Induction of Mutation Spectra by Complex Mixtures: Approaches, Problems, and Possibilities

David M. DeMarini

Genetic Toxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

More complex environmental mixtures have been evaluated for mutagenic activity at the hisD3052 allele of Salmonella, primarily in strain TA98, than in any other target or mutation assay. Using colony probe hybridization to detect a common hot spot deletion, followed by polymerase chain reaction and DNA sequencing, we have generated 10 mutation spectra from three classes of mixtures (i.e., urban air, cigarette smoke condensate, and municipal waste incinerator emissions). The mutation spectra are distinctly different among the three classes of mixtures; however, the spectra for samples within the same class of mixture are similar. In addition to the hot spot mutation, the mixtures induce complex mutations, which consist of a small deletion and a base substitution. These mutations suggest a mechanism involving misinsertion of a base opposite a DNA adduct followed by a slippage and mismatch. A role for DNA secondary structure also may be the basis for the mutational site specificity exhibited by the various mixtures. The results suggest that unique mutation spectra can be generated by different classes of complex mixtures and that such spectra are a consequence of the dominance of a particular chemical class or classes within the mixture. The problems associated with this type of research are discussed along with the potential value of mutation spectra as a tool for exposure and risk assessment. — Environ Health Perspect 102(Suppl 4):127–130 (1994).

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Introduction

Exposures to harmful substances that most people encounter tend not to be in the form of a single agent. Except for a few specialized industrial settings, most people are exposed to many potentially harmful agents at the same time, such as automobile exhaust (1). Exposures to such complex mixtures pose especially difficult regulatory and scientific problems. Considering the presence of mutagens alone in complex mixtures presents a challenge to the genetic toxicologist in terms of determining the genotoxicity of mixtures and identifying the genotoxic components of mixtures (2).

The earliest studies on the genotoxicity of complex mixtures began in the 1950s and involved mostly cytogenetic evaluations of mammalian cells in culture exposed to cigarette smoke (2,3). However, the study of the genotoxicity of complex mixtures can be said to have truly begun in 1974 with the report by Kier et al. (4) on the mutagenicity of cigarette smoke condensate in Salmonella. This important paper showed, for the first time, that an assay that had already been well characterized with a large number of single compounds also could be used to evaluate a complex mixture. Moreover, the authors paralleled previous studies in carcinogenesis by showing that chemical fractions of a complex mixture also could be evaluated for mutagenicity. Such a study thus provided insight into which chemical classes were responsible for the mutagenicity of the whole, unfractionated mixture.

During the 20 years since this important contribution by Kier et al., almost every type of environmental complex mixture has been evaluated for mutagenicity in the Salmonella assay (2). Combined with chemical fractionation and analysis (a procedure generally referred to as bioassay-directed chemical analysis), the Salmonella assay has permitted a detailed understanding of the chemical classes as well as single chemicals within our air, food, and water that are mutagenic and, potentially, carcinogenic (2,5–8). Collectively, these studies have been extremely useful in identifying mutagens in our environment and in improving risk assessment for exposure to complex mixtures (9,10).

Almost all types of mutational studies that can be performed with single chemicals also have been used to study complex mixtures. However, there is one important type of study that is currently performed with single chemicals that, until now, has not been attempted with a complex mixture. This is the molecular analysis of mutations induced by complex mixtures and fractions of complex mixtures and the subsequent generation of mutation spectra induced by complex mixtures.

The development of new techniques by which mutations can be examined at the molecular level has finally permitted molecular access to various genomes that were previously either unexamined or poorly understood at the molecular level (11). Because Salmonella has been used more than any other organism to evaluate the mutagenicity of complex mixtures, we have begun to apply some of the new molecular techniques to the analysis of mutations induced in Salmonella by a variety of environmental mutagens and complex mixtures. This paper provides a brief summary of the approaches used, the problems encountered, and the possibilities presented by such an approach to the study of the mutagenicity of complex mixtures.

Approaches

Of all of the Salmonella Ames tester strains, those containing the hisD3052 allele, especially strains TA1538 and TA98,
have been the strains used most for evaluating the mutagenicity of complex mixtures (2,7). Thus, our first approach was to develop techniques by which this allele could be analyzed easily and quickly at the DNA level. The hisD3052 allele contains a -1 deletion, and previous efforts at molecular analysis of mutations induced at this allele have been summarized (12,13). These studies revealed the presence of a hot spot for reverversion within the hisD3052 allele that consists of a deletion of a GC or CG within the sequence CGCGCGGC. As much as 50% of the spontaneous revertants of this allele, and frequently as much as 90% of revertants induced by various single compounds, are reverted by this pathway.

Recognizing this fact, Kupchella and Cebula (14) have developed a colony hybridization procedure that permits one to screen a large number of revertants of the hisD3052 allele for the presence of this hot spot mutation. This procedure is rapid and inexpensive and eliminates the redundant sequencing of the same mutation by standard DNA sequencing methods. The exquisite sensitivity of the oligonucleotide probe used to detect this two-base deletion was enhanced by the inclusion of an additional, unlabeled probe that hybridizes to the DNA sequence at a site that is adjacent and 3' to the labeled probe (14). This second probe prevents the formation of hairpin loops (that occur because of the quasi-palindromic sequence within this region), thus allowing greater access of the labeled probe to the region of interest, which is adjacent and 5' to the unla-beled probe. Because the frequency of this hot spot deletion can be extremely high, we routinely screen 400 revertants from any particular treatment. This usually provides 20 to 40 or more revertants that do not contain the hot spot deletion and that require DNA sequence analysis to determine what mutation such revertants contain.

The development of the polymerase chain reaction (PCR) has greatly facilitated the ability to sequence these revertants by providing a rapid means by which the region of interest can be amplified in quantities suitable for DNA sequencing. We (12) and others (14) have developed similar applications of PCR technology to amplify a region of the hisD3052 allele. Briefly, our approach first involves the purification of revertant colonies on minimal medium supplemented with biotin. Then, a colony is boiled for 10 min in 200 μl of TE buffer, centrifuged for 10 min, and 5 μl of the resulting supernatant is used to provide the Salmonella genomic DNA in an asymmetric PCR (12). The two amplification primers span a 328-bp segment that contains the hisD3052 allele mutation approximately in the center. Single-stranded DNA is then generated by asymmetric PCR using a 1:100 ratio of the primers and 40 cycles of heating and cooling. We have shown recently that excessive cycling beyond 40 converts the amplified fragment into random-length higher molecular weight fragments, resulting in a dramatic loss of the desired PCR product (15).

The excess amplification primers and deoxynucleotides are removed from the PCR mixture by ultrafiltration in a Centricon-30 microcentrator, and the amplified DNA is then dried and resuspended in 10 μl of dH2O. The amplified fragment is sequenced in a microtiter plate using dITP termination mixes because compression of the bands on the sequencing gel may occur with standard dNTPs due to the high (60%) GC content of this region of the Salmonella genome.

The approach to the molecular analysis described above is applicable to mutations induced at the hisD3052 allele by either single agents or complex mixtures. However, one additional step posed by complex mixtures involves the merging of this molecular analysis of revertants with techniques for bioassay-directed chemical analysis of the mixture. As discussed previously, bioassay-directed chemical analysis has proven to be highly useful in identifying the chemical classes and, in some cases, single chemicals in complex mixtures that are responsible for some or most of the mutagenicity of the mixtures.

Recently, we have begun to merge these two technologies for the purpose of identifying the mutations and generating mutation spectra induced by various types of complex mixtures, including an unfractionated and a neutral and base fraction of an ambient urban air sample (16), main-stream and side stream cigarette smoke condensate (17), and municipal waste incinerator particles (18). Our approach has involved the generation of mutation spectra from the unfractionated organic extract of a mixture as well as from selected mutagenic fractions from the mixture. The coupling of microsuspension mutagenicity assays with high pressure liquid chromatography (HPLC) to produce mutagrams (19,20) has afforded us an additional level of fractionation from which to generate mutation spectra.

**Problems**

There are a variety of issues that must be considered when attempting to generate mutation spectra with complex mixtures. A problem common to all studies on complex mixtures is the suitability and representative nature of the sample to be studied. Sample selection must be considered carefully before proceeding with a molecular analysis of the revertants. This is especially true for the present study because the time and expense required to generate a mutation spectrum precludes such an analysis from being performed routinely on a large number of samples. Thus, we have used well-characterized samples from standard sources to induce mutation spectra. These have included an extensively studied composite air sample from Boise, Idaho, that was collected as part of the US EPA's Integrated Air Cancer Project (IACP) (21), particular emissions from a municipal waste incinerator in North Carolina (20,22,23), and main stream and side stream cigarette smoke condensate generated from Kentucky 2Rl reference cigarettes on a cigarette smoking machine operated under standard puff conditions at the Institute for Tobacco and Health Research, Lexington, Kentucky (17).

As with single compounds, the mutagenic potency of a mixture is a critical factor because of the need to have an induced mutant yield that is adequately elevated relative to the spontaneous mutant yield. It is important to be able to distinguish the spontaneous mutations from the induced mutations in a mutation spectrum. Thus far, we have been able to achieve fold increases of 7 to 29 times over background with the whole or fractionated mixtures that we have studied. Our experience suggests that molecular analysis of revertants of the hisD3052 allele at fold increases less than five times over background might not yield a mutation spectrum that is sufficiently distinct from the spontaneous spectrum to permit any conclusion about what types of mutations were induced by the mixture.

A related phenomenon is that all of the complex mixtures (as well as nearly all single compounds) that we have evaluated at the hisD3052 allele induce a significant increase in the frequency of the hot spot two-base deletion (18). Most (>90%) of the revertants induced in TA98 by combustion emissions and air samples (and their respective fractions) contain the hotspot deletion (18). In this regard, these complex mixtures do not produce mutation spectra that are distinct from each other. In order to circumvent this problem posed by the presence of the hot spot in hisD3052, we have routinely screened 200 to 400 revertants at the highest fold over background that was achievable in order to have a sufficient number (about 40) non-hot spot revertants remaining to PCR
and sequence. Even though these revertants may account for only about 10% of the induced revertants, we have been able to show that the mutation spectrum composed of the mutations from these remaining non-hot spot revertants is unique for each complex mixture (unpublished results).

Although the target for reversion at the hisD3052 allele is at least 75 bases long and can be reverted by at least 200 different mutations (24), the hot spot region makes it a difficult allele to use to determine mutagenic specificity. A recent study by Koch et al. (25) showed that for 10 mutagens examined at the hisG46 allele in strain TA100, a unique mutational specificity was found for most of the agents. This is quite remarkable considering that the target for reversion of the hisG46 allele is extremely limited. Thus, size alone does not determine the ability of a DNA sequence to provide broad mutagenic specificity. Unfortunately, most complex mixtures, with the exception of drinking water extracts, are more mutagenic in strain TA98 rather than in TA100.

An essential consideration in the construction of a mutation spectrum involves the assurance that the mutants were independent in origin and did not arise from a single clone. With regard to the Salmonella plate-incorporation assay, we initially went to considerable lengths to assure the independence of the revertants that we analyzed. In our first study, we established separate cultures for each plate and picked only one colony randomly (from the center) from each plate (12). However, we have abandoned that approach, and we (13) and others (14) now use a single culture for a particular experiment and pick all (or most) of the revertants from a single plate. This approach is justified because revertants generated in the plate-incorporation assay are independent in origin because each arises from a single cell that is immobilized and physically isolated from other cells within the top agar. Thus, a mutation spectrum constructed from the mutant sequences of a set of revertants from a single plate can be assumed to be composed of mutations that are independent in origin.

It was conceivable that incubation of cells in a microsuspension assay could pose a problem in terms of the independence of the revertants resulting from such an assay. In order to investigate this possibility, we determined that during a 90-min incubation, the cells did not replicate in the absence of S9, although they replicated 1.5 times in the presence of S9 (26). Based on these results, we have concluded that the probability of sibling mutants occurring during the 90-min incubation is insignificant. Thus, we have proceeded with the construction of mutation spectra generated from revertants induced by HPLC fractions in the microsuspension assay. In these cases, we have picked most of the revertants from a single plate for molecular analysis. An additional consideration with both the plate-incorporation and microsuspension assays is the possibility of jackpot mutations within the starting culture. These, no doubt, occur, and one must interpret mutation spectra carefully, keeping in mind the possible influence of such mutations.

Needless to say, if a state-of-the-art molecular analysis is coupled to an inferior chemical fractionation scheme, then little of value will be learned from the resulting mutation spectra. It is critical that well-defined fractionation procedures be used and that these be coupled with suitable chemical analyses so that there is some understanding of the chemical nature of the fractions. Our experience with fractionating complex mixtures has indicated that different schemes may be necessary for different classes of complex mixtures (20-23,27). Thus, urban air samples may require different fractionation schemes than cigarette smoke condensates. However, mixtures of the same class (diesel exhausts from a variety of engines, for example) may likely be fractionated equally well by the same fractionation scheme.

**Possibilities**

The potentially important role that might be played by mutation spectra has been recognized for over a decade (28). By comparing a person’s mutation spectrum early in life (and viewing this as representing a background mutation spectrum) to spectra derived later in a person’s life (and viewing these as containing induced mutations along with the background), one might determine that the person had incurred a mutation induced by a particular environmental mutagen (28). Although this is still a distant goal, recent studies suggest that there may be some merit in thinking that certain environmental exposures may produce certain, distinctive, molecular signatures in DNA.

We have now generated 10 mutation spectra at the hisD3052 allele of TA98 with three classes of complex mixtures: cigarette smoke condensate (two spectra), urban air (two spectra), and municipal waste incinerator emissions (six spectra). Given the limitations and difficulties associated with producing mutation spectra at the hisD3052 allele, we have found that each of these three classes of mixtures produces a unique mutation spectrum with different hot spots. Furthermore, within a class, the mutation spectra are quite similar, showing the same hot spots and warm spots and similar mutations. Such features begin to make feasible the possibility that even mixtures as complex as these will produce unique spectra that may be predictive.

One of the conclusions that we have drawn from these spectra is that certain chemical classes must predominate in each mixture to produce the mutations unique to that mixture. Researchers have recognized for many years that complex mixtures such as air samples contain thousands of compounds, many of which are mutagenic to Salmonella TA98. Even the HPLC fractions that we have evaluated may contain hundreds of compounds. In addition, the mutagenic activity of various types of mixtures can frequently be accounted for by the presence of certain chemical classes within that mixture. For example, nitroaromatics account for much of the mutagenic activity of diesel exhaust; whereas aromatic amines account for much of the mutagenic activity of cigarette smoke condensate. Thus, it is not altogether surprising that the mutation spectra produced by a particular mixture is as unique as that produced by a single agent. Whether such a situation will prevail at other DNA targets in other organisms, and especially in humans, remains to be determined. However, these early results provide a basis to pursue such work in Salmonella as well as in other organisms, including humans.

The generation of mutation spectra induced by single compounds has permitted the inference of mutational mechanisms. Even with single compounds, however, the mechanisms may be complex. For example, we have shown recently that the relatively simple intercalating agent ellipticine produces two hot spots at the hisD3052 allele. However, several mechanisms may be involved, including intercalation, DNA adducts, DNA secondary structure, DNA slippage and mismatch, and possibly DNA gyrase (13). It is becoming clear that some mechanisms such as the slippage/mismatch of direct repeats can account for deletions and duplications in a wide variety of organisms, including Salmonella at the hisD3052 allele and humans at the HPRT gene. Other mechanisms may be unique to a particular organism or DNA sequence.

We have found that most mutations induced by complex mixtures at the hisD3052 allele can be accounted for by mechanisms that explain the induction of mutations by various single compounds. For example, the complex mutations induced by
4-aminobiphenyl in TA98 are similar to those produced by cigarette smoke condensate and urban air, and they are explainable by a mechanism in which an incorrect base is inserted opposite an added base, followed by a slippage and mismatch (29). The resulting mutation involves a small deletion of several bases and a base substitution. Thus, studies with single compounds are essential to understanding mechanisms of mutation by complex mixtures, and such studies should proceed in parallel. The next decade should permit a test of whether mutation spectra will be useful in identifying the types of mutagenic exposures encountered by people and whether mutation spectra will provide useful information for estimating the risk to health imposed by such mutations. Further advances in DNA sequencing and in the Human Genome Project should permit the generation of mutation spectra in people at genes other than HPRT and at a more rapid rate than is now possible. Comparison of these spectra to those generated in laboratory organisms might shed light on the causes of certain mutations and lead to a more informed estimation of health risks than is currently available.

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