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EPA 120/R-07/002-A
External Peer Review Draft
September 2007

Framework for Determining a Mutagenic Mode of Action for Carcinogenicity:

Using EPA's *2005 Cancer Guidelines* and *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens*

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Preface

This document is intended to help EPA risk assessors determine whether data support a mutagenic mode of action (MOA) for carcinogenicity. This document (*Framework for Determining a Mutagenic Mode of Action for Carcinogenicity)* deals only with MOA for carcinogenicity and not for other adverse endpoints that involve mutations. EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (or "*Cancer Guidelines*") emphasize using MOA information in interpreting and quantifying the potential cancer risk to humans (U.S. EPA, 2005a). EPA's *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (or "*Supplemental Guidance*") also relies on assessing the MOA. In particular, the *Supplemental Guidance* advises that age-dependent adjustment factors (ADAFs) be used with the cancer slope factors and age-specific estimates of exposure in the development of risk estimates if the weight of evidence (WOE) supports a mutagenic MOA. This default approach is used only when appropriate chemical-specific data are not available on susceptibility from early-life exposures (U.S. EPA, 2005b; see also discussion of analysis of the data in Barton et al., 2005). Given the potentially broad impact of judging an agent to have a mutagenic MOA, it is important that EPA's risk assessors approach the identification of a mutagenic MOA for carcinogenicity in a consistent, objective, transparent, and scientifically sound manner.

The analysis in this *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity* (or "*Framework*") expands and clarifies discussions found in the *Cancer Guidelines* and *Supplemental Guidance* on characteristics to be evaluated for a chemical's potential for a mutagenic MOA. It is meant to be used with those documents. Thus, the reader should be familiar with the framework for determining a mode of action in EPA's *Cancer Guidelines* as well as the discussion of the mutagenic MOA in the *Supplemental Guidance* before using this *Framework*. ***Nothing in this document should be interpreted as superseding either the* Cancer Guidelines *or the* Supplemental Guidance.** Related information on these documents can be obtained at www.epa.gov/cancerguidelines.

The members of the Risk Assessment Forum (RAF) Technical Panel on Mutagenic Mode of Action emphasize that the information offered here is neither a checklist nor specific set of criteria that must be met for determining if the WOE supports a mutagenic MOA. Instead it provides a framework for organizing data, determining relevance of those data and considering issues in determining a mutagenic MOA for cancer. It is also expected that this framework will result in greater consistency in Agency processes and decisions, and it describes some issues and points to consider in making those decisions. Additional (and perhaps more definitive) recommendations may be developed as Agency risk assessors gain experience in application of the *Cancer Guidelines* and *Supplemental Guidance*.

1.0 Introduction

1.1 Background

The two EPA documents that provide background, framework, and relevant information for assessing whether a chemical causes cancer by a mutagenic MOA are these:

1. *Guidelines for Carcinogen Risk Assessment* (or "*Cancer Guidelines,*" U.S. EPA, 2005a). The *Cancer Guidelines* describe how to weigh evidence on whether or not a chemical is carcinogenic for humans (hazard identification) and how to do a quantitative assessment of carcinogenic risk (dose response), when appropriate. The *Cancer Guidelines* also provide the framework for determining the mode(s) of action (MOA[s]) by which the chemical induces cancer. The *Cancer Guidelines* provides information on how the MOA may affect further analyses, such as low dose extrapolation.

2. *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (or "*Supplemental Guidance*," U.S. EPA, 2005b). The *Supplemental Guidance* evaluated the available data on differential effects of early-life exposure to carcinogens. One conclusion was a recommendation that, when supported by data, for compounds that cause cancer through a mutagenic mode of action, separate cancer potencies be calculated for early and later life exposure. For potential carcinogens determined to have a mutagenic MOA (and for which there are no chemical-specific data on early-life exposures), the *Supplemental Guidance* recommends a default procedure: that is, the use of age-dependent adjustment factors (ADAFs) to adjust the cancer potency estimated from adult animal exposures. These adjusted potency values, used in conjunction with age-specific exposure information, are used for **only** those exposures that occur during early-life. The procedures for these calculations are illustrated in section 6 of the *Supplemental Guidance.*

Another document that provides useful information related to whether a chemical causes mutations is the EPA's *Guidelines for Mutagenicity Risk Assessment* (or "*Mutagenicity Guidelines,"* U.S. EPA, 1986). The *Mutagenicity Guidelines* focus on heritable (germ cell) mutagenicity; that is, the risk of passing new mutations to future generations. While heritable mutation is a different adverse health outcome than cancer, both adverse health outcomes involve mutation as a part of their etiology. This document may be useful to the cancer MOA assessment because it provides a summary of endpoints that are useful in assessing the ability of a chemical to induce mutation.

The analysis in this *Framework* expands and clarifies discussions found in the *Cancer Guidelines* and *Supplemental Guidance* on characteristics to be evaluated to determine a chemical's potential for a mutagenic MOA. It is meant to complement the *Cancer Guidelines* and *Supplemental Guidance*. The reader should be familiar with the framework for determining a mode of action as presented in section 2.4 of the *Cancer Guidelines,* as well as the discussion of the mutagenic MOA as presented in section 2.2 of the *Supplemental Guidance* (and reproduced, below) before using this document. ***Nothing in this document should be interpreted as superseding either the* Cancer Guidelines *or the* Supplemental Guidance.** Related information on the implementation of these documents can be obtained at www.epa.gov/cancerguidelines.

In order to use the *Framework for Mutagenic MOA* properly, the chemical of interest must already have a WOE determination that indicates carcinogenic potential, i.e., a chemical that is carcinogenic to humans or likely to be carcinogenic to humans. The *Framework for Mutagenic MOA* does not provide an approach to hazard identification. Rather it gives information useful to determining whether MOAs by which the chemical causes cancer include mutagenicity as an early key event.

1.2 Regulatory Uses of Genetic Toxicology Data

There are several uses of genetic toxicology assays and data by regulatory agencies (Dearfield and Moore, 2005).

1. Genetic toxicity assays are used to screen chemicals for their ability to cause mutations or other types of genetic damage. This information may then be used to determine the potential of a chemical to induce human carcinogenicity. These assays, thus, contribute to hazard identification, either with or without long term assays in rodents. Often the decision that an agent produces some type of genetic damage may be used directly in risk management decisions; for example choices of conditions under which a pharmaceutical may be used in clinical trials. In this screening approach, genetic toxicity data are used to predict likelihood of adverse outcomes in the absence of information in animals or humans on this outcome. This is, thus, a type of hazard identification.

2. In EPA, and some other Agencies, analyses of genetic toxicity data may be included in a WOE assessment on whether a chemical is likely to induce some type of adverse health effect. Generally, these are hazard identification judgments for (a) heritable (germ cell) mutations that may be passed to future human generations, (b) reproductive effects, (c) developmental effects, and (d) cancer. In these applications, the intent is to make a judgment as to the likelihood of a

chemical's adverse effects for humans. Depending on the use of the risk assessment, the genetic toxicology data may have more or less weight. EPA sets out the parameters of its use of genetic toxicity data for cancer WOE judgments in the *Cancer Guidelines* (e.g., see section 2.3.5).

3. Genetic toxicity data may also be used in determining the MOA of a chemical. In this situation, the hazard identification has been done (see use #2, above), and the chemical has been assessed as having carcinogenic potential or some other effect. This *Framework* describes the use of genetic toxicity data to determine one specific MOA, i.e., a mutagenic mode of action for carcinogenicity.

While hazard identification and MOA judgments are related, they are, in fact, separate steps in the overall risk assessment process. This *Framework* focuses solely on the second of these steps. Furthermore this document provides approaches only for MOA involved in carcinogenesis. While the recommendations herein for organizing data may be useful when assessing other endpoints, the information on data interpretation or appropriateness of some data types is intended only for cancer MOA judgments.

EPA and other Agencies have established test batteries for the purpose of screening chemicals for their ability to cause mutation (for a recent overview see Cimino, 2006). The assays and strategies therein were designed to address the first uses above, i.e., hazard identification. However, these batteries and guidance for their interpretation provide generally accepted approaches to assuring adequate assessment as to whether a test chemical can induce mutation. Therefore, these recommended test batteries can provide high quality data useful in assessing MOA. EPA has published guidance for genotoxicity testing (described and referenced in Appendix B) as has FDA (for example, U.S. DHHS [2006a, b]).

1.3 Mutation as a Mode of Action (MOA) for Cancer

As defined in section 2.4 of the *Cancer Guidelines*, the term "mode of action" (MOA) encompasses a sequence of key events and processes, starting with the interaction of a chemical with a cell, proceeding through functional and structural changes, and resulting in cancer formation. A mutagenic MOA is discussed in both the *Cancer Guidelines* and *Supplemental Guidance*. This *Framework* addresses the assessment of mutation as a key event.

It is well established that mutations in somatic cells play a key, early role in cancer initiation and may also affect other stages of the carcinogenic process. Since all cancer cells acquire multiple mutations during carcinogenesis, mutation induction or acquisition can be key events at some stage in all cancers. However, in assessing evidence for a mutagenic MOA for cancer, there are a couple of important considerations: (1) when (in relationship to other key

events) the mutation occurs among the events that lead to cancer; and (2) whether the action of the carcinogen as a mutagen is a key event in its carcinogenic process. For a mutagenic MOA for cancer, mutagenicity is an obligatory early action, i.e., generally a very early key event for the MOA, of the chemical (or its metabolite). This is contrasted with other MOAs wherein mutations are acquired subsequent to other key events (e.g., cytotoxicity, regenerative proliferation). Consequently, for a mutagenic MOA for carcinogenesis, the chemical is expected to interact with DNA early in the process and produce changes in the DNA that are heritable.

The determination that a chemical carcinogen can induce mutation in one of a number of mutation assays is not sufficient to conclude that it causes specific tumors by a mutagenic MOA or that mutation is the only key event in the pathway to tumor induction. It is important to use the tools of weight of evidence and the *Cancer Guidelines* MOA framework in the determination of a mutagenic MOA. It should also be noted that there is no "default MOA." The *Cancer Guidelines* offer some default procedures to use when no MOA can be determined.

1.4 "Mutagenicity" in the Context of a Mutagenic MOA for Cancer

Genetic toxicologists have defined the term "mutagenic" in numerous, often incompatible ways. Moreover, the two terms "mutagenic" and "genotoxic" have, in different documents, been interpreted either to have different meanings, or to be synonymous. Some genetic toxicologists make a distinction between "mutagenicity" and "genotoxicity," considering the former a subset of the latter. In this sense, "genotoxic" includes all effects on genetic information, whether or not the chemical interacts with the DNA. The term "mutagenic," by contrast, implies interaction with DNA. These distinctions are not the focus of an analysis of data for a mutagenic MOA determination. Rather, it is more important to understand and report what mutagenic or genotoxic effect is being measured and how each effect enters into the WOE evaluation for mutagenic MOA.

EPA uses differing definitions of "mutagenicity" depending on the context. For example, EPA's *Mutagenicity Guidelines* (U.S. EPA, 1986) provide guidance primarily on assessment of germ cell mutagenicity. The *Mutagenicity Guidelines* describe different test endpoints as examples of "mutagenic" activity: point (gene) mutations, clastogenicity (structural breaks in chromosomes), and aneuploidy (numerical variations in chromosomes). Also included is damage to DNA, which can be observed in the following assays, among others: DNA adduct formation, unscheduled DNA synthesis, and sister chromatid exchange. All of these tests demonstrate effects on the genetic material that can have ramifications for subsequent changes in genetic content and/or expression. Moreover, the *Mutagenicity Guidelines* focus on heritable (germ cell) mutagenicity; that is, the risk of passing on new mutations to future generations of offspring. While heritable mutations can be an adverse health outcome, it may be by an MOA

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other than that described here. Thus, it is necessary to describe what is meant by a mutagenic MOA for carcinogenicity.

The text of the *Supplemental Guidance* describes effects that are indicators for determining a **mutagenic MOA for cancer** in the following words.

> "Key data for a mutagenic mode of action may be evidence that the carcinogen or a metabolite is DNA reactive and/or has the ability to bind DNA. Also, such carcinogens usually produce positive effects in multiple test systems for different genetic endpoints, particularly gene mutations and structural chromosome aberrations, and in tests performed *in vivo* which generally are supported by positive tests *in vitro*."

For purposes of this *Framework*, the term "mutagenic" will be considered **operationally** for mutagenic MOA for carcinogenicity as described in the *Cancer Guidelines* and *Supplemental Guidance.* The term "mutagenic," in this *Framework* is the capacity of either the carcinogen or its metabolite to react with or bind to DNA in a manner that causes mutations. In this context, mutagens usually (though not always) produce positive effects in multiple test systems for different genetic endpoints, particularly gene mutations and structural chromosome aberrations, both *in vitro* and *in vivo.*

This is an ***operational*** definition, and a very limited one. It is offered for weighing the evidence for a mutagenic MOA for carcinogenicity. **It is noted that not all carcinogenic chemicals that are capable of interacting with DNA will have a mutagenic MOA for cancer.**

2.0 Process: Determining if the Chemical Has a Mutagenic MOA for Carcinogenicity

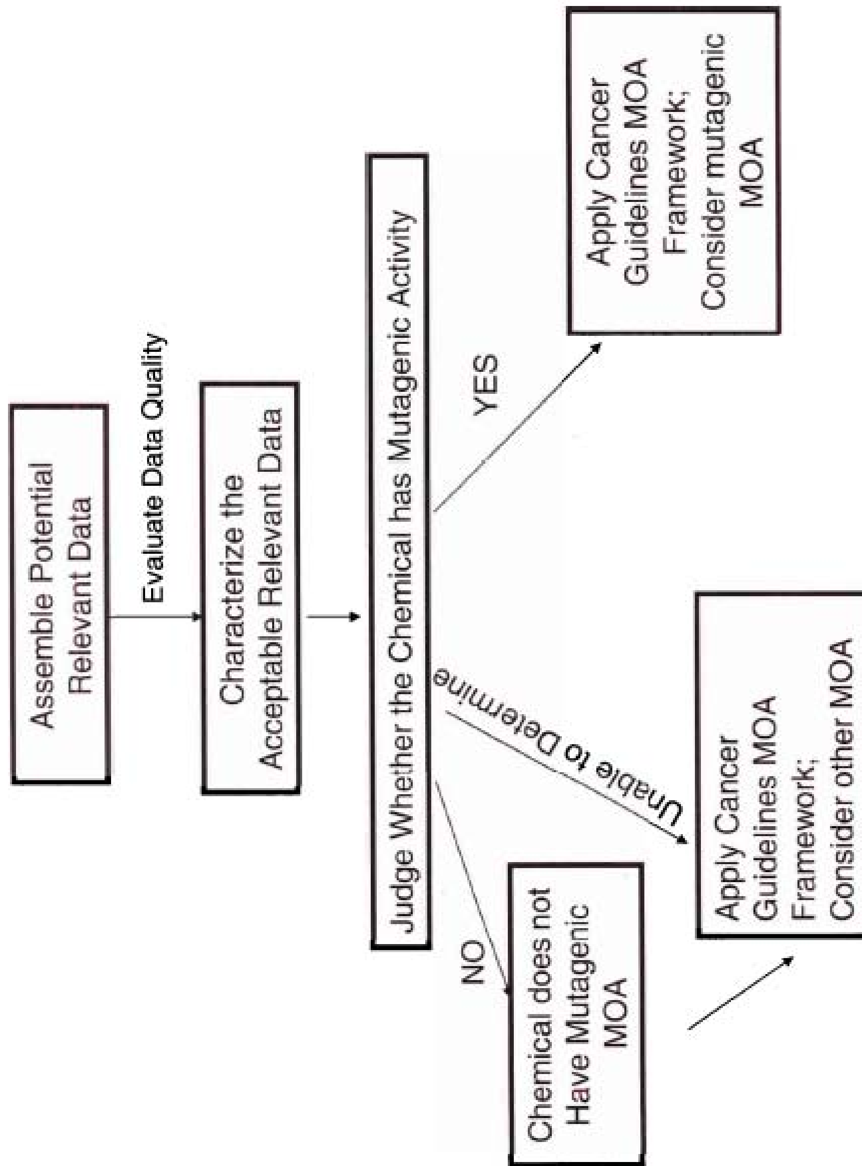
This document describes a general approach based on the *Cancer Guidelines* and *Supplemental Guidance* to determine whether a chemical carcinogen has a mutagenic MOA for induction of specific tumors. An MOA analysis may be performed for any chemical for which there is a positive finding of carcinogenicity; that is, one can do MOA for known, likely or suggestive WOE descriptors used in the *Cancer Guidelines*. This MOA analysis is a multi-step process, outlined in Figure 1 [N.B. Two versions of Figure 1 are presented on the next pages in this draft document. A final figure will be selected after external peer review of this document.] These steps are more completely discussed in the next sections.

1. Assemble all relevant data.
2. Evaluate the data against current acceptance and quality criteria.
3. Judge WOE that the chemical has mutagenic activity and if so, what type(s) of mutational activity.
4. For chemicals that can induce mutation, assess the evidence as to whether mutation is an early key event in the induction of tumors. This involves application of the *Cancer Guidelines* MOA framework.

2.1 Assemble the Relevant Data

No single mutagenicity test is able to detect the entire spectrum of induced mutagenic events. This is illustrated by EPA's TSCA and FIFRA programs and those of other regulatory bodies that use an established battery of mutagenicity tests for hazard identification of both somatic cell and germinal cell mutagens (e.g., Dearfield et al., 1991; ICH, 1995; Cimino, 2006; for a more complete discussion of EPA's required battery of tests, see Appendix B). While these testing schemes were designed with hazard identification in mind, they describe the range of genetic toxicity testing in common use that may be used in assessing mutagenic MOA.

It is important that all relevant, available information on mutagenic and genotoxic effects of a chemical is obtained to ensure as full a mutagenicity assessment as possible. The two primary sources are (1) data submissions to regulatory bodies and (2) the open literature. All available data that have been judged to be of sufficient quality are considered; EPA's *Cancer Guidelines*, the basis for this *Framework* has a discussion of data quality in sections 1.2.2, 2.3.5.4, 2.6, 4.3, 4.4, 5.1, and 5.4, including by reference EPA's document on information quality (U.S. EPA, 2002). In addition to any required test results submitted to EPA under FIFRA and TSCA, mutagenicity test results for the evaluation of a mutagenic MOA are often available from these sources: (1) other regulatory agencies; (2) the published, peer-reviewed literature; or (3) external databases. The peer-reviewed literature may also include summaries and evaluations of



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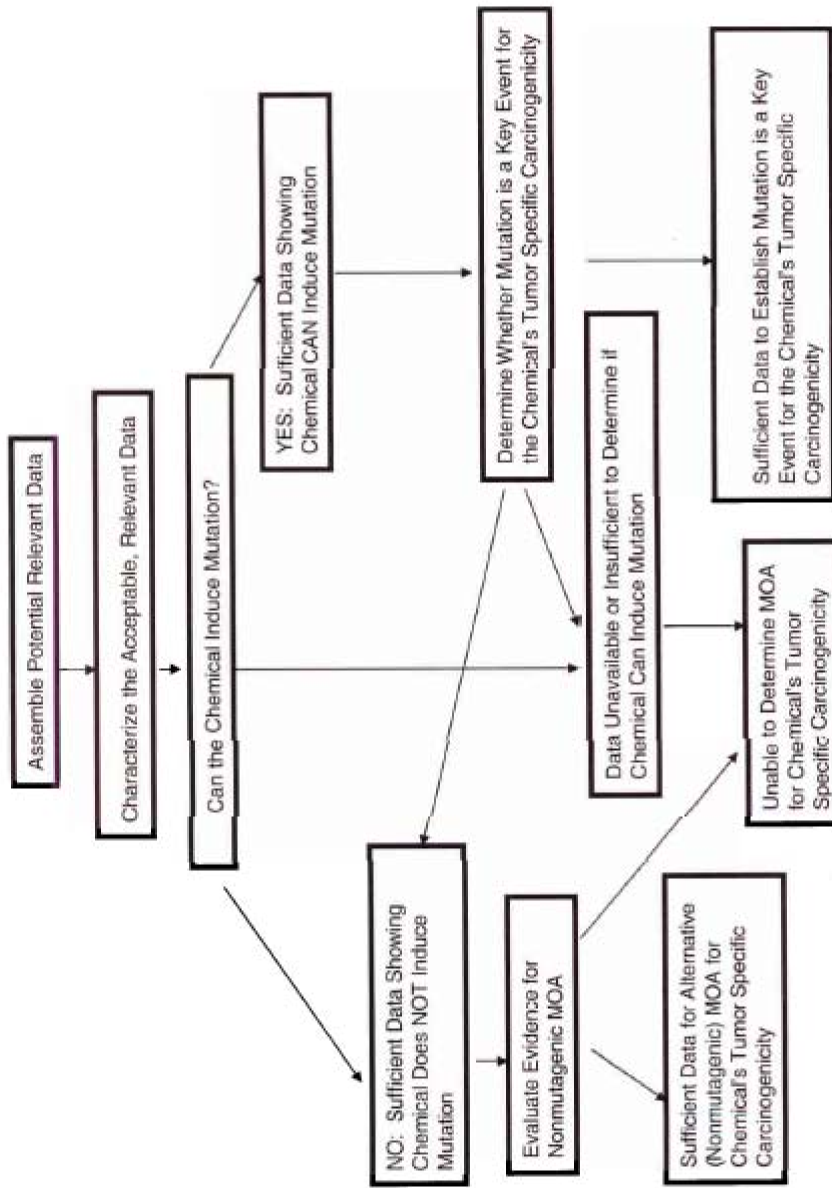

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Figure 1 v2 page 2

How to Weigh the Evidence as to Whether a Chemical Causes Specific Tumors by a Mutagenic Mode of Action (Mutation is THE Key Event)

(Listed in decreasing order of relevance/importance)

1. Cancer relevant oncogene/tumor suppressor gene mutations can be detected in the target tissue following chemical exposure
2. Surrogate gene mutations can be detected in the target tissue following chemical exposure
3. DNA adducts (known to be mutagenic adducts) can be detected in the target tissue following chemical exposure
4. Primary DNA damage can be detected in the target tissue following chemical exposure
5. Gene mutations and/or DNA adducts or other measures of primary DNA damage can be detected in vivo.
6. Evidence that the chemical can induce mutations, cytogenetic damage, DNA adducts and/or primary DNA damage in vitro.

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the mutagenicity testing of the chemical of interest. All of the major toxicity databases are searched to make an informed assessment of the mutagenicity data.

The assessment can often be enhanced by data other than those from mutagenicity tests, including these: (1) structure-activity relationships (SARs) with recognized mutagenic carcinogen(s); (2) structural analogues, such as the polycyclic aromatic hydrocarbons; (3) or the presence of certain structural alerts within the molecule, e.g., epoxides, aromatic amines, azo-structures, nitroso-groups, aromatic nitro-compounds, alkylating agents (see Appendix C). Such information can be particularly useful if the data on the chemical of interest are sparse, inconclusive, or contradictory. The MOA judgment will often be enhanced by consideration of the pharmacokinetics of the chemical. Genomic and proteomic analyses are likely to be useful in the future, as the ability to interpret these types of data matures.

2.2 Evaluate the Data against Current Acceptance and Quality Criteria

Once studies are assembled, they are judged against acceptability criteria and weighed for the appropriateness of their use. If a review article or other compendium is initially used for analysis of a chemical for which there are many results, evaluation of the data from the original publications may be necessary to ascertain the quality of the data. Older studies, for example, may have used conditions that are now regarded as unreliable. When data initially appear to be contradictory, a review of the quality of the data in the original report or article may resolve the issue.

Several publications may be consulted for criteria for judging acceptance of data. These include the following: the Organisation for Economic Cooperation and Development (OECD, 1997; 1998), the International Workshop on Genotoxicity Testing (IWGT, 1994; 2000; 2002; 2007), the International Conference on Harmonization (ICH, 1995; 1997), and those of other Federal agencies and their employees (e.g., DHHS, 2006; Cimino, 2006). Items to be considered include reporting of both positive and negative controls, numbers of replicate assays, and appropriate statistical treatments.

It is recommended that all studies be included on the data comparison tables described in the next section, with a note as to acceptability of the study.

2.3 Judge WOE that the Chemical Has Mutagenic Activity

This section deals specifically with mutagenic activity relevant to determining whether a putative carcinogen has a mutagenic MOA.

2.3.1 *Categorize the Data*

The data are best presented in a manner to facilitate the determination of a WOE for mutagenicity. Often, it will be useful to summarize the data in a table (for an example of a limited set of assays, see the table in Appendix A). The summary table includes all available negative, positive, and equivocal results, with comment on the adequacy of the study in light of current acceptability criteria (as described in section 2.2). The organization for each table may vary depending upon the amount and nature of the available data. In data-rich situations, each of the levels of conclusion listed below might be presented in a separate table.

- Present data on results from each study to judge its quality (as discussed below). Draw a conclusion regarding the results for that study.

- Present each type of assay (e.g., all *Salmonella* results), and determine a conclusion regarding the WOE for that type of assay.

- Present the findings for the type of effect (e.g., point mutation or clastogenicity), and determine a conclusion regarding the WOE for that type of effect.

- Present the totality of the database that, in some cases, may be a summary table for the table(s) mentioned above.

2.3.2. *Describe the WOE for Mutagenicity*

A decision that the chemical is mutagenic will be based on the overall WOE. Criteria for this judgment will be determined by the intended use of the risk assessment, i.e., in the situation described in this *Framework*, for determining a mutagenic MOA for carcinogenesis. It is recognized that the WOE for mutagenicity for other purposes, e.g., predicting endpoints such as developmental effects, may appropriately come to a different conclusion based on the same set of data.

2.3.2.1 Conclusions for Individual Assay Types or Endpoints

A consistent use of terms in describing the conclusions for the studies will facilitate communication. For example, the following terms may be used in judging the quality of individual studies and in rating conclusions for a specific test (when results from multiple assays using the same procedure are available) or mutagenic endpoint (when different assays examine the same endpoint).

Conclusions such as positive, negative, inconclusive, or contradictory are accompanied by rationales for these choices (See, for example Dearfield et al 2002). Some points to consider

for inconclusive and contradictory data sets are below.

- *Inconclusive* refers to the data set of test results that cannot be definitively termed sufficient or insufficient due to any of the following:
  - borderline responses;
  - insufficient number of test strains, or poor performance of the test organisms;
  - inadequate testing of exogenous metabolic activation for *in vitro* assays;
  - inadequate doses or concentrations (either too high or too low);
  - inadequate dose spacing;
  - inadequate sampling time(s);
  - positive responses observed only at unacceptable levels of cytotoxicity; or
  - statistical significance in the absence of biological significance.[1]

- *Contradictory* refers to test results that would lead to different conclusions that cannot be explained by the quality of the test performed or by other biological data. Some examples of contradictory results include those listed below:
  - differing responses in the same assay system from different laboratories (positive in one well-conducted study and negative in another well-conducted study of the same assay); or
  - differing results from different assays for the same endpoint, such as gene mutations in bacterial cells versus mammalian cells, where cell type is not expected to affect outcome (e.g., because differential transport into the cell would not be expected for the chemical under consideration); or chromosomal mutations *in vitro* versus *in vivo*, or in different cell lines or species, without a physiological explanation. For example, in some cases an *in vitro* test produces a biologically relevant positive result in contrast to a negative result in an *in vivo* assay (e.g., bone marrow micronucleus or cytogenetic tests).

Genetic toxicologists frequently perform and evaluate some tests that measure endpoints not useful for evaluating mutagenicity as it is defined in section 1.4; in other words data from some assays do not inform decisions on a mutagenic MOA for carcinogenesis. Two of these are described in EPA test guidelines or are discussed in other draft test guidelines.

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[1] OECD genetic toxicology guidelines stress this in evaluating and interpreting test results. For example, "[b]iological relevance . . . should be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance should not be the only determining factor for a positive response." (OECD guideline 476 for *In Vitro* Mammalian Cell Gene Mutation Test, section 32, adopted 21st July 1997)

1. *Morphological cell transformation assays.* The endpoint evaluated in these assays is not mutation, but rather changes in appearance of cells in culture that have been associated with some stages of neoplasia. The OECD explicitly addressed this issue when it referred to the various cell transformation assays in its draft detailed review paper for "Non-genotoxic Carcinogens."

2. In vivo *spermhead abnormality tests*. Changes in spermhead morphology may be caused by modification of the sperm's DNA content *or* by modification of its protein structure. Unless modification of proteins can be ruled out by a process that is not part of the standard test protocol, spermhead abnormality can not be definitively associated with mutagenicity. These tests were included for evaluation under the EPA GENE-TOX Program of the 1980s-1990s (Waters and Auletta 1981, Wyrobek et al. 1983a,b).

2.3.2.2 Evaluating Results across Endpoints

After evaluating results from one assay type or one endpoint, a WOE is evaluated across endpoints; for example, all conclusions on point mutations, DNA breakage, chromosomal aberrations can be summarized in tabular form and considered.

All WOE conclusions depend on professional judgment; these judgments are discussed in a clear and transparent manner. The presentation of WOE includes rationales and decisions supporting a judgment that the data are either sufficient or insufficient to proceed with an evaluation of a mutagenic MOA. The conclusion can be discussed in the overall evaluation of MOA for the chemical, as described in the *Cancer Guidelines*. The analysis of the WOE for mutagenic activity may benefit from the distinction made in the *Cancer Guidelines* between differing and conflicting results. As the *Cancer Guidelines* state:

> "… conflicting evidence. . . [occurs when] . . . some studies provide [positive] evidence … but other studies of equal quality in the same [test system] … are negative. *Differing results*, that is, positive results in some studies and negative results in one or more different experimental systems, do not constitute *conflicting evidence,* as the term is used here."

When using the operational definition presented in section 1.4 to assess endpoints as supportive of a mutagenic MOA, some positive results contribute more to the WOE than others, i.e., while some results may indicate mutagenic activity, the nature of this activity may not support a mutagenic mode of action for cancer. For example, aneuploidy is a common occurrence in certain tumor types, but such changes in chromosome number may not occur through the same mechanisms that produce other mutations. Similarly, positive results in cell transformation assays may be associated with chemical carcinogens, but not solely with those

that have a mutagenic MOA. Positive responses in both of these assays have been shown to occur for some chemicals that are negative in multiple tests for gene mutations, chromosome mutations (clastogenicity), and DNA effects. Positive results *only* in these assays, therefore, are less likely to support a WOE determination for a mutagenic MOA for carcinogenesis, as described in the *Cancer Guidelines* and *Supplemental Guidance.*

2.3.2.3 WOE Conclusions for Mutagenic Activity

In this step the risk assessors decides if there is justification for further considering a mutagenic mode of action. If there is not sufficient evidence of mutagenic activity, then other modes of action can investigated instead.

There are several types of WOE conclusions for evidence of mutagenic activity relevant to a mutagenic MOA. They can include those presented below.

- The data are sufficient for a judgment of negative. The chemical has been tested in acceptable studies and all or most of the acceptable assays are negative.

- The data set is inadequate; that is, there are insufficient tests upon which to make a determination. For example, inadequate data may be the absence of one or more of the basic tests for the common genetic endpoints, such as point mutation or clastogenicity.

- The data are of questionable quality. For example, the data may not meet criteria for acceptability (see section 2.2) according Agency standardized protocols and guidelines such as described in Appendix B.

- The data are equivocal. Sufficient and appropriate tests were performed, but the overall evaluation of the data is neither convincingly negative nor positive. Or the results are not consistent or are not coherent (see the description of *Cancer Guidelines* MOA framework, below). For example, the tests results are of borderline significance (statistically or biologically) by comparison to the concurrent negative (or solvent) control.

- The data are positive. The WOE is sufficient to judge that the agent has mutagenicity activity and to consider further whether the chemical has a mutagenic MOA.

From the array of usable tests for mutagenicity, the risk assessor can describe the nature of the mutagenic and/or genotoxic effect; for example, gene mutation or clastogenicity. It may

be useful to describe the type of interaction (e.g., DNA adduct) and what kind (e.g., O^6 methylguanine adducts).

2.4 Apply the MOA Framework

Once it is decided that the chemical or metabolite is mutagenic, the next step is to determine if this activity is critical to its MOA for cancer. This section discusses the MOA framework described in the *Cancer Guidelines* as it pertains to a mutagenic MOA. The *Cancer Guidelines* present a framework for evaluation of all of the relevant data to "judge whether available data support a mode of carcinogenic action hypothesized for an agent" (U.S. EPA, 2005a). The framework can also be used to identify data gaps and to suggest research to help fill those gaps. Details can be found in the *Cancer Guidelines* section 2.4.2. There is no "default MOA." All MOA determinations used by EPA address the various parts of the framework described in the *Cancer Guidelines.* The major components, as they pertain to mutagenic MOA, are presented below.

- A description of the hypothesized mutagenic MOA and its sequence of key event(s). Even though the criteria for mutagenicity within the context of a mutagenic MOA for carcinogenicity (see section 3.2) may be the major, or even the sole, key event, additional key events for the chemical under consideration are included to the extent they can be documented. This provides the context for a discussion of the experimental data.

- A discussion of the experimental mutagenicity data: that is, the manner in which these data support the key event(s) and how other relevant associated data support the hypothesized mutagenic MOA.

    - An analysis of all available information concerning cellular interactions, whether for the parent chemical or for its metabolite(s), for determining what is known about the MOA.
        - An analysis of the absorption, distribution, metabolism, and excretion (ADME) aspects of a chemical exposure. Physiological, cellular, and biochemical differences among species can be illuminated by physiologically based pharmacokinetics (PBPK). Pharmacokinetics describes the nature and time course of ADME.
        - An evaluation of the toxicodynamics, and the consequences of the interactions between the chemical (or its metabolite) and the target cell, tissue, or organ.

    - An analysis of the strength, consistency, and specificity of association between genetic events and outcomes.

  - o An analysis of dose-response concordance.

  - o A discussion of data on the temporal relationship.

  - o An analysis of the biological plausibility and coherence of the database.

- A consideration of the plausibility of other MOA(s), and the sufficiency of the biological support for those alternative MOA(s).

- Conclusions on the hypothesized mutagenic MOA. These may include, but are not limited to, answers to these questions:
  - Is the hypothesized mutagenic MOA sufficiently supported in the animal models?
  - Is the hypothesized mutagenic MOA relevant to humans?
  - Are there populations or life stages particularly susceptible to the hypothesized mutagenic MOA?

The steps above are applicable to any MOA analysis. The sections below, however, provide a framework for evaluating what sort of data and types of considerations are particularly pertinent to determining a mutagenic MOA for cancer. The information offered here is neither a checklist nor specific set of criteria that must be met for determining if the WOE supports a mutagenic MOA.

Generally, for a chemical to be considered to have a mutagenic MOA for carcinogenicity, the data will include positive responses from one or more *in vivo* studies that are generally supported by *in vitro* gene mutation or cytogenetic assays. Supportive information may include interaction of the chemical with DNA, for example, gene mutation, DNA strand breaks, unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE), and/or DNA adduct formation. A high degree of consistency and biological significance in the findings across test systems would generally be expected for chemicals considered mutagens in the context of the *Cancer Guidelines* and *Supplemental Guidance*. This includes chemicals (or metabolites) that are DNA-reactive or have the ability to bind DNA and cause mutations, as demonstrated through effects in multiple test systems for different endpoints. *In vivo* data are generally supported by positive findings *in vitro*. These chemicals typically are systemic[2] mutagens. One would generally expect positive results in more than one organ or tissue, as well as positive *in vivo* test results from more than one phylogenetically distinct species. Situations where only one type of activity or only one site or species is affected would be explained and well documented. Examples might include highly reactive chemicals (or metabolites) that cause cancer at the portal

[2] Systemic mutagens cause mutations in places that are distal to the portal of entry, regardless of route of exposure.

of entry or the site of metabolism.

The following is presented to facilitate the consideration of mutagenicity as a MOA for cancer. It is neither a checklist, nor a list of requirements, but rather an indication of some of the types of data that would lend support to a WOE determination for mutagenicity for a mutagenic MOA.

- Consistency of the same effect across different assays supports the WOE for that specific mutagenic effect.

- Induction of more than one type of genetic effect strengthens the support for the WOE for a mutagenic MOA.

- Observation of effects *in vivo*, as contrasted with *in vitro*, tends to provide greater support, while the reverse may be less convincing. Evaluation of such data may benefit from the distinction made between "differing" and "conflicting" results, as discussed in the *Cancer Guidelines* and in section 2.3.2.2, above. In a report of a meeting of the Expert Working Group (of the International Association of Environmental Mutagen Societies) on Hazard Identification and Risk Assessment in Relation to *In Vitro* Testing (Thybaud et al., 2007) discuss this situation in the context of hazard identification. They note the following:

> "In some cases a clear and reproducible positive in vitro result is seen, yet the other assays . . . including any required in vivo test, are negative. The in vitro result is not automatically overruled by the negative in vivo result."

Additional testing may be recommended, when possible, to assist in understanding such results. The concern for the *in vitro* result lessens as the number and types of *in vivo* negatives rise, or as a biological explanation can account for the discrepancy. For example, if more than one, well-conducted *in vivo* study using different tissues as the target are negative for mutagenicity, the WOE includes a discussion on why other *in vivo* information about the chemical gives support to one of the following positions.

  - The conditions that caused the chemical to produce positive results *in vitro* are known to differ significantly from the conditions that occur *in vivo*. In this case, the WOE for a mutagenic MOA is decreased.

  - The target tissue for the observed tumors is not one of the tissues tested for mutagenicity *in vivo*, **and** *in vitro* mutagenicity data are well understood and

occur through interaction with DNA. These conditions may support continued consideration of a mutagenic MOA.

- Mutations seen in the presence of no or low cytotoxicity increase the WOE.

- Kinetochore staining may be used to look for possible aneuploidy when micronucleus assays are positive to determine if the chemical's effect is binding to proteins, such as microtubules of the spindle apparatus that would not be considered mutagenic *per se*.

- Determining whether a gene mutation is a base substitution or frameshift may help describe how the gene's expression.

- SAR information consistent with the data from mutagenicity testing increases the WOE.

Other cellular functions, such as enzyme induction or hormone level shift, may contribute to the tumorigenic process. Changes in such cellular functions may result from the same chemical exposure that caused the mutations. For certain chemicals, these cellular functions may enhance the role of mutagenicity in the carcinogenic process. The relevance and interpretation of all of these activities is part of the MOA analysis discussed later in this document.

Even in the absence of an extensive data base for a chemical, a decision may be made on a case-by-case basis to proceed with an evaluation of a mutagenic MOA in these situations. Examples of these situations include:

1. no *in vivo* data are available for the chemical of interest
2. substantial *in vivo* and *in vitro* data are available on the mutagenicity of a structurally similar group of chemicals (or, where appropriate, their reactive metabolites)
3. information on the toxicokinetics for the chemical of interest support formation of the reactive species

2.4.1 Key Events

Cancer pathogenesis can be described operationally as proceeding from initiation to promotion and progression. Mutation can occur in one or more of these stages of pathogenesis. Certain mechanisms may recur several times during the process (for discussion of some key events related to mutagenic MOA, see for example, Preston 2007 and Preston and Williams 2006). It is important to decide if the effect of the chemical is: (1) contributing directly to mutation through DNA reactivity (that is, has the potential for a mutagenic MOA); or (2) altering or disrupting other mechanisms in a sequence of events [such as polymerization of the mitotic spindle (McCarroll et al., 2002)] that eventually occurs after prolonged insult indirectly affects DNA damage and ultimately advances to mutation. For a mutagenic MOA, mutation is the first

step which initiates a cascade of other key events such as cytotoxicity or cell proliferation that are key to the carcinogenesis process.

The mutagenicity data and mutagenic events that are key for tumor induction are analyzed in light of data on other properties of the chemical. Obviously, one key event will be DNA changes resulting in a mutation or mutations. Other key events associated with the tumor response (e.g., enzyme induction, cell death, or cell proliferation) are presented and arranged in a manner to facilitate analysis. Additional relevant toxicological effects, while not specifically associated with tumor formation, may also be mentioned.

The experimental data and analysis supporting the WOE determination that the chemical is mutagenic is included in the MOA analysis under the framework in EPA's *Cancer Guidelines*. The MOA analysis uses these data and conclusions, along with other relevant data that support the other key events to develop the hypothesized mutagenic MOA.

As stated in the *Cancer Guidelines*, different tumors observed in the same animal, from exposure to one chemical, may arise from different MOAs. In MOA analysis, each site-specific tumor is to be analyzed separately. However, if tumors at different sites are induced by the same MOA, they may be analyzed together. There may be dose-dependent and/or age-of-exposure-dependent changes in MOAs. Thus, the contribution of the key events of a particular MOA toward tumor induction (and, therefore, the MOA itself) may vary with conditions of exposure. Delineating the contributions of these events may be important for guiding dose-response analysis, low-dose extrapolation, and risk characterization when more than one MOA has been demonstrated.

The critical question posed at this stage in the evaluation is this: "Is mutation an early key event in this chemical's induction of cancer?" **For a chemical to act by a mutagenic MOA, either the chemical or its direct metabolite is the agent inducing the mutations that initiate cancer**. This is contrasted with a MOA wherein mutagenicity occurs as an indirect effect of another key event in carcinogenesis occurring later in the process.

Examples of the properties for mutagenicity as the key event for a mutagenic MOA in the target tissue (or, at minimum, in a relevant surrogate tissue) may include the following that are illustrative, but not comprehensive, examples.[3]

- There is direct DNA reactivity.

[3] Additional information may be obtained from the *Cancer Guidelines*, including section 2.4.1. For example, that section states, "multisite and multispecies tumor effects … are often associated with mutagenic agents."

- DNA of the target cell or tissue is damaged.

- Mutation is an early event in the carcinogenesis process, rather than a by-product or observation at later stages.

- The target cell/tissue is exposed to the ultimate DNA-reactive chemical (parent chemical or metabolite). A demonstrated pathway exists for the chemical to reach the target cell (or surrogate tissue) or, if the DNA reactive chemical is a metabolite, it has been observed to be produced in or distributed to the target or surrogate tissue.

- Termination of treatment does not reverse the carcinogenic effect; for example, in a recovery experiment in which the post-exposure observation time is purposely shorter than the normal expression time. In the parlance of the initiation-promotion testing protocols, the chemical is an initiator.

- Tumors are observed in multiple sites, in multiple species, and from multiple routes of exposure.

- An increase in tumor-bearing animals, an increase in tumor multiplicity, or a decrease in time to tumors is observed after a short-term exposure to the chemical followed by exposure to tumor promoters.

- The chemical belongs to a chemical group[4] comprised of carcinogens already established to have a mutagenic MOA, including those named as having a mutagenic MOA in the *Supplemental Guidance*.

- Tumor responses generally occur early in chronic studies (e.g., within 52 weeks).

- Mutations by the chemical (or its metabolite) observed in genes that affect carcinogenesis (e.g., tumor suppressor *p53*, *Rb*) increase the WOE. This does not refer to the general characterization of mutations found in tumors but rather to mutations that can be specifically associated with exposure to the chemical being assessed. At the time of this writing the ability to detect these rare mutational events is limited.

[4] Section 2.2.3 of the *Cancer Guidelines* discusses this issue more generically and states, “Analogue effects are instructive in investigating carcinogenic potential … and potential functional class effects or modes of action.”

2.4.2 *Strength, Consistency, and Specificity of Association*

These characteristics link key events, precursor lesions, and the tumor response. For a mutagenic MOA, the precursor effects will typically be indications of reaction with DNA, DNA repair, and mutations. Therefore, much of the strength and consistency of the WOE for a mutagenic MOA will depend on the strength and consistency of the data on mutagenicity. Additional data for the other key events are also presented. Important tests of the *specificity* of the association include recovery studies showing reduction (or absence) of subsequent events (including tumors) when a key event, for example, blocking conversion to the mutagenic metabolite, is diminished (or blocked). Consistent observations in a number of such studies with differing experimental designs increase the WOE. *Consistency*, which addresses repeatability of key events in the postulated MOA for cancer in different studies, is distinguished from *coherence*, which addresses the relationship of the postulated MOA with observations in the broader database.

Data other than those for mutagenicity, such as ADME, may be useful for determining a mutagenic MOA. For example, if a direct metabolite of the chemical is mutagenic, the analysis may proceed as if the original chemical were mutagenic. Similarly, knowledge about distribution of the chemical may demonstrate that the chemical or its metabolite does (or does not) have the potential to reach the tissues in which the tumors were observed. In addition, pharmacokinetic studies may provide information on coherence. Such studies, for example, might provide information on species-specific or dose-specific differences in site of tumor formation.

2.4.3 *Site Concordance between Mutagenicity and Cancer*

Data demonstrating site concordance between mutagenicity and the observation of tumors in the same species can strengthen the determination of a mutagenic MOA. Appendix D presents a selection of the available literature showing concordance between specific mutagenic assays and tumors.

Lack of site concordance, however, may not necessarily indicate that a mutagenic MOA is not operating. Chemicals with a mutagenic MOA are frequently observed to cause cancers in many sites in one species, as well as to have different sites of tumor formation in different species. Other factors, such as the magnitude of the dose or the timing of doses, may affect tumor site(s) for chemicals with this MOA.

2.4.4 *Dose-response Relationships*

For a mutagenic MOA, the key issue is whether the observed dose-response relationships of the initial mutagenic events correspond with the dose-response relationship for tumors.

Ideally, increases in the incidence of the initial key event correlate with increases in incidence or severity (e.g., lesion progression) of other key events occurring later in the process, and ultimately with tumor formation. Comparative tabular presentation of incidence data for key mutagenic events and tumors may be helpful in examining dose-response concordance. Note that none of these statements concerning concordance refer to the shape of the dose-response curves. These are expected to differ for mutations, intermediate lesions, and tumors. For example, not every mutation would be expected to initiate carcinogenesis. Since mutations may affect other stages of carcinogenesis, occurrence of multiple mutations may facilitate carcinogenesis. Thus, the ratio between mutations and tumors may vary with dose.

Dose-response data may also suggest that the chemical does not act by a mutagenic MOA. For example, if mutations occur only above doses that produce cytotoxicity or other impaired cellular functions, the observed mutations may be determined to be secondary to the other toxic effects. Similarly, since *in vivo* mutagenic activity would generally be expected at doses lower than those that result in tumors, the absence of mutagenicity at doses lower than those that cause cancer may suggest that mutagenicity is a secondary effect and, therefore, may suggest an MOA other than a mutagenic MOA.

2.4.5 Temporality

For a mutagenic MOA, a key mutagenic event is observed early in the process of carcinogenesis. Such a finding adds considerable weight to decisions that the data support a mutagenic MOA, as many steps occur after mutation before the process of carcinogenesis is complete.

Often, adequate data sets are not available to address the criterion of temporality. To the extent that the mutagenic events occur earlier than or at lower doses than the tumors, however, the WOE for a mutagenic MOA is strengthened.

2.4.6 Biological Plausibility and Coherence

At this step, the analyst considers all of the data, as well as any data gaps, to determine if the data conform to the postulated MOA. For the MOA to be accepted, the postulated MOA and the key events must be supported and based on an understanding of the biology underlying carcinogenesis.

Mutagenesis is routinely accepted as part of the carcinogenic process. Consideration of the biological plausibility for a mutagenic MOA, therefore, is that the chemical (or its metabolite) caused the mutations that initiated the formation of the observed tumors. Thus, all of the data that support or negate this hypothesis are examined. Comparison to a chemical that has been determined to have a mutagenic MOA for carcinogenesis may guide the risk assessors in determining if there is sufficient evidence to justify a mutagenic MOA conclusion, even if data that might be useful are not available. This discussion also considers whether the database on the chemical is internally consistent in supporting a mutagenic MOA, including that for relevant non-cancer toxicities. Part of this analysis includes consideration of other potential MOAs that have significant biological support (see next section).

2.4.7 Other Modes of Action

If, through use of the MOA framework, alternative MOAs are found to have significant biological support, they may either argue against a mutagenic MOA or may be analyzed in conjunction with a mutagenic MOA. In general, the finding that another MOA is equally likely or more likely reduces the WOE that a chemical is operating by a mutagenic MOA. In some cases, however, both a mutagenic MOA and another MOA can operate simultaneously. If more than one MOA has significant biological support, they may be analyzed through to risk characterization and presented to the risk manager (as stated in the *Cancer Guidelines*).

2.4.8 Uncertainties, Inconsistencies, and Data Gaps

Uncertainties include those related to the biology of tumor development as well as those inherent in the data base on the chemical of interest. *Inconsistencies* for either a particular mutagenicity assay, or for a mutagenic endpoint, or for mutagenicity as a whole, are noted and, where possible, explained in the context of other data. Inconsistencies are flagged and *data gaps* identified. The discussion includes some indication of whether the identified data gaps are significant with regard to supporting a mutagenic MOA.

For any data set there will always be some uncertainties, inconsistencies, or data gaps. Thus, the analysis of biological plausibility and coherence for a mutagenic MOA will rely on some degree of inference. If such inferences are based on close similarity to a well studied chemical, they may be sufficient to overcome the limitations in the data base. In other cases, the link between the chemical of interest and data on other chemicals is too tenuous to overcome the deficiencies, and the MOA cannot be determined.

It is expected that many chemicals will not have data on some elements of the MOA that could be useful for supporting a particular MOA. Through use of the MOA framework, a determination is made whether there are ***sufficient*** data to support the hypothesized mutagenic

MOA or whether the uncertainties, inconsistencies, and data gaps are such that the WOE is ***insufficient to support the hypothesized mutagenic MOA***.

2.4.9 Is the Mutagenic MOA for Carcinogenesis Supported in Animals or In Vitro*?*

This step summarizes the data supporting the hypothesized mutagenic MOA. These data will most often be from animals or animal cells (*in vitro* or *in vivo*) or from human cells *in vitro*. A WOE analysis is used to determine that the data support a mutagenic MOA. As such, the conclusions for each part of the MOA analysis (discussed in the previous section) are clearly stated, and the observations on which the conclusions rely are presented in a manner that promotes understanding by those who will rely on the analysis. Thus, though all WOE analyses rely on professional judgment, the scientific basis for each conclusion, as well as the overall evaluation, will be transparent.

Data that may be useful for such a consideration include evidence of mutagenesis at the site of tumor formation; a discussion with examples is presented in Appendix D. For establishing a mutagenic MOA, *in vivo* data are most useful when genetic damage is examined in the target organ (e.g., observation of mutations in liver, liver Comet or liver UDS assay in the species showing liver tumors in the carcinogenicity studies). These organ-specific data are particularly convincing in establishing mutagenicity as the MOA. Certain assays may be better suited for specific tissues. For example, the micronucleus test has shown positive results for chemicals inducing neoplastic effect on the hematopoietic system, while DNA damage, DNA adduction, and transgenic mutational systems have shown results in liver and some other target organs. It is important to note that the currently accepted (see section 2.2) whole animal genetic toxicology assays are not designed to detect the specific mutation(s) that initiate the carcinogenic process. Rather these are surrogate tests that may demonstrate that the carcinogen is a systemic mutagen and that a mutagenic MOA is plausible. Also useful are data on induction of mutation in the target tissue using any one of several surrogate gene mutation assays. This provides plausible evidence that mutation is a key event. It should be noted that mutations obtained using the target organ or tissue may be used to address the issues of dose response and site concordance, and possibly temporal associations.

Chemicals with a mutagenic MOA for carcinogenicity frequently exhibit several types of mutagenic effects, such as point mutations and DNA strand breakage. Observation of only one mutagenic effect, however, does not rule out a mutagenic MOA.

If a chemical has been demonstrated to be a systemic mutagen, it is generally assumed that a mutagenic MOA is at least one of the operative MOAs.

2.4.10 Is the Mutagenic MOA for Carcinogenesis Supported in Humans?

In this step, the relevance of the MOA to human carcinogenesis is assessed. Human data, particularly epidemiology data, simplify this analysis. For most chemicals, however, there are few usable human data to support the association between exposure and carcinogenicity. Data from animal experiments may also inform an assessment of ADME. All available, relevant, and quality data should be used to evaluate whether a mutagenic MOA is supported for humans. In the absence of data, however, if a chemical has a mutagenic MOA for carcinogenesis in animal models and the chemical is a systemic mutagen, it is assumed that the chemical will operate through a mutagenic MOA in humans. This assumption is based on the knowledge that the key events, target tissues, and other related toxicokinetic and toxicodynamic processes related to a mutagenic MOA in humans are qualitatively similar to those observed in laboratory animals. Thus, unless data exist that suggest otherwise, it can be assumed that the key events in a mutagenic MOA for carcinogenesis will be the same across species.

Chemical-specific information that supports the relevancy of animal data to humans may include, but are not limited to, the following:

- *non-tumor data, such as ADME (that may identify metabolites, distribution of metabolites and/or parent compound, and excretion products) for both humans and the laboratory animal(s) used.* These data can be compared for similarities or differences.

- *observation of animal tumors at sites remote from the portal of entry*. Highly reactive chemicals may have a mutagenic MOA and produce tumors only at the portal of entry. Chemicals with a mutagenic MOA that act systemically would be expected to cause tumors at multiple sites. Generally, if tumors are observed only at one site, including only at the point of contact, a more thorough characterization of the MOA is presented than if the tumors occur as a result of systemic distribution. This characterization will include data (likely based on properties other than mutagenicity) of why tumorigenicity at only that site is consistent with a mutagenic MOA. In the absence of cancer data from human exposures, this discussion will support the relevance of these tumors for humans. Some very reactive chemicals with a mutagenic MOA are known to react only with tissues at the point of contact.

Evidence that the chemical induces mutations in cancer-relevant genes found in humans (e.g., *ras*, tumor suppressor *p53*, *Rb*, etc.) in the target tissue soon after exposure and prior to the development of the tumor can be very useful. While the techniques to detect and/or quantitate these rare mutations are beginning to be developed (McKinzie and Parsons, 2002), they are not currently available to detect all the possible mutations in the actual pathway(s) that results in tumors. It should be noted that demonstration of mutations in oncogenes or tumor suppressor

1 genes in tumor tissue is not sufficient evidence that mutation is a key event. However, data that a
2 specific chemical can induce mutations in relevant human cancer genes, does add to the WOE
3 that a mutagenic MOA is operative in human cancer.
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3.0 Implications of Determining that the WOE Supports a Mutagenic MOA for Carcinogenicity

Determination that a chemical has a mutagenic MOA for carcinogenesis invokes use of the *Supplemental Guidance*. The *Cancer Guidelines* and *Supplemental Guidance* state that chemical-specific risk estimates incorporating life-stage susceptibility are developed when data are available. In the absence of early-life studies on the specific chemical under consideration, early-life susceptibility is assumed for carcinogens operating through a mutagenic MOA. If appropriate chemical-specific data are not available on susceptibility from early-life exposures, the dose-response assessment for carcinogens operating through a mutagenic mode of action indicates that the ADAFs are used with the cancer slope factors and age-specific estimates of exposure in the development of risk estimates, as illustrated in section 6 of the *Supplemental Guidance*. A strong, biological rationale is provided for any decision to deviate from the *Supplemental Guidance*.

4.0 Glossary and Acronyms

ADAF(s)	age dependent adjustment factors(s)
ADME	absorption, distribution, metabolism, and excretion
aneuploidy	additions or deletions of a small number of whole chromosomes
cell transformation	assay in which cells in culture, with a limited ability to divide, are altered by chemicals to display increased potential for division
CHO	Chinese hamster ovary
Comet assay	alkaline single cell electrophoresis assay
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
gene mutations	small-scale change in the nucleotide sequence of a DNA molecule
genomics	the study of genes and their biochemical function in an organism
HPV	High Production Volume
IARC	International Association for Research on Cancer
ICH	International Conference on Harmonization
IWGT	International Workshop on Genotoxicity Testing
microarray	set of miniaturized chemical reaction areas used to test DNA fragments, antibodies, or proteins
MOA	mode of action
NTP	US National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
OPP	Office of Pesticide Programs
OPPT	Office of Pollution Prevention and Toxics
PBPK	physiologically-based pharmacokinetics
PMN	Premanufacture Notice for new chemical
POD	point of departure
RtK	EPA Chemical Right-to-Know program for High Production Volume chemicals
SAR	structure-activity relationships
SIDS	Screening Information Data Set
site concordance	mutagenicity and tumors observed in the same tissue or organ; tumors observed in the same tissue or organ in laboratory experiments and in humans
toxicodynamics	process of interaction of chemical substances with target sites and subsequent reactions leading to adverse effects
toxicogenomics	the collection, interpretation, and storage of information about gene and protein activity in order to identify toxic substances
toxicokinetics	the absorption, distribution, metabolism, storage, and excretion of toxic chemicals
TSCA	Toxic Substances Control Act
WOE	weight of evidence

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1 **Appendix A. Suggested Format for Organizing the Assays of Mutagenicity**
2
3 These examples are illustrative rather than comprehensive. The assays included, as well
4 as the organization thereof, depend on the quality and quantity of assay available.
5

In vivo **Assays Test System**	**Doses**	**Duration of Exposure**	**Tissue or Organ Affected**	**Results**	**Reference**
Somatic cell mutation: mammalian					
Transgenic assay					
Chromosome damage: mammalian					
Dominant lethal assay (germ cell)					
Micronucleus formation					
Chromosomal aberrations					
DNA Effects: mammalian					
DNA single strand breaks					
Unscheduled DNA synthesis					
Sister chromatid exchange					
Comet assay					
DNA adduct analysis					
Other in vivo assays					
1.					
2.					

6
7
8

In vivo **Assays Test System**	**Doses**	**Duration of Exposure**	**Tissue or Organ Affected**	**Results**	**Reference**
Chromosome Mutations					
Mammalian					
Dominant lethal mutation assay					
Micronucleus formation					
Chromosomal aberrations					
DNA Effects					
Mammalian					
DNA single strand breaks					
Unscheduled DNA synthesis					
Sister chromatid exchanges					
Comet assay					
DNA adduct analysis					

***In vitro* Assays** **Test System**	**Concentrations**	**Duration of Exposure**	**Results** With metabolic activation (+ S9)	**Results** Without metabolic activation (- S9)	**Reference**
Gene Mutation					
Bacterial					
Salmonella, reverse mutation					
E. coli, reverse mutation					
Mammalian					
CHO gene mutation, *hprt* locus					
Mouse L5178Y, *tk* locus					
Chromosome Mutation					
Mammalian Genotoxicity					
Micronucleus assay					
Chromosomal aberrations					
DNA Effects					
Mammalian					
Unscheduled DNA synthesis					
Sister chromatid exchanges					
Comet assay					
DNA adduct analysis					
Lower Eukaryote					
Saccharomyces cerevisiae, gene conversion					

Appendix B. Mutagenicity Testing Schemes in Use at EPA

There are multiple uses of genetic toxicology data. Two discussed in this *Framework* are these:1) in hazard identification either as a screen for an effect (such as cancer or heritable mutation) or as part of the weight of evidence (WOE) for an effect; 2) in assessing the mode of action (MOA) of a chemical. The EPA and other regulatory agencies have designed testing batteries largely to address the first (hazard identification) use of genetic toxicity data. While the focus of either a battery or tiered approach is on hazard identification, the testing schemes describe the range of genetic toxicity assays that can be useful in determining MOA.

As discussed in several papers (Dearfield et al., 1991; Cimino, 2002, 2006), the existing EPA test battery is a three-tiered system used by the Office of Pesticides Programs (OPP) and the Office of Pollution Prevention and Toxics (OPPT) under the auspices of the EPA Office of Prevention, Pesticides and Toxic Substances (OPPTS). Specific mutagenicity data requirements can be found in 40 CFR Parts 152 and 158, Proposed Rule, March 11, 2005, (OPP) Pesticide Assessment Guidelines, Subdivision F, Toxicology Data Requirements. The main statutory mandates for mutagenicity testing for other EPA offices also reside in these Toxics and Pesticides programs.

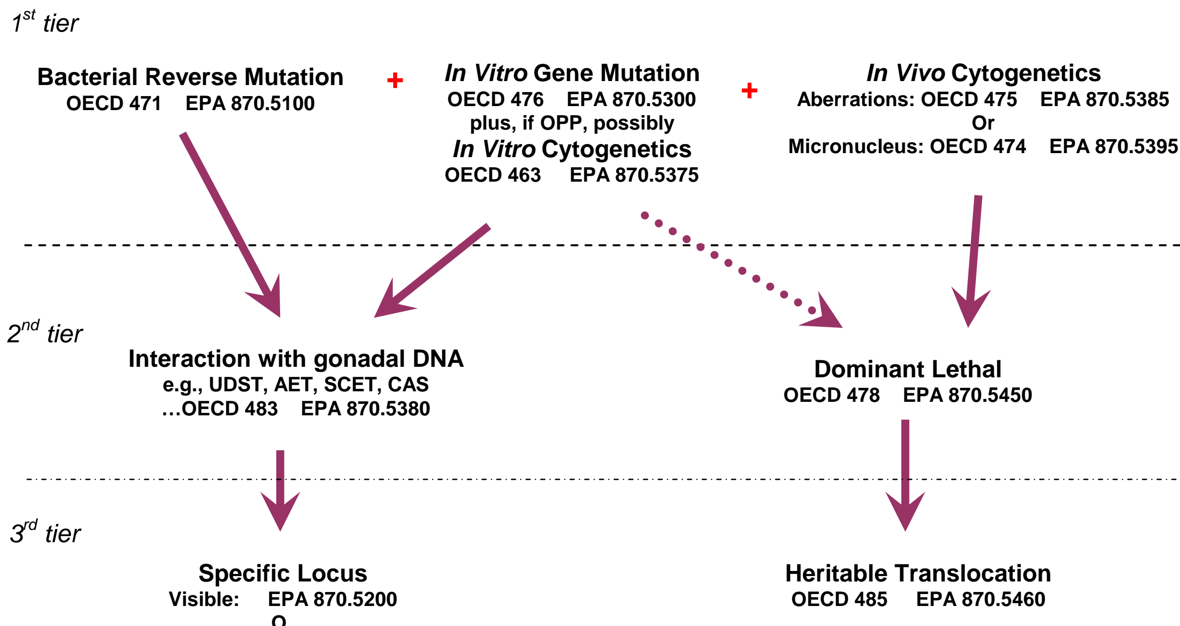
The current EPA test battery (Figure B-1) is a multi-test, three-tiered system, designed to address mutagenicity in both somatic and germinal cells. Because of the wide variety of genetic events that can occur, no single test is able to detect the entire spectrum of induced mutation(s). Therefore, the following comprise the first tier of the test scheme: (1) the bacterial reverse mutation assay for gene mutations (generally in *Salmonella typhimurium* and *Escherichia coli*), (2) the *in vitro* mammalian cell gene mutation assay (generally in mouse lymphoma cells), and (3) either the *in vivo* mammalian chromosome aberration or the *in vivo* micronucleus assay (Dearfield et al., 1991). For the Toxic Substances Program, two positive responses in mutagenicity assays, at least one of which is in an *in vivo* assay, is a trigger for consideration of a lifetime whole-animal bioassay for chemicals in a chemical class that has been identified to be carcinogenic. However, potential exposures and other factors would also influence decisions on the need for a two-year bioassay. Other combinations of responses, including a single positive response in one assay, or positive responses in the two *in vitro* tests but not in the *in vivo* test, may result in a "data review." This review, which occurs before a decision is made to require further testing, considers all available information from submitted studies and published articles, including metabolism and toxicokinetics, other test results, structure-activity relationships (SAR), production volume, and extent of potential exposure.

Chemicals that are negative in all first-tier tests generally require no further testing, unless exposure data, SAR, or other factors suggest that additional investigations are warranted. However, if positive result(s) are detected in the first tier of tests, a second tier of *in vivo* tests (Figure B-1) is used to assess mutagenic interactions with germinal tissue. Such tests may be the *in vivo* unscheduled DNA synthesis (UDS), alkaline elution (AE), sister chromatid exchange (SCE), and/or chromosomal aberration assays (all in testicular tissues), and the rodent dominant lethal assay. Other tests that have also been proposed as second tier tests for somatic and/or germinal tissue include: Comet (single cell gel electrophoresis) assays, transgenic assays, tests with "knock out" animal models, or DNA adduct studies (e.g., Dearfield et al., 2002). Gene and/or chromosome mutagens positive in second tier gonadal assay(s), after a data review, may be tested in third tier specific locus and/or rodent heritable translocation assays, respectively. Harmonized test guidelines have been developed for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations (U.S. EPA, 2005c).

For certain regulatory programs under TSCA, abbreviated test batteries are accepted. For new chemicals that meet specified criteria for potential volume and exposure, EPA requires a two-test battery, generally comprising the bacterial gene mutation and *in vivo* mouse micronucleus assays (Figure B-2). For certain chemical classes (e.g., acrylates, vinyl sulfones), there is a specialized requirement for *in vitro* gene mutation data in mammalian cells, specifically the mouse lymphoma test. For testing under the EPA Chemical Right-to-Know (RtK) program for High Production Volume (HPV) chemicals and the SIDS (Screening Information Data Set) program of the Organisation for Economic Co-operation and Development (OECD), a two-test battery is employed comprising the bacterial gene mutation test, and either the *in vivo* or *in vitro* chromosome aberration assay or micronucleus test.

Other regulatory agencies generally recommend batteries similar to the EPA battery, with some center-specific modifications (see Cimino, 2006, for a review). For human pharmaceuticals, the U.S. Food and Drug Administration (FDA) recommends three tests: (1) bacterial gene mutation, (2) *in vitro* mammalian cell assay (either mouse lymphoma or cytogenetics), and (3) *in vivo* cytogenetics (aberrations or micronucleus). This is the test battery proposed by the Fourth International Conference of Harmonization (ICH) for mutagenicity testing of pharmaceuticals (Muller et al., 2001). Other centers of the FDA generally recommend batteries similar to or the same as the ICH or EPA batteries, with some center-specific modifications.

1



2
3
4

5 Figure B-1. Mutagenicity testing scheme for EPA OPPTS for new chemicals. This three-tier
6 scheme applies to existing chemicals (e.g., test rules) and for pesticide registration. Test
7 guideline numbers for OECD and OPPTS guidelines ("OECD" and "EPA" respectively) are
8 indicated where appropriate. Abbreviations: UDST = unscheduled DNA synthesis in testicular
9 cells *in vivo*, AET = alkaline elution in testicular cells *in vivo*, SCET = sister chromatid exchange
10 in testicular cells *in vivo*, CAS = chromosomal aberrations in spermatogonial cells *in vivo*. The
11 guideline number under "Interaction with Gonadal DNA" applies specifically to the CAS test.
12 Modified from Dearfield et al. (2002).

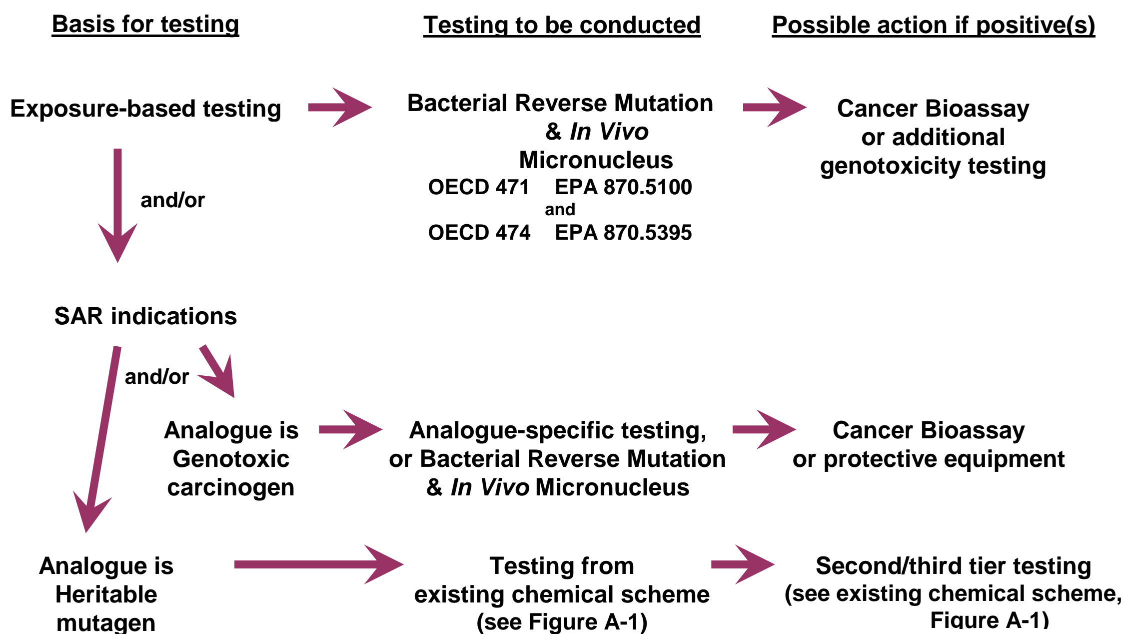


Figure B-2. Mutagenicity testing scheme for the EPA OPPT for new chemicals. This scheme displays the various conditions under which mutagenicity testing is required under Section 5 of TSCA, and the testing required under each condition. Test guideline numbers for OECD and OPPTS guidelines (as "OECD" and "EPA" respectively) are indicated where appropriate. Modified from Dearfield et al. (2002).

Appendix C. Examples of the Use of Structure-Activity Relationships in Assessing Mutagenicity

Mutagenicity assessment by EPA and other agencies frequently uses structure activity relationships (SAR) and analogue information. For example, the New Chemicals Program under the Toxic Substances Control Act (TSCA) does not mandate toxicity testing results be part of the initial data set provided in a Premanufacture Notification (PMN)[5] to the Agency. Therefore, the initial hazard evaluation on proposed new chemicals routinely involves SAR considerations, chemical categorization, and test data available either on substances analogous to the PMN chemical, on key potential metabolites of the PMN chemical, or on analogues of those metabolites. Furthermore, if mutagenicity testing is required for a PMN chemical based upon a New Chemical review or upon exposure-based requirements, and if an appropriate carcinogenic analogue has been tested in the same mutagenicity assay(s) as those required for the PMN chemical, this chemical is generally included in the genotoxicity test as an additional concurrent positive control.

Similarly, in the FIFRA Pesticides Program, if inconclusive or contradictory results occur among the submitted assays in the initial test battery, confirmatory testing or other relevant information may be required to clarify the potential mutagenicity hazard of the chemical. For example, additional *in vivo* cytogenetics testing may be required to address considerations from SAR.

For industrial chemicals under TSCA, where exposure data, SAR, or other factors warrant, existing chemicals negative in all three baseline mutagenicity tests[6] may be subject to additional testing. For both the Existing Chemicals and Pesticides Programs, two positive responses for mutagenicity in first tier testing, with at least one being *in vivo,* constitute an automatic trigger to a lifetime whole-animal bioassay. Any other combination of responses results in a "data review" before final decision is made to require further testing, and this data review considers all available information, including SAR (see Dearfield et al., 1991, and Figures B-1 and B-2 in Appendix B).

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[5] Before a manufacturer or importer introduces a new industrial chemical in the United States, TSCA Section 5 requires that it notify the EPA of its intent to do so via a Premanufacture Notification (PMN).

[6] These three tests are: the bacterial reverse mutation assay for gene mutations (generally in *Salmonella typhimurium* and *Escherichia coli*), the *in vitro* mammalian cell gene mutation assay (generally in mouse lymphoma cells), and either the *in vivo* mammalian chromosome aberration or the *in vivo* micronucleus assay.

DISCLAIMER: THIS INFORMATION IS DISTRIBUTED SOLELY FOR THE PURPOSE OF EXTERNAL REVIEW UNDER APPLICABLE INFORMATION QUALITY GUIDELINES. IT HAS NOT BEEN FORMALLY DISSEMINATED BY THE EPA AND SHOULD NOT BE CONSTRUED TO REPRESENT ANY AGENCY DETERMINATION OR POLICY.

The FDA has also employed SAR information in its decision-making (Matthews et al., 2006). Its Informatics and Computational Safety Analysis Staff (ICSAS) has provided *in silico* predictions for regulatory and research activities for food contact substances for the Center for Food Safety and Applied Nutrition (CFSAN), and for contaminants in pharmaceutical preparations for the Center for Drug Evaluation and Research (CDER). SAR has also been proposed as useful in screening for drug discovery and chemical selection.

In the United Kingdom, the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) has recommended a four-stage process in its draft Guidance on a Strategy for the Risk Assessment of Chemical Carcinogens (November 2003. One stage includes the identification of hazard based upon SAR.

In summary, SAR can be useful for providing supplemental information, either to confirm or complement, the database for a chemical regarding either mutagenicity or mutagenic MOA for carcinogenicity.

Appendix D. Concordance Between Specific Mutagenicity Assays and Observation of Cancer Outcomes

The following discussion cites some of the available literature showing concordance that, of necessity, refers to specific assays using particular chemicals. These discussions are considered as illustrative examples only. Mention of these assays and these chemicals does not imply that EPA considers these compounds to be carcinogenic through a mutagenic MOA. These examples are meant to illustrate concordance only. Furthermore, this Appendix is not intended to be an exhaustive review of the literature on concordance or on the chemicals mentioned.

Bone Marrow Chromosomal Aberrations/Micronucleus Induction

Ethylene oxide studies show micronucleus induction in mouse and rat bone marrow (Farooqui et al., 1993; Applegren et al., 1978) and rat spleen cells as early as 3 months after inhalation exposure to 600 ppm 1 hour/day, 5 days /week (Hochberg et al., 1990). There are also positive results from bone marrow chromosome aberration studies with this chemical (Farooqui et al., 1993; Ribeiro et al., 1987). The use of bone marrow chromosome aberration assays is appropriate to judge concordance between mutagenicity assay and cancer outcome since ethylene oxide is associated with malignancies of the lymphatic and hematopoietic system. Other investigators have found that the micronucleus test is a useful *in vivo* assay for chemicals that cause neoplasms in the hematopoietic system or when the lung is the target tissue for carcinogenesis (Morita et al., 2000).

Studies on DNA adducts

A strong correlation between DNA adduct formation and tumor incidence has been reported by Ottender and Lutz (1999) in their review of 27 genotoxic carcinogens including: methylating and ethylating chemicals such as n-methyl-N-nitrosourea (MNU) and diethylnitrosamine (DEN); chemicals forming adducts through epoxidation such as ethylene oxide and styrene; aromatic amines and nitroarenes such as 4-aminobiphenol, 2-acetylaminoflurene (2-AAF) and 2-nitrofluorene (2-NF); and polycyclic aromatic hydrocarbons such as benzo[a]pyrene. Results of studies that examined DNA adduct formation in tissue (primarily liver) harvested from rodents treated for 10 days with the test chemicals were reviewed. There was a 100% correlation reported between adduct formation and tumors. Some

of the carcinogens (i.e., MNNG, DMN, 2-NF), that induce tumors at multiple sites in rodents, were found to form DNA adducts not only in the liver but also in other tissues.

Alkaline Single Cell Electrophoresis Assay (Comet assay)

The alkaline single cell electrophoresis assay (Comet assay) has been used by several investigators to compare genetic toxicity in multiple organs of mice and rats with carcinogenicity. Sasaki et al. (1997) showed that the direct acting mutagen and carcinogen, ethyl nitrosourea induced DNA damage in liver, lung, kidney, spleen, and bone marrow cells of mice 3 and 24 hours after treatment, while the liver carcinogen, p-dimethylaminoazobenzene, was only positive in the mouse liver. In a later publication, Sasaki et al. (1999) presented data from Comet assays with 30 aromatic amines. The results indicated that carcinogens such as benzidine and β-naphthylamine caused DNA damage in multiple tissues including the liver, which is consistent with their induction of liver tumors in mice. The chemical β-naphthylamine, which also causes urinary bladder tumors in rats, was positive in the Comet assay with mouse urinary bladder. The rodent hepatocarcinogen, 4,4'-methylenebis(2-chloroaniline), was also positive for DNA damage in several tissues including the liver. Evidence of DNA damage in multiple tissue sites and correlation with tumor sites was found for 13 of the 30 compounds. All ten of the noncarcinogens included in this study were negative, and no cancer data were available for four other negative compounds. The rat carcinogen, p-chloro-o-toluidine was negative for DNA damage, while 2,4-diaminoanisole showed DNA damage in the mouse liver but not in any specific target tissue in either rats or mice.

Sekihashi et al. (2002) studied 30 chemicals listed by NTP and IARC as mouse carcinogens, rat carcinogens, or rat and mouse carcinogens in Comet assays by examining multiple organs. They found that the 23 mouse and/or rat carcinogens were positive in at least one mouse organ. Of the seven Comet assay-negative chemicals, four were not carcinogenic in rodents and three were not mutagenic. From these results, the investigators concluded that carcinogens that induce positive results in the Comet assay in at least one organ show a good concordance between the positive response and carcinogenicity in rodents. Although the study authors did not consider the concordance high between positive Comet assay results and tumor target organs, the study design was only for a single dose administration and a short exposure time (3, 4, or 24 hours).

In vivo Transgenic Models

The ability to detect somatic mutations in whole animals, made possible through the use of *in vivo* transgenic models, offers a potentially powerful tool for establishing site of action

concordance. Dean et al. (1999) reviewed the data from transgenic mouse mutation assays with 14 potent site-of-contact carcinogens that are also mutagenic including benzo[a]pyrene, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ); 7,12-dimethylbenz[a,h]anthracene (DMBA); dimethylnitrosoamine (DMN); N-methyl- N'-nitro-N –nitrosoguanidine (MNNG); 1-methyl-1-nitrosourea (MNU); 4-nitroquinoline-N-oxide (4-NQO, and β-propiolactone. They reported a good correlation between mutations in specific tissues and primary tumors at the same site. For example, increased mutation frequencies were seen in the forestomach and liver of *lac*I transgenic mice treated with MeIQ for 12 weeks; these findings are consistent with the induction of forestomach tumors in mice. The potent skin carcinogen, DMBA also increased the frequency of mutations in the skin of *lac*I and *lac*Z mice as well as inducing skin papillomas following topical application to TG.AC transgenic mice. In agreement with the findings of Otteneder and Lutz (1999) demonstrating positive Comet assay results throughout the gastrointestinal tract with MNNG (an inducer of glandular tumors), increased mutation frequencies were observed in the glandular stomach of *lac*Z transgenic mice. Additionally, Thier and Bolt (2000), confirming the findings of Walker and Skopek (1993), showed induction of mutations at the HPRT locus in the T-cells of B6C3F1 male *lac*I transgenic mice 2 or 8 weeks post-treatment with 0, 92, 183, or 399 mg/m^3 ethylene oxide 6 hours/day, 5 days/week for 4 weeks.

Use of Toxicogenomic Data

Toxicogenomics is a comparatively new scientific subdiscipline that combines the emerging technologies of genomics and bioinformatics to identify and characterize mechanisms of action of toxicants. Application of toxicogenomics can be expected in the extrapolation from experimental *in vitro* to *in vivo* systems and across the species barrier by further understanding specific molecular events underlying the mode of action of toxicants. Toxicogenomics is anticipated to serve as a powerful tool for evaluating the exposure to and effects of environmental stressors, and offers a means to simultaneously examine a number of response pathways including genotoxicity pathways. This in turn facilitates better understanding of the mode of action and hazard characterization of a chemical (Sarrif et al., 2005).

Several investigators have explored the use of microarrays to discern characteristic gene expression profiles for genotoxic carcinogens. Ellinger-Ziegelbauer et al. (2004) reported upregulation, i.e., an increase in the expression, of genes associated with the tumor suppressor protein and DNA damage in the livers of rats treated with four mutagenic hepatocarcinogens [DMN, 2-NF, aflatoxin B1, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone] at tumorigenic doses. Toxicogenomics/gene expression profiling was also used in a method attempting to distinguish genotoxic and non-genotoxic chemical carcinogens based on their mode of action (van Delft et al., 2004). The authors have demonstrated, by Pearson correlation analysis of gene

expression profiles from treated cells, that it is possible to correctly discriminate and predict genotoxic from non-genotoxic carcinogens. van Delft et al. (2005) have also demonstrated that several supervised clustering methods can separate certain genotoxic from non-genotoxic carcinogens by gene expression profiling. From the above examples, toxicogenomics holds great promise for its use in understanding the genotoxic mechanism and hazard identification of a chemical. If toxicogenomics data are available that can assist in either (1) prediction of the toxic properties of a chemical based on modulation of gene expression profiles or (2) discrimination between mutagenic MOAs and alternative MOAs, then such data is included in the WOE determination.

Toxicogenomic data may also be used for the detection of biomarkers (i.e., molecular indicators of a specific biological property that measure the progress of disease or the effects of exposure to carcinogens). For example, Ember et al. (1998) reported changes in the expression of genes associated with carcinogenesis (n-*ras* and *p53*) in the white blood cells of 20 hospital workers with exposure to ethylene oxide that could otherwise not be demonstrated.