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MUTATIONAL SPECTROMETRY IN ANIMAL TOXICITY TESTING

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INTRODUCTION

Radiation or chemical mutagens produce unique distributions of point mutations with regard to kind and position when studied in homogeneous cell populations. It should therefore be possible to learn if a tested substance has had a genetic effect on a particular animal or even human tissue by examining this distribution before and after treatment. If one had the *means* to observe such distributions of mutations, called mutational spectra, one could evaluate the effects of proposed pharmaceuticals, pesticides, cosmetics, or food additives by using concentrations within the range of intended use and with any of the test animal tissues as might be desired.

Each cell within a tissue contains identical DNA sequences in frequencies of one to ten thousand copies per cell. Each tissue in a small rodent contains from 10^6 to 10^8 cells. An analytical approach that could use all of the cells and all of the DNA copies would have the statistical advantage of requiring small numbers of animals to obtain specific information about the genotoxicity of any substance. Since the time of genetic fixation of damage is dependent on the amount of cell division in each tissue, experiments with young growing animals might be as short as a month. Yet the quality of the information derived should exceed that obtained by lifetime studies with several hundred animals. Because several oncogenes are now known to be associated with tissue-specific tumors in the form of specific point mutations, it should also be

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possible to study mutational spectra induced in one or several oncogenes on a tissue-by-tissue basis.

The scope of this review and discussion is to acquaint pharmacologists and toxicologists with the general history of mutational spectra, refer to illustrative observations to date in single cell systems amenable to the techniques of microbial genetics, and introduce recent technological advances which in combination have made it possible to consider the direct studies of mutation in animal tissue as suggested above. Also within the scope of this review are the delineation of limitations of present theory and technology. Students should know at the outset that while progress has recently accelerated, there is much more to do than has been done.

Mutational Spectra

A *mutational spectrum* is the set of all mutations found in a defined DNA sequence in a population of homogeneous cells or viruses. Point mutations (base-pair substitutions, small insertions, and small deletions) and larger events (large deletions, insertions, rearrangments, transpositions, etc) are all part of the ensemble of genetic changes that characterize response to mutagens. However, this chapter focuses on point mutations exclusively because it is the opportunity to exploit this subset that has motivated most recent research.

The beginning of mutational spectrometry may be fairly traced to Benzer & Freese' 1958 paper "Induction of specific mutations with 5 bromouracil" (1). This paper demonstrated that spontaneous mutations in the rII genes of the bacteriophage T4 differed from 5 bromouracil-induced mutations both with regard to the position of mutations and the kind of mutations observed. The ability to make these observations was based on Benzer's exploitation of direct genetic recombination among T4 mutants and conditional rII mutants which would not form plaques on *Escherichia coli* lambda lysogens. In 1955, Benzer (2) had first created fine structure map of the rII region, while Freese recognized the fact that each point mutant could be tested for identity with another on the basis of its reversion rate after various mutagen treatments. Their basic approach has been used without essential variation until the present day, although there have been several facilitating improvements. Miller (4, 5) developed an ingenious set of microbial genetic techniques to facilitate identification of any of a set of base-pair substitutions leading to stop codons in the lac I gene of *E. coli*. Skopek (7) combined direct DNA sequencing methods after selecting individual mutants in the cI gene of bacteriophage λ.

In a typical experiment, viruses, bacteria, yeasts, or animal cells are grown as homogeneous populations and variants in a phenotypic trait associated with a particular DNA sequence are selected as plaques or colonies. One may use

an experimental design such that only one variant or mutant is taken from each of a series of independent populations to assure that each mutant examined represents an independent mutational event. The position and/or kind of DNA change is then determined for each isolated mutant.

Of particular importance is the point that in these experiments mutant viruses or bacteria are isolated on a mutant-by-mutant basis. The process of examining hundreds of individual colonies placed a severe burden on individual researchers. In fact, even though Benzer & Freese were recognized as having made important observations, no additional laboratories reported comprehensive chemical mutational spectra studies between the early 1960s and Miller's first paper in 1977.

Although DNA-sequencing technology has led more laboratories to use mutational spectra, it was Miller's lead in the continuing series of papers from 1977 that breathed new life into this important area of research. In fact, so many papers have been published in the last few years on mutational spectra that several scholarly manuscripts could be created comparing and contrasting the observations. Readers interested in procedural aspects and specific observations for individual mutagens are referred to the recent work of Calos (8, 9), Drinkwater (10, 11), Fuchs (12, 13), Glickman (14–17), Hutchinson (18, 19), Miller (20–22), Ripley (23–26), Skopek (27–29), and Tindall (30–32), with their respective collaborators.

Each of these papers reveals a painstaking effort to avoid experimental biases. Spontaneous spectra are determined by growing a large number of small cultures and selecting one and only one mutant from each. This use of independent mutants reduces to zero the probability that two mutants characterized for a spectrum have arisen from the same mutational event in a common ancestor (sib selection). This process also reduces the effect of jackpots, early mutations in single small populations, which afterwards constitute a large fraction of mutants within that particular population. In many, but not all cases, reconstruction experiments have been performed to discover if any particular mutant phenotype has a selective advantage or disadvantage in the experimental protocol.

Mutagen-induced spectra are obtained with similar care. Either large numbers of small independent cultures are treated separately, or a single large culture is treated and immediately subdivided into small cultures before cell division could create sibling mutants. Special care is taken to assure that induced mutants are numerically predominant over background mutants.

In one respect, however, almost all these reports must be considered preliminary in nature for reasons of statistical imprecision. The problem with all but some of the first papers from Miller's laboratory (e.g. 5) is that the drudgery of colony-by-colony or plaque-by-plaque analysis has sapped the energy of even the most dedicated researchers before the information poten-

tially available was wrung out of the experiments. As B. W. Glickman recently put the problem in discussing his laboratory's experiences, "You can get a graduate student to sequence 1000 clones but you can't get him to do it again." Miller, appalled at the large and growing volume of published observations of too low precision for confident discussion of mechanism, mutters about "sequencing shops." Both of these gentlemen have a good point.

The problem lies in the variety of possible mutations, the generally large (>500 bp) genes coding for selectable markers, and the fact that phenotypic variants represent a small fraction of all of the possible genotypic mutants in any population.

A thought experiment may illustrate these problems. Imagine that a gene of 500 bp codes for an enzyme that converts a nontoxic drug to a toxic drug intracellularly. Mutations within the 500 bp that lead to an inactive gene product lead to recognizable colonies when individual cells are allowed to attempt to grow in the presence of the nontoxic selective agent. Ignoring large deletions, insertions, etc and focusing on point mutations, we note that there are three possible single base-pair substitutions for each of 500 bp; there are also a set of ± 1, ± 2, ± 3 . . . small insertion deletions that may formally be considered to occur at the base-pair immediately 5' to the event. Considering single base-pair substitutions and ± 1 insertion deletions only, we can calculate 2500 different mutations of these kinds are possible within the 500 bp gene. On average, each possible mutation would constitute a mutant fraction of 4×10^{-4} if each occurred with equal probability.

However, if one particular mutation occurred with *100 times* the average rate it could constitute 4×10^{-2} of the total mutants. Analysis of 1000 independent mutants would be expected to yield 40 ± 13 (95% c.l.) mutants of that kind and position, which would constitute 4% of all the mutants isolated and sequenced. Few of the papers cited involve studies of 1000 or more colonies in a single experiment, the vast majority involve fewer than 100 clones, and many report results on the basis of a few dozen isolates.

This shortcoming does not detract at this early stage from the excitement of identifying mutations of exceptional high frequency (hotspots). Not much has really changed since Benzer (3) noted "that the distribution is non-random leaps to the eye" for one site he mapped in the rII region of T4 had 500 of 1612 spontaneous mutants. As Singer et al (33) pointed out, Benzer (34) knew that mutants at one of his hotspots had differing reversion frequencies and thus represented at least two separate mutations mapping very close together; Singer et al report reexamination of Benzer's spontaneous hotspots by direct DNA sequencing and confirming that each hotspot contained several different mutations.

At this stage of research one must avoid the too facile conclusion that the

most prominent hotspots constitute all that we should want to know about mutational spectra. If mutational spectra are to be of real use in mechanistic or diagnostic studies, numerical methods of analysis must be developed which can first distinguish nonrandom from random distributions and then test the probability that two spectra are or are not identical.

Which of several forms such analyses may take is immaterial at this point. For the purpose of this discussion it will be posited that even though one wishes to maximize the number of mutants observed, one would be content (for the time being) to be able to use information from any hotspot that represented 1% or more of the mutants present in a population. Roughly speaking (subject to completion of an analysis in progress), a set of mutations will be required approximately ten times larger than the number of base pairs in the selected DNA target. In the case of Couloundre & Miller's magnum opus (5), the spectra involved fewer than 100 selectable base-pair changes so that apparently similar spectra induced by the alkylating agents ethyl methane sulphonate and methyl nitro nitrosoguanidine could be distinguished statistically with *only* 600–900 mutants for each mutational spectrum. [Analysis was by X^2 of all mutations exceeding 5 mutants.]

Using this suggested rule of thumb, one would require an experimental approach yielding 1000 mutants for every 100 bp in a selected target gene. The novitiate has three choices: continue the present mode and dutifully publish at intervals of 1000 mutants; automate the process so that more mutants may be sequenced in a shorter period of time; or devise an approach to observe all of the mutants simultaneously.

If by any path the researcher perseveres, he or she will have a high probability of success so long as studies are confined to single cell or virus cultures. It is in following this line of experiments from petri dishes to experimental animals and humans that the ratiocination and cleverness of microbial genetics meets an apparently insurmountable obstacle.

THE GENERAL PROBLEM OF STUDYING MUTATION IN EXPERIMENTAL ANIMALS AND MAN

At the outset, it should be noted that the laboratories of R. J. Albertini (35) and A. A. Morley (36) have independently demonstrated that one may sample large numbers ($\sim 10^7$) of circulating peripheral T cells, induce them to undergo sufficient cell division in cell culture to produce macroscopic colonies and observe that a small fraction of such cells ($2–10 \times 10^{-6}$) will form colonies in the presence of the selective agent 6 thioguanine. Such 6-thioguanine–resistant T cells do not express an active form of the enzyme hypoxanthine guanine phosphoribosyl transferase, coded by the X-linked ***hprt*** gene.

Using the same approach, work in several laboratories has indicated that approximately 10% of these selected mutants have suffered a large deletion of all or part of the *hprt* gene. As many as 90% therefore appear to be point mutations effecting control of transcription, mRNA splicing, protein structure, etc and may be expected to be distributed as point mutational spectra.

Unfortunately, the approach of Albertini and Morley, an extension of microbial selection techniques, depends on the tissue sampled (blood) containing sufficient numbers of cells capable of dividing 15 consecutive times to produce a macroscopic colony. Secondly, the number of colony forming T cells in 10 ml of human blood does not exceed 10^7 so that mutant fractions of $2–10 \times 10^{-6}$ limits practical studies to 20–100 *hprt*-deficient mutants. Promoter, reading frame, and intron-flanking sequences in which mutations could effect HPRT function contain about 1000 bp, so the number of mutants available falls far short of the $10 \times 1000 = 10{,}000$ desired for a spectrum in which hotspots constituting 1% of the population might be used in analysis. Thus our present technology for mutation measurements in humans is limited to one-cell type and by the small number of mutations occurring in a practical sample.

For the sake of argument, however, let us imagine that one could miraculously know the sequence of any and all copies of a DNA sequence of any 100 bp sequence of an animal's genome for any tissue. Obviously this would overcome the problem of limitations caused by the requirement of cell growth. I posit that the means to do so have been developed and turn to the question of obtaining sufficient numbers of mutants to constitute a point mutational spectrum in which all events of 1% frequency or higher are known with reasonable (± 30%) precision.

To explore this question we will assume that the observation of Albertini's (35) and Morley's (36) laboratories regarding T cells are valid for other tissues and, as a consequence, for *hprt*-null mutations a point mutant fraction of 5×10^{-6} is distributed as a spectrum over 1000 bp. Thus, we may expect a mutant fraction of 5×10^{-7} to be distributed over 100 bp. Furthermore, the number of actual point mutations (genotype) will far exceed the number of observed mutations (phenotype) and we will assume a ratio of 7:1, subject to further observation. This is the same as assuming a total point mutation fraction of 3.5×10^{-6} for a 100-bp sequence. If we want 1000 mutants we will need $1000/3.5 \times 10^{-6} \equiv 3 \times 10^8$ copies of such a 100-bp sequence in our tissue sample.

Approximately 3 gm of liver would contain 3×10^8 cells and may be taken as a rough estimate of the tissue sample size if one were to choose a 100-bp DNA sequence present once per diploid genome such as the *hprt* gene in male cells. *But there is no requirement that we should choose such a single copy sequence!* We may halve our sample size simply by choosing an autosomal

sequence present in two copies per diploid genome. One might even choose sequences such as the ribosomal RNA genes found in hundreds of copies per cell, mitochondrial sequences present in thousands of copies, or even subsets of highly repetitive nuclear DNA families of unknown physiologic function. As long as the set of multicopy sequences did not contain inherited variations that could be confused with postconception somatic mutations, any of these possibilities would be numerically equivalent and would drop our requirement of 3×10^8 cells for a single copy sequence to as low as 3×10^5 cells (1000 copies/cell.)

We know such multicopy sequences exist and have assumed pro tempore that one can obtain the point mutational spectrum for certain 100-bp sequences. Thus, one has also assumed that one could obtain the mutational spectrum from any tissue or organ in an experimental animal from which the DNA of 3×10^5 or fewer cells could be isolated. This includes any available tissue of cellular mass greater than 3.0 mgm and permits us to consider nearly any tissue, including germ tissue of experimental rodents.

An experiment to determine if a test substance induced significant mutation in a tissue would be performed as follows: A group of test animals is exposed to the substance. Concentration and duration of exposure are experimental variables as are the sex, and age of the animals. Matched controls consist of unexposed animals and animals exposed to subtoxic doses of mutagen such as X-rays, which are expected to induce mutations in all tissues.

At such time as it is known from prior studies that tissue mutant fractions have reached a stable maximum (which depends on species, tissue, sex, and age) the animals are sacrificed, DNA isolated from all available tissues and the *total number* and *distribution* of point mutations is determined for the particular 100-bp sequence studied. Assuming the concurrent positive and negative controls exhibit behavior consistent with historical control observations, the treated tissues may be statistically compared to the concurrent and historical negative control values for total mutant fraction and distribution of mutations. If the total mutant fraction has been significantly increased by treatment *and* the spectrum of mutations in tissue of identically treated animals is significantly different from that of matched controls, it may be concluded that the treatment induced point mutations in that tissue and that the difference spectrum observed is the mutational spectrum of the test substance in vivo with regard to that tissue's cell type.

Consider the problems of interpretations of toxicity testing which are addressed directly by this stratagem.

NUMBERS OF ANIMALS To avoid "jackpot" mutations during organ development, more than one animal must be used for each experimental condition. However, three animals would be sufficient to avoid this statistical

artifact. This may be compared to the number of animals required for measurement of cancer induction (hundreds) and induced heritable change (thousands).

AMOUNT AND DURATION OF EXPOSURE The sensitivity of the test may be expressed in terms of distinguishing induced mutational spectra from that seen in the untreated animals. Roughly speaking, an induced mutant fraction three times the background fraction can be induced in human cell cultures by essentially instantaneous subtoxic treatments with heat and ultraviolet light or by treatment for several days with subtoxic concentrations with chemical mutagens (37, 38).

One may expect, therefore, to observe mutation in vivo at exposure levels well below thresholds for acute toxicity.

One problem is the choice of maximum daily concentration to use in exposures continuing for several weeks. The maximally tolerated dose might serve as a starting point but the physiologic perturbation occasioned by such treatments would make it equally prudent to observe animals at significantly lower doses, certainly including the concentration range of intended use or expected human exposure.

In any case, it may be expected that tissues would be available for analysis within 60–90 days of treatment unless in vivo genotypic fixation of mutation is delayed for a longer period. No experiments have ever addressed this issue.

XENOBIOTIC METABOLISM AND DNA REPAIR In situ mutational spectrometry automatically includes the variation of DNA repair physiology of each tissue type in the animal and simultaneously integrates drug metabolism within the tissue studied with metabolism of the test substance by other tissues. Thus, metabolism of parent compound A in organ 1 to compound B, which becomes a mutagen when metabolized by organ 2 to compound C, would be detected in organ 2 and in tissues subsequently exposed to compound C. In addition to providing a firm analytical basis for reaching conclusions of mutagenicity, the spectra derived from such studies would also provide hypotheses on tissue-specific metabolic pathways and/or DNA repair cascades. Such information combined with DNA-adduction patterns would permit separation of effects from metabolism and repair. Furthermore, nonlinearities in dose responses might lead to the discovery of induction thresholds or saturation phenomena useful in design of optimal therapeutic regimens for cancer chemotherapy.

CANCER At this writing, two kinds of gene sequences—the *ras* family and the *neu* gene—are known to be associated with tumorigenesis by somatic point mutations. Experiments to date have not yet distinguished between

induction of these point mutations by applied chemical carcinogen of selection of such mutants either by (*a*) creating selective conditions conducive to growth or (*b*) inducing other necessary mutations before or after *ras* or *neu* mutations occur. Experiments to make such a distinction are in progress. It would clearly be of value to know if treatment with a test substance induced any of the specific oncogenetic changes necessary but not sufficient to give rise to tumors. The technology of measuring mutational spectra could in fact be focused on these gene sequences, although numerical limitations would arise because each cell carries two copies of wild-type sequences and some 1.5×10^8 cells per tissue per animal would be required for an analysis to yield a meaningful negative result. Again, roughly speaking, organs of 1.5 gm mass or greater would be required—not a problem in larger experimental species but a real limitation in mice, for instance.

However, each and every available tissue would be available for analysis in a much shorter time than required for tumor appearance. The fact that animals might succumb to systemic toxicity would not mask the underlying oncogenetic changes as in present testing protocols.

GERMINAL MUTATIONS With regard to recognizing point mutations in germ cells, no real differences exist from the study of somatic cells. Precision and sensitivity are functions of the number of homogeneous cells derived from dissection of the animal and the number of identical copies of the desired DNA sequence in each cell. For sperm or spermatogonia, cell numbers of 10^6–10^7 are generally available, which means that 100-copy genes such as the ribosomal RNA genes could be used. In oocytes numbers of 10^3–10^5 may be encountered which would require use of mitochondrial sequences or even repetitive DNA family subsets for analysis.

EFFECTS OF AGE, SEX, DIET OR SYNERGY WITH COMMON PHARMACEUTICALS The organs of any animal should be available for genetic study and allow direct evaluation of the many host factors known to effect responses to carcinogens.

GENETIC CHANGE AS A SUBCHEMICAL MARKER For a test substance to imprint its mutational spectra on a tissue, its pharmacodynamic pathway must include distribution to the tissue, metabolism to a reactive intermediate(s), reaction with DNA, and creation of a heritable change in DNA sequence. Because such changes can now be detected as subtoxic concentrations, such spectra would be prima facie evidence of the chemical's presence and of one kind of specifically related untoward biologic effect. One might wonder if such genetic signals could be used as warnings of organ-specific toxicity even if changes in oncogenes were in fact not themselves induced by a particular substance.

NECESSARY ADVANCES IN TECHNOLOGY: A PROGRESS REPORT

The two previous sections have established the fact that point mutational spectra have been obtained by microbial geneticists from phage, bacteria, rodent, and human cell systems and that *if* one could obtain such spectra with reasonable precision from organs of experimental animals, certain practical questions could be directly addressed in pharmaceutical/toxic agent testing. In these discussions it has been posited that such technology exists and this section outlines its essential elements.

Technical Summary

The method though powerful is essentially easy to understand and perform in its present stage of development. First, the cell type of interest is isolated as a homogeneous population and DNA is isolated by standard techniques and cut with suitable restriction enzymes. The restrictions digest is run on a gel electrophoretic system and the desired fragment removed from the gel along with many other genomic fragments of similar size. These fragments are boiled briefly and allowed to cool slowly so that the single DNA strands arising during boiling reanneal with antiparallel partners. This step, however, causes mutant sequences to form reannealing partners with normal antiparallel sequences because the normal sequences are present in vast numerical excess. This boiled and cooled mixture, typically containing 1% of the total DNA isolate, is loaded on a denaturing gradient gel and normal homoduplexes are separated from mutant-containing heteroduplexes by 1–15 cm in the course of 12–16 hours.

The separated heteroduplexes are eluted en masse from the denaturing gradient gel and subjected to DNA amplification by a Mullis polymerase chain reaction (46) modified to substantially and necessarily increase the fidelity of the amplification reaction. During this step, radioactive or chemical labels are added to amplification products only. The amplification products are again boiled and reannealed in the presence of excess normal DNA sequence so that all mutant sequences are again in the form of mismatched DNA duplexes (heteroduplexes). This mixture is now loaded on a second denaturing gradient gel to accomplish separation of mutant-containing heteroduplexes from both normal homoduplexes and from each other. Use of autoradiography in the case of ^{32}P-labelled-DNA reveals a series of bands—usually visible as doublets—the intensity of which is proportional to the number of particular mutant DNA sequences in the original sample.

While the positions and intensity of these bands are themselves a mutational spectrum, this pattern is easily translated into particular point mutations of the normal DNA sequence by further elution of individual bands, DNA amplification, and DNA sequencing.

The process is straightforward, easily learned, and seems amenable to automation that would use some modification of existing systems for amplification, separation, and DNA sequencing. The sensitivity of the present protocol is estimated to be 100 molecules of mutant sequence among 10^6–10^{10} total sequences. In practice, mutant fractions of 10^{-8} appear to be detectable as long as 100 molecules of mutant sequence are present.

This level of sensitivity and the attendant precision when 100 molecules of mutant are present should be more than sufficient for the animal-testing protocols anticipated in the previous sections.

TECHNICAL NOTES

Denaturing Gradient Gel Electrophoresis

This technology was developed by Fischer & Lerman (39) as a means to separate mutant DNA molecules on the basis of the stability of the DNA to thermal melting or melting in the presence of solutes such as urea and formamide which compete with base pairs' hydrogen bonds. In this they took advantage of the condition of cooperative equilibria in DNA melting revealed in the statistical mechanics treatment by Scheraga & Poland (40). In brief, any continuous double-stranded DNA consists of a series of sequences which melt as a single unit if one slowly raises the temperature (or urea/formamide concentration). The melting temperature (Tm) of such a sequence, or isomelting domain, may vary from about 60–105°C, depending on the sequence itself. Knowledge of the GC content is insufficient to predict Tm. The entropy of the melting transition pathway is absolutely dependent on the order of the bases, and the enthalpy is dependent both on stacking energy between neighboring bases as well as the better appreciated hydrogen bonding across the DNA double helix as accounted by Gotah & Tagashira (41).

The fact that *any* point mutants could be separated from wild-type sequences was enormously exciting to us who felt that the future of genetic toxicology lay in the use of the abundant specific information available in mutational spectra (42). It seemed that if single base-pair changes caused sufficient changes in Tm to allow separation of a fair fraction of all possible substitutions as homoduplexes then the relative destabilization of the helix in heteroduplexes formed by mutant/wild-type annealing would be great enough to separate *all* possible point mutations on these wonderful denaturing gradient gels (43). This idea of using heteroduplexes on gradient denaturing gels was in fact soon reduced to practice by Myers et al (44), separating β-thalessemia mutants as heteroduplexes with normal human sequences.

Our laboratory proceeded to probe the limits of sensitivity for detecting low frequencies of mutants that would be encountered in cell culture or animal experiments. Based on the current limitations in signal detection, we found that mutant fractions lower than 10^{-2} or mutant numbers fewer than 10^5

molecules could not be detected (44). We had to face the fact that we expected to have to detect one mutant in 10^7 copies when as few as 100 mutant copies were available and that we were therefore about 10^5-fold away from that goal. Perceiving the problem in engineering terms of signal-to-noise ratio, we proceeded to solve the copy number problem by growing the human B cells (increased signal) in the presence of selective agent which killed off nonmutant cells (decreased noise). We also studied a number of signal amplification systems used by molecular biologists and astrophysicists to increase the sensitivity of data acquisitions from gels.

In the meantime, Mullis, while reputedly immobilized in Bay Area traffic, conceived the PCR technology that has revolutionized the practice of genetics (46).

DNA Amplification by Polymerase Chain Reaction

The work of Mullis is probably familiar by now to most readers: a desired DNA sequence of hundreds to several thousands of base pairs may be uniquely and exponentially increased in number by alternatively boiling and cooling it in the presence of short antiparallel oligodeoxy nucleotides which demark the 3' and 5' boundaries of the desired sequence. These oligodeoxy nucleotides serve as primers for DNA synthesis in the presence of a DNA polymerase. The beauty and simplicity of this concept is striking: exponential increase of a desired DNA sequence by the same strategy employed in life here imitated by science. The practical consequences are staggering and well beyond the scope of this chapter.

However, upon reading the first available publication of this technique (47), we noted that a DNA polymerase, Klenow fragment of DNA polymerase I of *E. coli* had been used. We recalled that others (48) had noted replication error rates for this enzyme of the order of 10^{-4} errors/base replication. We calculated that a 10^6-fold amplification of 100-bp fragment would involve 20 doubling and that a 10^{-4} error rate per base copy would lead to $10^{-4} \times 20 \times 100 = 0.20$ mutations for every 100-bp duplex at the finish. Obviously such amplification-induced mutants would swamp our expected signal of 5×10^{-6} mutants for a 100-bp duplex. This problem has now been satisfactorily addressed by devising conditions that allow use of higher fidelity DNA polymerases in DNA amplification. (49, 50). Using the high fidelity amplification procedure (Hi Fi PCR), we found in reconstruction experiments that we could now detect mutant fractions as low as 10^{-4} only 1000-fold away from the necessary sensitivity for human studies (51).

Combination of Denaturing Gradient Gels and Hi Fi PCR

At this stage (summer 1988), the procedure was to amplify DNA from the sample, run the boiled and reannealed products on denaturing gradient gels

and observe mutant bands. The third exon of human *hprt* gene in TK6 diploid human lymphoblasts was chosen because it contained a naturally occurring low-melting domain of 104 bp adjacent to an 80 bp high-melting domain and was thus appropriate for separating low-melting domain mutants as heteroduplexes on denaturing gradient gels. The mutagens methyl nitroso guanidine, ICR-191 (a substituted acridine) ultraviolet light, benzo[α]pyrene diol epoxide, X-rays, hyperbaric oxygen, and hydrogen peroxide all induced reproducable hotspots of 6-thioguanine resistant mutations within this exon that were different from the spontaneously arising hotspots (W. G. Thilly, manuscript in preparation).

However edifying this progress, it was clear to us that one could not use the microbial trick of selecting mutants from a tissue sample in order to eliminate the large number of nonmutant sequences in the sample. Here a discussion with other students of the *hprt* locus (52) proved to be a stimulus to clearer thinking which has led Phouthone Keohavong and me to conceive the protocol outlined in the beginning of this section, the practicality of which we have now demonstrated to our own satisfaction: a crude restriction enzyme digest is boiled and reannealed and then run on a denaturing gradient gel. This step removes nearly all of the wild-type homoduplexes while permitting recovery of about half of the mutant sequences as heteroduplexes for subsequent HiFi PCR amplification and a second separation and enumeration of mutant DNA molecules on a denaturing gradient gel (49). Side by side comparison of analyses of the same untreated human cell population with or without 6-thioguanine selection reveals clear discernible bands that we estimate to represent mutant fractions of about 10^{-8} each. Background 6-thioguanine–resistant mutant fraction was about 2×10^{-6} in our cultures and certain intense bands from the 6-thioguanine–resistant population represent about 4% of the total 6-thioguanine–resistant DNA, while bands less than 1/10 as intense are easily seen on the second denaturing gradient gels prepared from the total unselected population. $(2 \times 10^{-6})(4 \times 10^{-2})(10^{-1}) < 10^{-8}$, Q.E.D.

This demonstration has considerable meaning for those of us who have worked together to devise means to observe and characterize mutations as they occur in nature, especially in humans. That the methods turn out to be so simple and yet so sensitive is a bonus for which we are thankful.

PRESENT CAPABILITIES AND LIMITATIONS

It appears that point mutations lying in low-melting domains in restriction fragments containing adjacent high-melting domains may be detected at frequencies as low as 10^{-8} or perhaps lower if needed.

We expect that mutant fractions of approximately 10^{-7} will be encountered

at hotspots of spontaneous and induced mutation in humans and that higher fractions and different distributions of mutations will arise when man or experimental animals are treated with chemicals inducing mutation frequencies above this background level.

We expect that certain multicopy sequences such as the ribosomal RNA genes or mitochondrial DNA will contain suitable sequences for our method of analysis with interference from inherited polymorphisms easily factored in the analyses of data. Thus we expect that very small tissue samples containing as few as 3×10^5 to 3×10^6 cells would be processed in application in pharmaceutical/toxic agent testing.

In particular, the protocol would seem an appropriate means to evaluate potential genetic effects in the cohort of human subjects required for registration of certain products such as proposed food additives.

The present limitations also require comment, further analysis, and of course, more work.

1. The present method is labor intensive and requires a high degree of technical competence in what would be a highly repetitive task. Automation of the entire process after tissue dissection is, however, a feasible goal.
2. The original data base for this method is zero. No experiments in man or experimental animals have been completed although two are in progress. Our optimism is based on observations of particular point mutations in human lymphoblasts in culture.
3. Although pattern recognition algorithms exist in other fields, we have found no treatment both applicable to statistical analysis of mutational spectra which would also be understandable and thus useful for biologists who would use these methods.
4. Despite the expectation that more precise and organ-specific information may be obtained from animal testing models by this approach, the central problem of biochemical specificity among species is not addressed at all by this technology nor is the known pharmacogenetic variation within human populations. This new technology will not be a contribution to extrapolation of responses in animals to expected response in man. It may, however, serve to demonstrate that such extrapolations are impossible.
5. Although at its present level of sensitivity the protocol outlined should be able to detect and enumerate certain point mutations associated with oncogenic activation, it does not determine whether such mutation is or is not rate limiting and thus a risk factor in tumor formation in the particular organ studied. Such interpretation must rest on other studies showing whether or not spontaneous or induced tumors of the particular organ involve such oncomutations.

6. Clonal growth is exactly what happens in tumor formation. In animal studies, selective advantage of a certain kind of mutant would be observed as a time-dependent increase in its frequency compared to unselected mutational hotspots that maintain the same relative frequency. These and other possibilities require serious future consideration. The problem of jackpots and sib selection as well as clonal expansion or extinction in vivo cannot be avoided by the microbial strategy of selecting a single mutant from each tissue of many animals. Jackpots may be recognized as strong signals that occur only once in replicate animals; sib selection is overcome by the large numbers of mutants but does require that cells of an entire tissue sample be used as well as mixed samples.

SUMMARY

The microbial geneticists who pioneered the study of mutational spectra have shown us how rich a source of mutagen-specific information it can be for the pharmacologist and toxicologist. Contributions by physical chemists and molecular biologists have led to a practical means to observe mutation spectra directly from the DNA of cultured human cells and this review suggests that the same methodology may be applied successfully to the study of mutations and mutational spectra in tissues of experimental animals and humans.

In toxicological testing, the new field of mutational spectrometry offers a previously unattainable level of sensitivity and specificity as well as new tools for dissecting mechanisms of pharmocologic and toxic action.

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