DNA probes. A convenient in vitro micronucleus assay protocol has been developed by Fenech et al. (1999) which enables those cells that have completed one mitotic division to be identified and the daughter nuclei are kept together in a binucleate cell as is illustrated in Fig. 1. This is the basis of the cytokinesis-blocked micronucleus assay, which employs the inhibitor of actin polymerisation, cytochalasin-B, to retain the products of a mitotic division in a single cell. In addition to the assessment of micronuclei in such cells, chromosome non-disjunction in the main sister nuclei can be measured by FISH using chromosome specific centromere probes. The in vitro micronucleus assay was used to determine the dose–response relationships of the induction of chromosome damage in the AHH-1 cell line using the model mutagen MMS. These data are illustrated in Fig. 2,which demonstrate that in the case of MMS exposure no increases in chromosome damage as measured by micronucleus induction could be detected at concentrations below 0.75 μg/ml.

## The quantitation of induced gene mutation in mammalian cells

To enable the direct comparison on mutation data both in vitro and in vivo, ideally one should make use of a methodology which can be utilised in both situations. Currently, the most suitable system is the HPRT methodology. This assay is based upon the mutation of the gene coding for the hypoxanthine/guanine phosphoribosyl transferase gene, which is on the X chromosome of mammals. The HPRT enzyme phosphorylates guanine and inosine in what is considered to be a salvage pathway. However, in the presence of 6-thioguanine and 8-azaguanine HPRT enzyme activity results in the production of highly toxic nucleotides, which are lethal to mammalian cells. The basis of the assay is the induction of mutations in the HPRT gene which inactivates the enzyme thus conferring resistance to 6-thioguanine and 8-azaguanine. The basic assay

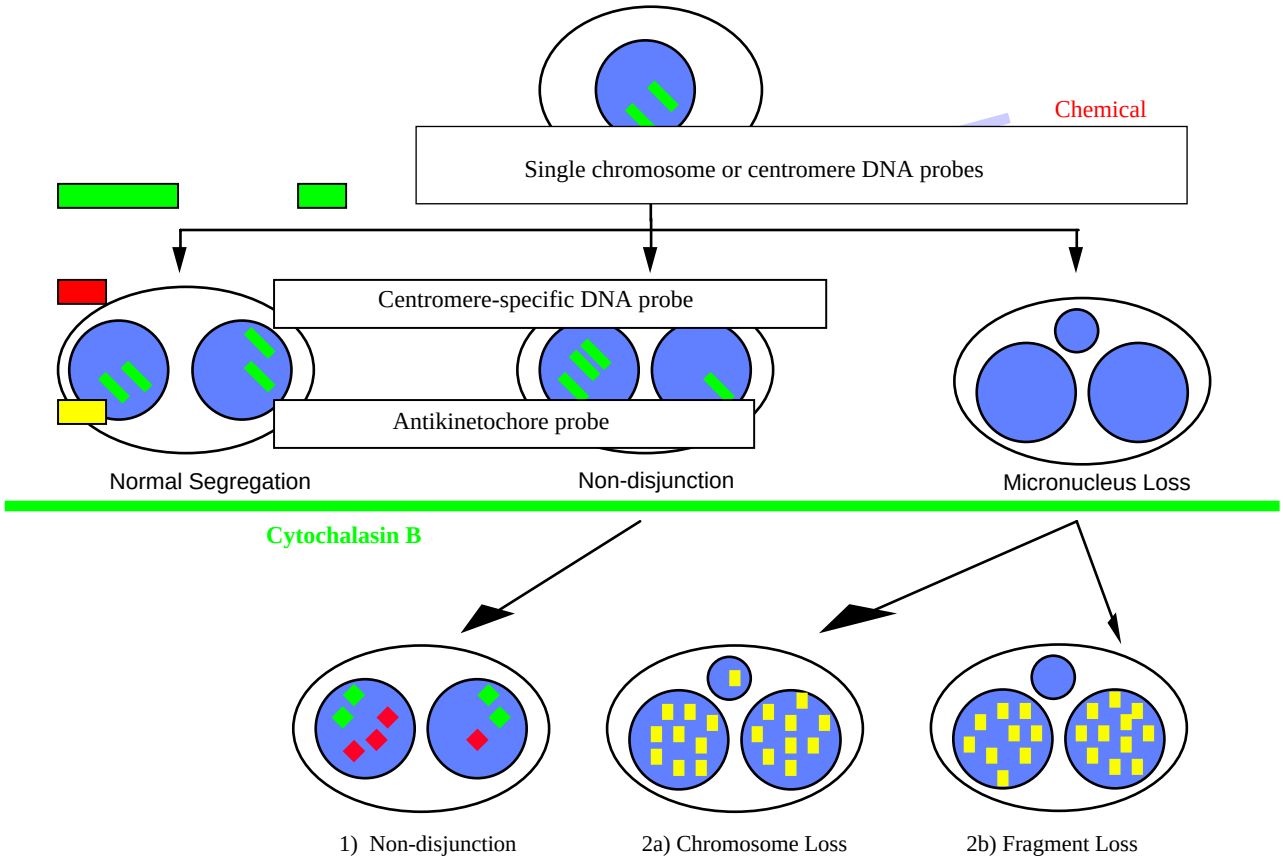


**Fig. 1.** The in vitro interphase binucleate cell chromosome segregation and micronucleus assay.

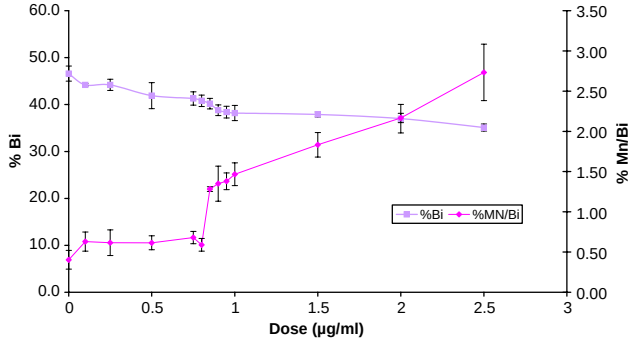


**Fig. 2.** Micronucleus induction in human AHH-1 cells by methyl methane sulphonate (MMS), ■ % binucleate cells, ● % micronucleated binucleate cells.

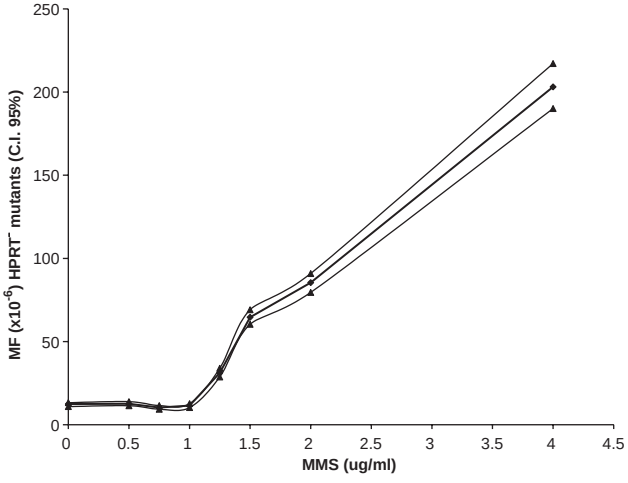


**Fig. 3.** Mutation induction at the *hprt* gene in human AHH-1 cells by methyl methane sulphonate (MMS), data shown for triplicate studies.

methodology has been reviewed in detail by O'Neill et al. (1987) and Aidoo et al. (1997) and can be applied both in vitro and in vivo thus allowing quantitative comparisons to be made between cultured cells and intact mammals.

The AHH-1 male cell line carries a single copy of the HPRT gene and has been used to illustrate here the nature of the dose response relationship of induced gene mutation in vitro using the model compound MMS. The dose–response relationship of MMS in the HPRT assay is shown in Fig. 3. The data in Fig. 3 demonstrate that in the case of MMS no increase in mutation frequency could be detected at exposure concentrations below 1 µg/ml.

The data shown here for the model mutagen MMS demonstrate that the in vitro gene and chromosome

assays can be used to produce detailed dose–response information.

## Evaluation of genotoxin exposure in vivo

In situations when exposure has occurred to potential genotoxic chemicals and mixtures, the range of human tissues that have been evaluated have been limited and often unrepresentative of those tissues and organs in which the consequences of exposure may occur. Examples of human material which has been analysed for potential genotoxin exposure include lymphocytes cultured in vitro from blood samples, nasal and buccal smears, exfoliated cells in urine and sperm samples.

The micronucleus assay in human biomonitoring studies is most commonly performed on cultured peripheral blood lymphocytes, the most easily obtainable and least invasive sample. These cells are in $G_0$ and can be stimulated to divide in vitro so that any chromosome damage can be expressed. For acute exposure and for chronic exposure where the exposure has ceased, the extent to which the DNA lesions persist in lympohocytes between exposure and blood collection is a critical determinant in the level of chromosome damage detected in vitro. The frequency of DNA damage in lymphocytes decreases with increasing time between exposure and sampling due to DNA repair processes and the loss of heavily damaged cells by apoptosis or necrosis.

Following the completion of detailed in vitro dose response data the next stage in mechanistic studies will be to undertake studies in suitable models using intact animals.

Reviews such as that of the UK Advisory Committee on the Mutagenicity of Chemicals describe a range of methods available for the analysis of in vivo mutagenicity (COM, 2000). These methods can be classified as follows:

1. Measurements of induction of DNA lesions, i.e. measures of exposure, uptake and reactivity to DNA. These methods include Comet assays, $^{32}$P-postlabelling assays, covalent binding to DNA, DNA unwinding assays.
2. Measurements of the repair of DNA lesions such as liver UDS measurements.
3. Measurements of induction of genetic changes including assays for point mutations and chromosomal aberrations and micronuclei primarily in erythrocytes.

The assays outlined above were developed primarily for the confirmation that the genotoxicity of a chemical that has been detected in vitro is also reproduced in vivo and have mainly been used to assess oral and/or interperitoneal exposures. To undertake the analyses of exposures to potential genotoxic chemicals and mixtures it has been necessary to develop dedicated assays. Currently, the most promising method is that developed in the rat by De Boeck et al. (2003). These workers have exposed rats to hard metal mixtures of tungsten and cobalt via the lungs and analysed genotoxic effects (using the Comet and micronucleus assays) in type II pneumocytes from lungs, lymphocytes from the peripheral blood and a mixture of cells from broncho-alveolar lavage (BAL). The principles of the De Boeck assay are shown in Fig. 4 and the authors successfully demonstrated the ability of the methodology to characterise genetic damage to the lungs. If

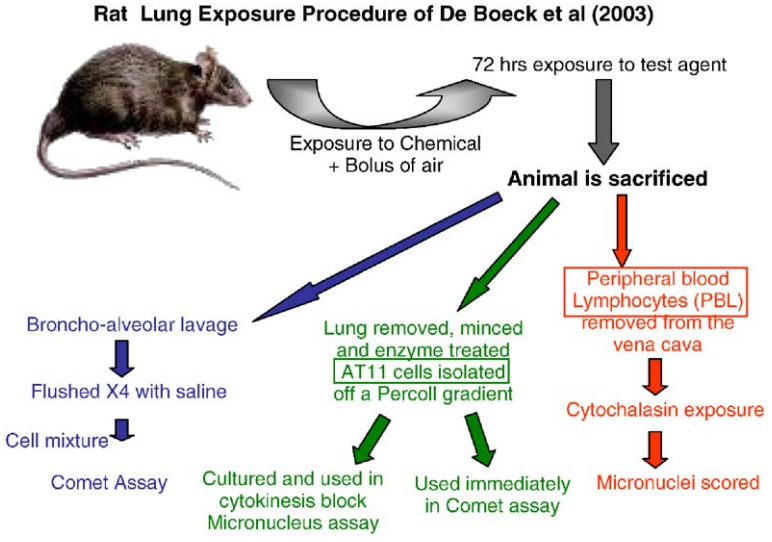


**Fig. 4.** Procedure for the assessment of genotoxicity in rat type II epithelial lung cells.

combined with a gene mutation assay the De Boeck rat assay has considerable potential for evaluating mixtures in the respiratory systems of an experimental mammal species, particularly one whose metabolic profile has been well characterised.

Analogous genotoxicity protocols could be developed in humans using laryngeal and bronchial biopsies using the methodologies described by Merkle et al. (2000) and Toma et al. (2005). Samples obtained by this method could be analysed using the Comet, micronucleus and HPRT assay.

## Assessment of the qualitative mutagenic effects of mutagenic chemicals and mixtures

Thus far, we have discussed the determination of the quantitative effects of potential genotoxins. When mutants produced by chemical exposure are sequenced it can be seen that base changes fall into two types of changes: the transitions (such as AT to GC and GC to AT) and the transversions (such as AT to CG, GC to CG, etc.). However, DNA reactive chemicals may react at characteristic sites within the genetic material, for example our model chemical MMS acts primarily at the N7 site of guanine and the N3 site of adenine. The site of chemical interaction and adduct formation has a major influence upon the type of mutation produced. Experimental systems such as the HPRT assay can provide data on the profile of mutations produced by specific chemicals.

The relationship of mutation type and sites of mutations within a gene can have a major influence upon the activity of the gene's product. Thus, the sites and types of mutations have been extensively studied in human cancers as a potential guide to identifying potential causal agents responsible for cancer initiation. The pattern of mutations identified in tumours can be considered the profile of the specific cancer and is based upon at least two factors i.e. the initial sites of chemical interaction, mutation formation and fixation and the influence of selection upon the survival of particular mutant genes in tumours.

To undertake the analysis of mutation profiles of both chemicals and tumours it is necessary to have comprehensive data, which can rarely be obtained from typical individual studies. Thus efforts have been made to develop databases, which contain all the published data in standardised and accessible formats. The most convenient source of information concerning mutations in tumours can be found at the Web site of the International Agency for Research on Cancer in Lyon, France at http://www-p53.iarc.fr/index.html. This database contains an extensive range of information on the mutations identified in the p53 tumour suppressor gene which can be used to produce mutation profiles of cancers such as those of lung and liver.

To analyse the profiles of mutations produced by specific chemicals and mixtures information can be obtained from the Mammalian Gene Mutation Database, which is operated from Swansea at http://lisntweb.swan.ac.uk/cmgt/index.htm. This database contains the collected and standardised published information available on a wide range of chemicals and exposures in test systems including the Sup F-plasmid which is capable of growth in both bacterial and mammalian cells, bacterial and bacteriophage genes such as LAC and CII in transgenic mice and the HPRT gene. It should be noted that we do not have comprehensive data concerning the induction of mutations in the p53 in the database. This is due to the current lack of a suitable selective experimental system that allows the identification in experimental systems without very large DNA sequencing efforts. Thus, in this paper we illustrate some of the mutation profiles that we have constructed from the data on a range of chemicals and exposures in which mutations in the HPRT gene have been identified and sequenced.

The HPRT gene contains nine exons and is illustrated in Fig. 5. The size of the gene has meant that the sequencing of mutants requires major effort to produce information which illustrates the most mutable sites within the gene. In this paper, we illustrate the patterns of mutation type that have been identified both in vitro and in vivo and in both humans and experimental rodents. In the reference list we have provided information on all the studies used in our analyses.

Figs. 6 and 7 illustrate the combined data of a number of studies which involved the analysis of the HPRT mutations identified in human populations. Fig. 6 illustrates the analysis of HPRT mutations present in the lymphocytes of both non-smoking and smoking individuals. In this study there was no evidence of any differences in the types of base change mutations in the non-target tissue (for cancer induction) between the two populations. Fig. 7 illustrates the data for the types of mutation in a number of populations. The main

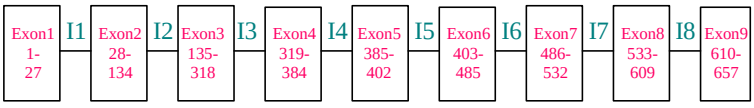


**Fig. 5.** Structure of the human *hprt* gene.

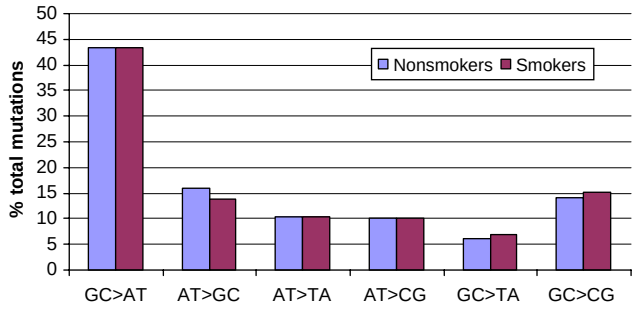


**Fig. 6.** The distribution of mutations in the *hprt* gene of human smokers and non-smokers.

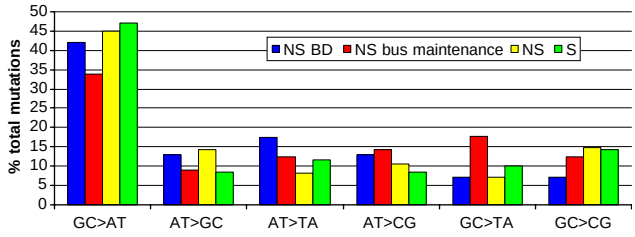


**Fig. 7.** Distribution of mutations in the *hprt* gene of non-smoking humans occupationally exposed to 1,3-butadiene or diesel exhaust compared with smoking and non-smoking controls. Key: NS BD non-smoking males occupationally exposed to 1,3-butadiene, NS bus maintenance; non-smoking males exposed to diesel exhaust, NS non-smoking males, pooled data, S smoking males, pooled data.

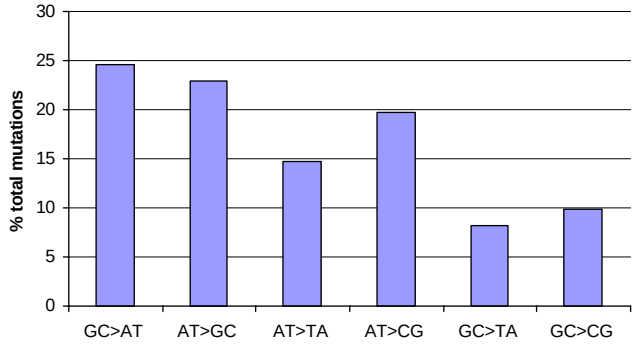


**Fig. 8.** The distribution of spontaneous mutations induced in the *hprt* gene of rat in vivo.

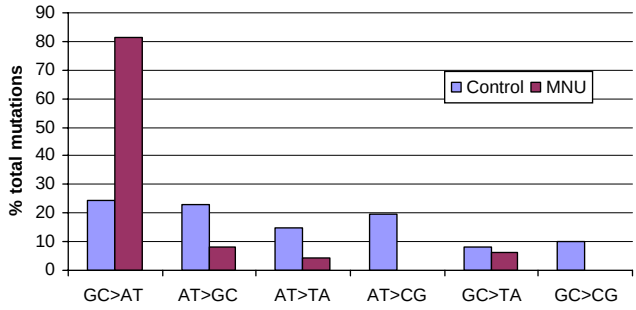


**Fig. 9.** The distribution of mutations induced in the *hprt* gene of rat in vivo following exposure to *N*-methyl-*N*-nitrosourea (MNU).

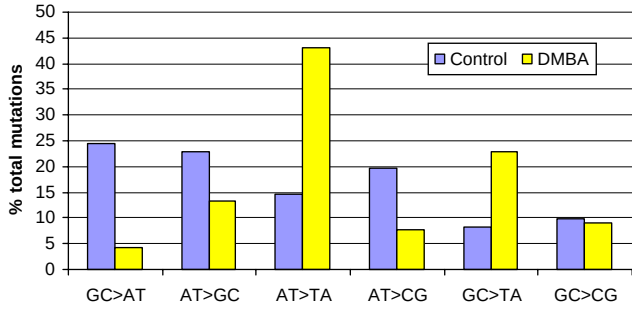


**Fig. 10.** The distribution of mutations induced in the *hprt* gene of rat in vivo following exposure to Dimethylbenz(a)anthracene (DMBA).

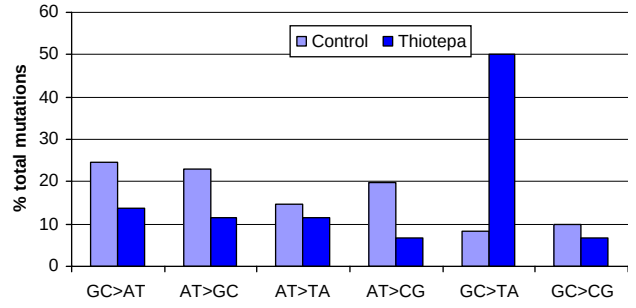


**Fig. 11.** The distribution of mutations induced in the *hprt* gene of rat in vivo following exposure to Thiotepa.

difference in this dataset is the higher levels of GC to TA transversions in the lymphocytes of non-smoking individuals exposed to diesel exhausts.

In an earlier section, we have described the development of a suitable rat model for the study of respiratory exposures. Thus far, this model has not been used for the study of HPRT mutations. However, a substantial number of studies have been performed in other rat models in a variety of tissues and we illustrate here a range of the mutation profiles obtained following exposure to a variety of chemical types which can be compared with the profile of mutations found in untreated rats shown in Fig. 8.

Fig. 9 illustrates the effects of the potent alkylating agent *N*-methyl-*N*-nitrosourea, which induces a substantially higher level of GC to AT transitions than the control levels. In marked contrast, the chemical 7,12-dimethylbenza(a)anthracene induces high levels of AT to TA transversions compared with the control levels, as is shown in Fig. 10. A similar increase in transversions (in this case of GC to TA) is seen following exposure to Thiotepa as is shown in Fig. 11. These data sets

demonstrate the considerable potential of rat models to provide information on the mutation profiles of specific chemicals and mixtures.

## Conclusions

Here, we have demonstrated that, following exposure to potentially genotoxic chemicals and mixtures there is a need to understand both the qualitative and quantitative relationships between DNA lesion induction and genetic endpoints induced.

In vitro models and assay systems such as the in vitro micronucleus and the HPRT gene mutation assays allow us to produce detailed qualitative and quantitative data and these models allow us to identify NOELs, mechanisms of action and modifying factors.

When comprehensive in vitro data has been produced this can be used to determine the priorities for in vivo analyses. These priorities include confirmation of similarities of mechanisms, in vitro and in vivo relative concentrations and genetic profiles.

Currently, the most appropriate in vivo exposure model is one which involves the rat where the respiratory system can be used as the target for both exposure and consequence. It would be extremely valuable if studies were undertaken in vitro in rat cells to produce detailed dose response data, which can be used to guide future in vivo work.

## Acknowledgements

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*Data on human studies involving exposure to butadiene and diesel exhaust*

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