# TWO-DIMENSIONAL GEL ELECTROPHORESIS FOR THE DETECTION OF HERITABLE MUTATIONS--NORMAL QUANTITATIVE VARIABILITY RELATED TO AGE

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SUMMARY

Heritable mutations as quantitative alterations in protein expression can be detected by using two-dimensional gel electrophoresis (2DE) coupled with computer-assisted data analysis. Ideally, one would like to monitor all of the proteins seen in a 2DE pattern for mutations, thereby allowing the examination of several hundred different genetic loci in a single individual. The background variability of each protein in the 2DE patterns, however, dictates the magnitude of change that can realistically be detected and thereby determines which proteins can be monitored for mutation events that influence protein quantity. Thus, an understanding of the factors that influence the normal quantitative variability of proteins is imperative so that conditions can be optimized for the maximum number of proteins. Using software developed at Argonne National Laboratory for the analysis of large numbers of 2DE gel patterns, we have assimilated data on the effects of the age of individuals sampled on quantitative variability.

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Abbreviations: CV: coefficient of variation; 2DE: two-dimensional gel electrophoresis; ENN: N-ethyl-N-nitrosourea; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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## 1 INTRODUCTION

The power of 2DE for genetic monitoring lies in its ability to resolve over 1000 different proteins in an individual sample, thus presenting the possibility of monitoring large numbers of gene loci in a single assay (1). In addition, the gene loci monitored by the 2DE technique are obviously coding regions, thus targeting mutations in functional DNA as opposed to the random sequences monitored by DNA sequencing methods in the absence of specific probes. Several studies have demonstrated that point mutations induced by alkylating agents can be detected as obvious qualitative protein changes in 2DE patterns (2-5). In heterozygous carriers, such mutations have been detected as the appearance of a new protein in the 2DE pattern or as the quantitative loss of a normally expressed protein in the presence of an induced protein variant (5). The use of 2DE for the detection in heterozygous carriers of mutation events that result in the loss of a gene copy (i.e., deletion mutations) rather than alteration of a gene sequence, however, remains to be demonstrated. Such deletion events in structural genes are assumed to result in a 50% reduction in protein expression in heterozygous individuals (1). Our interest in detection of mutations induced by ionizing radiations of low versus high linear energy transfer (i.e., neutrons versus gamma rays), assumed to cause deletion events more frequently than point mutations, necessitates optimization of the 2DE technique and data analysis for the detection of such quantitative changes.

Our current approach to the detection of quantitative alterations in protein expression relevant to mutation events is to search the data for spots with integrated densities (spot volumes) that are more than 2.5 times the standard deviation from the mean spot volume. We have chosen to assess the quantitative reproducibility of protein spots in 2DE patterns in terms of the coefficient of variation (CV) for each protein in a data set. The CV is defined as the standard deviation of the spot volume for a particular protein spot divided by the mean spot volume of the same protein spot across a specific data set and is expressed as a percentage. While screening the offspring of male

mice mutagenized with N-ethyl-N-nitrosourea (ENU), we found that a coefficient of variation (CV) of 20% or less was required for detection of decreases in protein expression equal to approximately 50%, and 15% or less was required for alterations of less than 50% (5,6). For example, of four ENU-induced mutations that resulted in the expression of a normal and a variant form of a specific protein, only three were detectable because of the decrease in abundance of the normal protein (5). Of those three, one had a CV of 22% and a reduction in spot volume of 54%, while the other two had CVs of 6% and 15% with 37% and 48% reductions, respectively. The fourth mutation resulted in a 39% reduction in a protein spot with a CV of 18% and was thus undetectable because the normal between-individual quantitative variability was large enough to mask the less-than 50% change in abundance of the normal protein. Among the matched spots (420 average) compared between 372 patterns from female and 425 patterns from male offspring from ENU-treated and gamma-ray irradiated sires, approximately 50 protein spots had CVs of 15% or less and were therefore stable enough to allow for reliable detection of mutations as quantitative alterations in protein expression in the range of 30-50% (5). Thus, not all proteins in a 2DE pattern are actually suitable for genetic monitoring in studies that involve quantitative rather than qualitative protein changes. If the sources of background quantitative variability can be identified and controlled, however, the number of proteins that can be monitored will be maximized as will the potential of 2DE for genetic monitoring.

The observed quantitative variability has both experimental and biological components, the former being related to sample preparation and electrophoresis/image acquisition methods and the latter to the biological system being monitored. Previous work from our laboratory showed that among 137 proteins detected in all patterns, 74 mouse liver proteins had CVs of less than 10% when 20 replicate gels of the same sample (7) were compared. This represents the variability introduced by electrophoresis, staining, and image analysis techniques. In our mutagenesis study, which represents analysis of several

hundred different mouse liver samples, of the 50 proteins with CVs of 15% or less, only 21 had CVs of 10% or less (5). Thus, analysis of multiple individuals introduces considerable quantitative variability. Although some variability due to sample preparation is assumed, we believe that individual biological variability contributes most appreciably to the background variability observed. We report here on the relationship of quantitative variability of mouse liver proteins to the ages of individuals sampled.

## 2 METHODS

### 2.1 Samples

Approximately 150 mg of mouse liver is homogenized in 8 volumes of a solution containing 9 M urea, 4% Nonidet P40, 5% 2-mercaptoethanol, and 2% ampholytes (pH 9-11). The homogenates are centrifuged for 5 min at approximately 435,000 x *g* in a Beckman TL100 ultracentrifuge. The supernatants are then stored at -70°C until 2DE analysis.

### 2.2 Two-Dimensional Electrophoresis

Isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are done as described previously (5). Gels are stained with Coomassie Blue R250 in 2.5% $H_3PO_4$ and 50% ethanol and destained in 20% ethanol.

### 2.3 Computer-assisted Data Analysis

Wet gels are digitized by using an Eikonix 785 scanner. The raw data are processed, and spot models are generated as previously described (8). The large number of patterns generated necessitates the use of software (GR42, developed at Argonne National Laboratory) designed for intercomparison of data from multiple 2DE patterns. This software includes all of the spot editing and matching capabilities originally described for the Tycho system (8), but allows incorporation of up to 500 individual patterns into a single data set while providing options for analysis of quantitative data through the use of vector

operations. Standard statistical tests (e.g., Student's T test) have been incorporated into the operations.

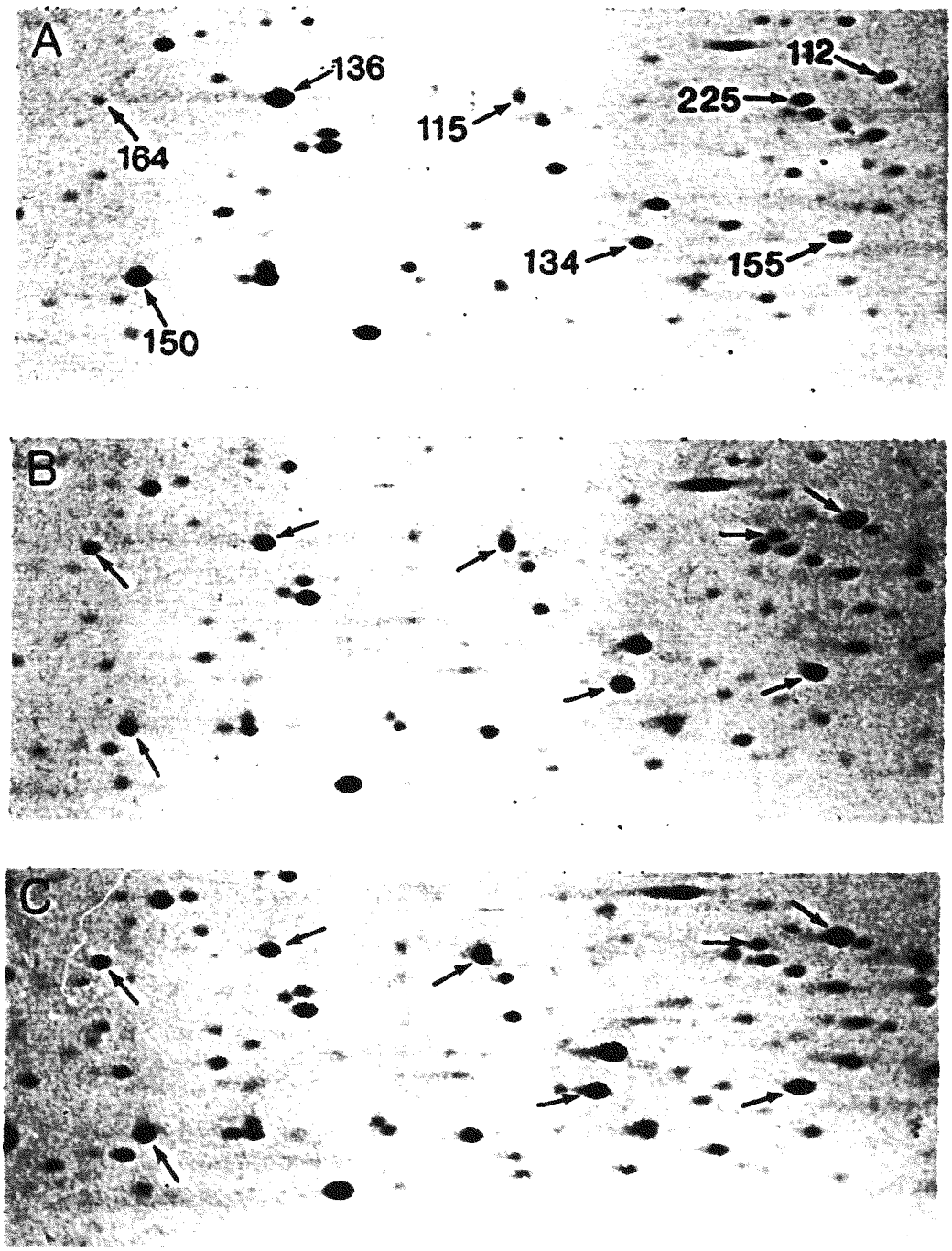
## 3 RESULTS

### 3.1 Specific Protein Differences Observed in Mice of Different Ages

At seven weeks of age mice are entering puberty (9), suggesting that sex hormones could influence the quantity of specific liver proteins and thus introduce individual variability into the system. Therefore, a comparison of liver protein expression in control mice at different ages was done. Figure 1 shows the 2DE patterns of liver proteins from control male mice at two, six, and ten weeks of age. Dramatic quantitative differences in the protein patterns are obvious between two and six weeks. Between six and ten weeks, the differences are more subtle but still significant, as is shown by the data from selected spots summarized in Table 1. Although more than the eight proteins listed have quantitative differences when liver proteins from mice of different ages are compared, the proteins listed in Table 1 are among those found to be more quantitatively stable (i.e., with CVs of 15% or less) in older than in younger mice. Of these eight proteins, the expression of proteins 115, 134, and 155 is altered by castration of seven-week-old males (data not shown). The expression of these proteins in castrated males is maintained at a level comparable to that seen in control two-week-old males. These results suggest that sex hormones do affect the expression of a subset of male mouse liver proteins, and that more mature animals might provide a protein population with a larger proportion of quantitatively stable proteins than do seven-week-old individuals.

### 3.2 A Comparison of Mouse Liver Proteins from 7- and 13-Week-Old Individuals

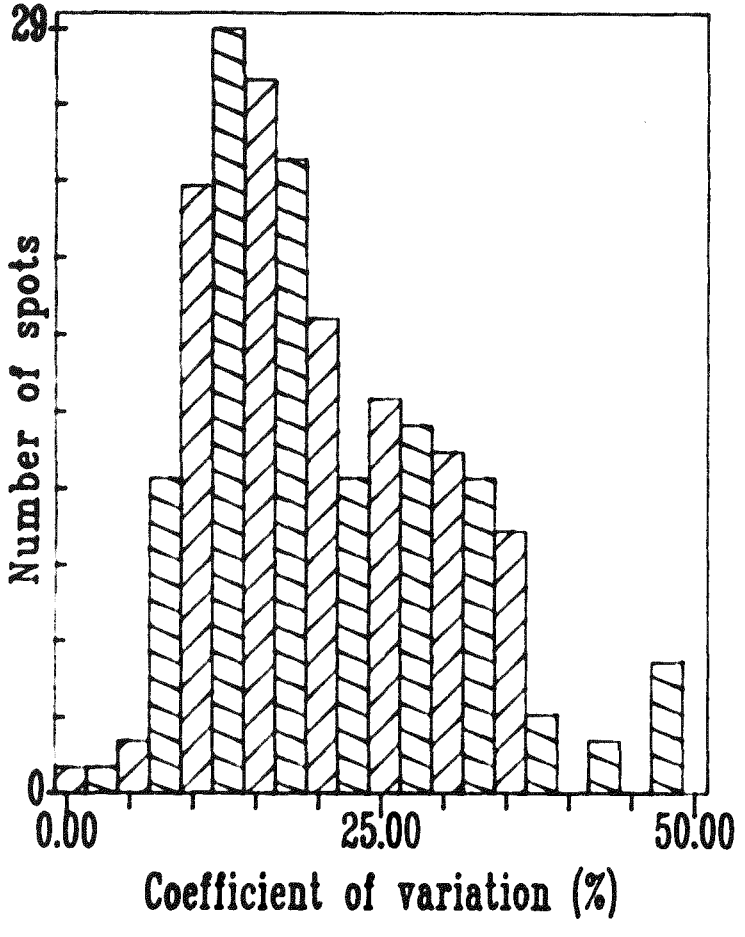
Figure 2 shows histograms of CV values for liver proteins detected in 95% of the 2DE patterns from 7-week-old male mice (222 protein spots in 164 patterns) and 13-week-old male mice (212 protein spots in 111 patterns). In this experiment, 74 proteins had CVs of 15% or less in patterns from 7-week-old mice and 35 of those proteins had CVs of 10% or less. In contrast, 95 proteins




## DISCLAIMER

Figure 1. Two-dimensional electrophoresis patterns of liver proteins from control male mice at (A) two, (B) six, and (C) ten weeks of age. The patterns are oriented with the acidic side to the left and the basic side to the right.

with CVs of 15% or less were observed in 13-week-old mice. Thus, using the older animals resulted in approximately a 25% increase in the number of proteins that can be efficiently monitored for quantitative decreases in the range of 30-50%.


29
0
Number of spots
0.00
25.00
50.00
Coefficient of variation (%)

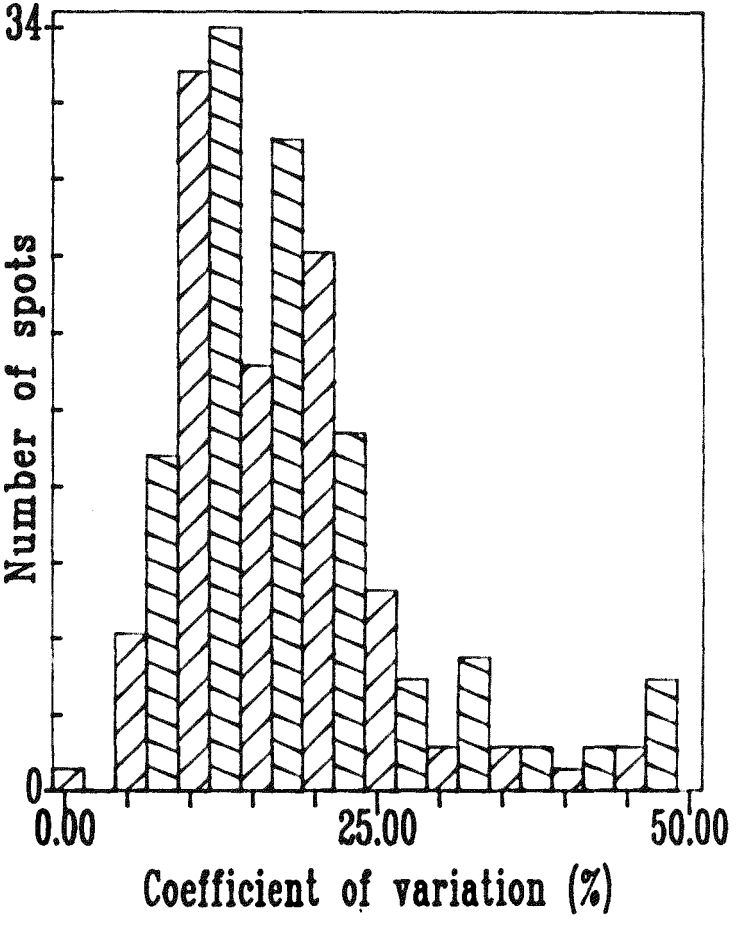

34
0
Number of spots
0.00
25.00
50.00
Coefficient of variation (%)

Table 1. Mouse liver proteins showing quantitative differences related to age of individual sampled. (A) Two weeks old (N=10), (B) six weeks old (N=11), (C) ten weeks old (N=10).

| Spot Number | Mean Spot Volume | | | Coefficient of Variation | | |
|---|---|---|---|---|---|---|
| | A | B | C | A | B | C |
| 112 | 9132 | 18705 | 22861 | 28 | 19 | 11 |
| 115 | 5931 | 8152 | 10372 | 25 | 24 | 13 |
| 134 | 9987 | 13385 | 18642 | 17 | 21 | 12 |
| 136 | 19837 | 10614 | 11957 | 34 | 22 | 13 |
| 150 | 13098 | 8224 | 9212 | 15 | 21 | 14 |
| 155 | 10164 | 10317 | 16290 | 13 | 22 | 13 |
| 164 | 2782 | 5175 | 7129 | 22 | 22 | 15 |
| 225 | 10846 | 4997 | 5946 | 17 | 20 | 14 |

Figure 2. Histograms of CV values for liver proteins detected in at least 95% of the 2DE patterns evaluated from (A) 7-week-old and (B) 13-week-old male mice. Data from 222 proteins in 164 patterns (A) and from 212 proteins in 111 patterns (B) are summarized.

## 4 CONCLUSIONS

In a typical 2DE pattern of mouse liver proteins stained with Coomassie Blue R250, 600 distinct protein spots can be detected. By using the Tycho software for data manipulation, approximately 70% of these spots (i.e., 420 proteins) can be matched accurately in a majority of the patterns, the remainder being members of poorly resolved protein complexes or minor proteins close to threshold that are inconsistently detected. Of the 420 matched proteins, approximately 200 are consistently matched in 95%, and 150 are matched in 100%, of the mouse liver patterns used for our mutagenesis studies, without a significant investment of interactive effort. For mice sampled at an age when liver protein expression has stabilized relative to maturation criteria, our data indicate that almost 100 liver protein spots have the quantitative stability (i.e., CV values are 15% or less) to allow detection of a 30-50% decrease in abundance in searches for quantitative outliers. Therefore, assuming that each of the 100 liver proteins monitored represents a distinct structural gene, 1000 mice are required for assessment of mutation rates per 100,000 gene loci. The technique has potential for becoming more efficient as other sources of background quantitative variability are identified and controlled. In the context of human genetic monitoring projects, the number of quantitatively stable proteins in human samples remains to be explored. Our experience with mouse studies indicates that careful selection of sample material and definition/documentation of donor history will be critical to the success of such studies when quantitative data are to be analyzed.

## 6 ACKNOWLEDGEMENTS

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