N. Leigh Anderson
Sharron L. Nance
Sandra L. Tollaksen
Frederic A. Giere
Norman G. Anderson

Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Agronne, IL

Quantitative reproducibility of measurements from Coomassie Blue-stained two-dimensional gels: Analysis of mouse liver protein patterns and a comparison of BALB/c and C57 strains

Using the ISO-DALT system for two-dimensional (2-D) electrophoresis and the TYCHO system for computer analysis of the resulting protein maps, we obtained high quality quantitative protein abundance data from Coomassie Brilliant Blue-stained gels of mouse liver samples. High resolution gels allow more than 100 proteins to be measured with coefficients of variation less than 15 %. A comparison of results from two mouse strains (C57BL/6 and BALB/c) and the cross between them (BCF_1) shows that a large number of qutive polymorphisms can be detected, and that, as expected, the amount of protein produced in the heterozygote is intermediate between the parental values. The system described is shown to be capable of reliably detecting decreases in protein abundance such as those expected to result from radiation-induced deletion of one copy of a gene. The implications of these results for the study of gene regulation are discussed in relation to applications in genetics, toxicology, and differentiation.

1 Introduction

Protein mapping by means of two-dimensional (2-D) electrophoresis allows the separation and quantitation of large numbers of proteins in a single analysis [1]. From the viewpoint of biochemical genetics, such an approach offers the possibility of systematically surveying a large set of expressed genes and thereby increasing the number of markers available [2, 3]. By looking at a large number of gene products, we can obtain much more data per individual, and a better picture of the overall genetic properties of the population can be obtained. Likewise in toxicology [4] and differentiation, the examination of a large number of markers allows much more precise definition of the effects of xenobiotic treatment and/or developmental events on the constitution of complex organisms. Since the advent of high-resolution 2-D electrophoresis in 1975, numerous investigations of its usefulness in genetics have been described. Klose has demonstrated that the technique can be used to address fundamental questions in mouse genetics and mutagenesis, even without the advantage of fully computerized analysis [2, 5–7]. Recently, Marshall *et al.* [8] have also used the 2-D approach to screen for chemically induced mutations in the mouse. Baier *et al.* [9] have examined the effects of radiation-induced deletions at the mouse albino locus, and Silver *et al.* [10] have examined a series of mouse t-haplotypes. Naturally occurring polymorphisms in the mouse have been examined by several groups [11–14], and a series of positional polymorphisms has been described. In addition, a series of studies has shown that 2-D protein mapping is useful in detecting interesting mutations in human cells lines [15–17]. Almost all of these studies have concentrated on the discovery and analysis of positional variants, assumed generally to result from changes in charge stemming from amino acid sequence alterations caused by a point mutation in the appropriate structural gene. Those studies that have employed some quantitation [5, 9, 10] have still used primarily visual inspection to select proteins of interest and have not emphasized statistical analysis of the quantitative data. The study described here is thus the first to

Correspondence: Dr. N. Leigh Anderson, Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA

Abbreviation: 2-D, two-dimensional

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use quantitative densitometry to look for abundance changes and polymorphisms in large numbers of proteins and to investigate the reliability of such data.

In this paper we have described two simple investigations to serve as a basis for the general application of quantitative mouse liver protein mapping as a test system for use in mutation research, basic genetics, and toxicology. First, we analyzed the same sample on a series of gels to measure the quantitative reproducibility of the combined gel and computer system. Second, we analyzed the quantitative and qualitative differences between protein patterns derived from the livers of two widely used laboratory strains of mice (BALB/c and C7BL/6) and the BCF_1 animals generated by crossing them. The data set generated when multiple animals are used in each group allows a detailed analysis of the statistical accuracy achievable in quantitative measurements generated through protein mapping. The results reveal several interesting features of the genetics of quantitative (probably regulatory) strain differences and illustrate the usefulness of the method for a wide range of investigations.

2 Materials and methods

2.1 Preparation of samples

Mice were bred in the colony of the Division of Biological and Medical Research, Argonne National Laboratory (strains designated BALB/cJANL and C57BL/6JANL). For analysis, mice were killed by cervical dislocation and the apical end of the left lateral lobe of the liver was immediately removed. (Liver samples can also be removed surgically without killing the mouse, thus allowing it to be maintained for further genetic analysis when required.) Approximately 200 mg of liver tissue was homogenized in eight times the tissue weight of 9 M urea, 2 % Nonidet P-40 detergent, 2 % mercaptoethanol and 2 % LKB pH 9–11 carrier ampholytes, final pH 9.5. Samples were then centrifuged to remove insoluble material at 100 000 rpm (435 000 *g*, max) for 5 min (total run time, including acceleration and deceleration) in a Beckman TL-100 ultracentrifuge. The supernatant was frozen at -80 °C. The extraction of whole tissue with a powerful solubilizing mixture is intended to eliminate the possibilities for protein modification and differential extraction that may accompany extraction with water or simple buffers.

2.2 Two-dimensional electrophoresis

Gels were run on either the 18 × 18 cm or 20 × 25 cm ISODALT 2-D gel systems [18, 19]. Isoelectric focusing gels contained a 1:1 mixture of wide-range Servalyt and Biolyte carrier ampholytes. Focusing was performed for 12 000 or 25 700 Volt-hours (18 cm and 25 cm gels, respectively). Slab gels were prepared with a computer-controlled gradient pumping device ("Angelique") and redesigned gel-casting boxes holding 23 gels each. The computer controller, based on a Digital Equipment Co. Q-bus Falcon computer programmed and loaded through Vax-based MicroPower Pascal software, will be described in detail elsewhere. This system allows large series of gels to be made more reproducibly than by hand. Gels were stained overnight in 0.125 % Coomassie Brilliant Blue R-250 ("CB"; Serva Blue R) in 50 % ethanol/2.5 % phosphoric acid, and destained through several changes of 20 % ethanol in water.

2.3 Computer analysis

Gels were scanned at 100 microns resolution and 8-bits grey level information (0–1.2 OD typical) with an Eikonix model 785 linear CCD-based device modified to allow scanning of large gels submerged in water. A null image was recorded and subtracted from each scan to remove light box inhomogeneities, and the gel images were processed with the TYCHO software system [20] running on a VAX 11/780 (Digital Equipment Co.) with two AP-120B array processors (Floating Point Systems, Inc.) and a DeAnza IP8500 (Gould, Inc.) image display system. For each experiment, the resulting spotfiles were edited and matched together as a set through use of the GR42 interactive spotfile management software system (constructed by J. Taylor of this laboratory), and stretched into the same coordinate system. Spots were selected for inclusion into the final data set if measured unambiguously on all gels (experiment Mouse 9b) or on 19–20 gels (experiment Mouse 3b) in the experimental set; any single gel missing data was filled in as the average of other measurements for the spot in the appropriate group. The procedure for selecting unambiguous matches is based on physical position of each spot in an object pattern with respect to spots in a master gel pattern, and has been described [21]. While this selective approach reduces the total amount of data, it increases its quality by ensuring accurate matching; ongoing improvements in gel registration make possible a continuous increase in the number of spots found reliable by these procedures. Data from different gels in the same experiment were scaled together by setting the sum of the selected spots to a constant (linear scaling). Tests on gels of the same sample at different protein loadings indicate that linear scaling applies over a wide range with CB-stained gels (data not shown). The effect of such scaling is to eliminate differences due to variation in sample loading; in the experiments described here this variation was within ± 10 %. Calculation of statistical tests and preparation of statistical plots were carried out with Lotus 1-2-3 (Lotus Development Corp.) on an IBM PC/XT personal computer, according to the methods described elsewhere [22]. Because the standard deviation (and hence variance) is not the same for spots of different abundance, the normal t-test (which assumes equal variances) cannot be used. Instead we have used an approximate, two-tailed t-test based on a weighted average of the variances in two sample groups [22; pp. 411, 412]. Financial spreadsheet programs turn out to be extremely useful in exploratory data analysis because of their flexibility, user friendliness, and capabilities for viewing, manipulating and storing the entire mathematical model used to treat the data.

3 Results

3.1 Quantitative reproducibility of protein maps

We ran a series of ten 20 × 25 cm 2-D gels of the same mouse liver sample (Experiment Mouse 9b, patterns as exemplified by Fig. 1), and selected 137 unambiguously measured spots present on all gels. A fundamental statistical question to be asked before analyzing such data is whether the errors in spot integrated density measurement are distributed in a Gaussian

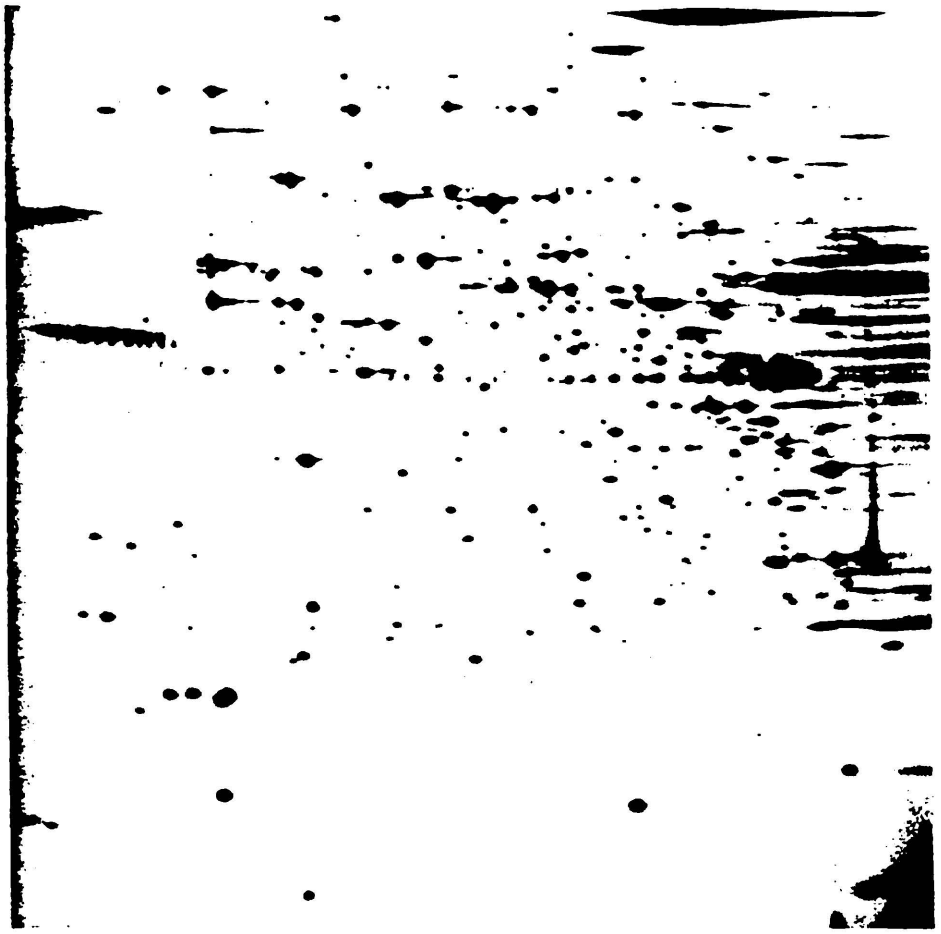

Figure 1. A CB-stained 2-D gel of proteins from the liver of a male BCF_1 (BALB/c male × C57BL6 female) mouse. The horizontal line of evenly spaced spots across the pattern halfway down are creatine phosphokinase charge standards [25]. The resolution of this gel is 15 800 when measured as described previously [26].

manner. As shown in Fig. 2, the normalized deviations of individual values closely approximate the expected Gaussian distribution (Chi-squared = 26.36), indicating that the distribution is not significantly non-Gaussian. The analysis of variance (ANOVA) assumes that errors are distributed in this manner, and thus this method can be used on the data obtained here.

A second fundamental question concerns the relationship between the amount of error variation and the magnitude of the protein spot. Is the standard deviation (SD) similar for all spots, or is it greater for large spots, as might be expected intuitively? Fig. 3 shows a plot of the log of standard deviation versus log total spot integrated density (protein abundance)

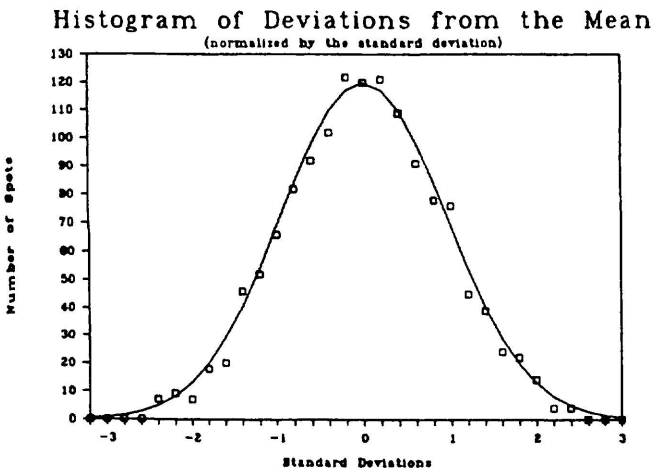

Figure 2. Hostogram of measured standardized deviates. Standardized deviates were calculated by subtracting the mean value of a spot from each of the individual gel measurements, and then dividing these values by the standard deviation for the spot. Such deviates may be expected to approximate a Gaussian distribution describing the error associated with protein measurement in this system. The continuous curve represents a true Gaussian selected to fit the data. The histogram uses centered bins.

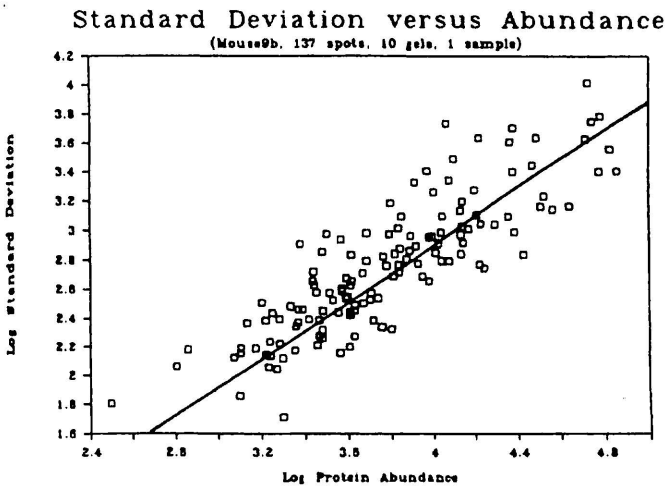

Figure 3. A plot of the log average protein abundance (mean spot volume) *versus* log standard deviation for all the spots measured in experiment Mouse 9b. The data are consistent with the assumption of a linear relationship between abundance and SD, as indicated by the line of slope 1. It is therefore most appropriate to express statistical error in abundance measurements as a coefficient of variation (CV), the ratio of SD to abundance.

for all 137 spots. It is clear that standard deviation is proportional to protein abundance, and, as a result, we decided to express errors for individual spots in terms of a coefficient of variation (CV; standard deviation divided by the mean value) rather than an absolute standard deviation. As a consequence of the relationship between SD (or variance) and abundance, we cannot assume equal variance for measurements of a given protein under different conditions or in different strains. It is therefore necessary to use an approximate *t*- test involving a weighted average of the variances in two sample groups, rather than the classical Student *t*-test with a single variance.

Finally, we asked how reproducible the measurements of individual spots were. Since spots may differ in measurement precision (because of differing physical properties of the proteins or because of overlap with neighboring spots), the result is a histogram of values for the set of spots, rather than a single value. This histogram, shown in Fig. 4, indicates that almost half the proteins (74/137) have CVs less than 10 %, and more than three quarters (106/137) less than 15 %. A major source of imprecision, and hence large CVs, appears to be overlap with surrounding spots; hence the use of well-separated, unambiguously matched proteins seems to lead to better data, albeit on fewer proteins. By selecting spots for good quantitative reproducibility, quite accurate comparative measurements can be made.

3.2 Differences between strains

To explore the application of quantitative measurement to real differences, we analyzed twenty 18 × 18 cm gels of livers of individual male mice (experiment Mouse 3): seven of the C57BL/6 strain, seven BALB/c, and six BCF_1 (the cross between C57 females and BALB/c males). Only males were used to avoid any contribution of sex differences to the strain comparison. These differences will be dealt with in a subsequent paper. By matching all gels to a heterozygote master gel, we selected 135 spots that were either unambiguously well measured, or else were unambiguously absent, on at least all but one gel of the set (as described in Section 2.3). An approximate Student's *t*-test (see Section 2.3) was used to evaluate the likelihood that the measurements for a given spot from the two parental strains arose from the same distribution (*i. e.*, that the amounts of the protein present in the two strains were identical). We compared the results with a two-tailed *t*-distribution with 6 degrees of freedom, a more conservative approach than the use of 12 degrees of freedom appropriate when all variances are equal. A histogram of the t-values for all 135 spots is shown in Fig. 5; values exceeding 2.45, 3.71 and 5.96 provide evidence against the null (no strain difference) hypothesis at 5 %, 1 % and 0.1 % levels of confidence, respectively. A total of 22 spots (more than 15 % of the total) show differences significant at the 0.1 % level, and a total of 32 at the 1 % confidence level. Using the *F*-statistic from a one-way ANOVA to analyze all three groups for any significant pairwise difference, we obtained similar results.

Once the *t*-test had been used to select a set of spots showing significant strain differences, it was possible to investigate the appearance of the heterozygote (BCF_1) with respect to the parental patterns. We examined two classes of proteins: those that were detected in all strains, and hence represent purely quantitative (regulatory) differences, and those that were undetected in one parent, and hence may represent products of one allele (the other appearing perhaps elsewhere on the gel). Fig. 6 shows the mean protein abundances in all three strains for the eight proteins showing the statistically most significant parental strain differences of the first type (regulatory). Fig. 7 shows analagous data for the eight proteins showing the most significant differences of the second type (probable, qualitative' changes). Spots 106 and 142 in Fig. 7 are likely to represent a pair of alleles related by a simple charged amino acid substitution. Fig. 8 shows the locations of these proteins in the 2-D pattern, and summarizes the statistical results for all of the proteins found to differ significantly between strains. Using a

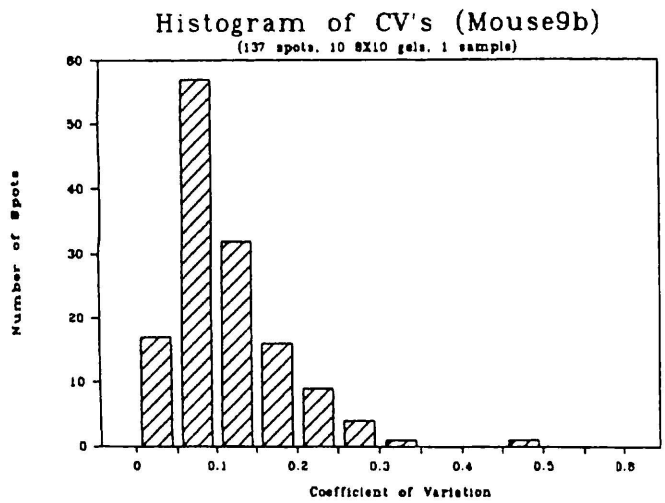

Figure 4. A histogram of coefficients of variation for the spots measured in Mouse 9b. For each spot, the standard deviation of the 10 measurements was divided by the mean to yield the CV. Each bar in the histogram indicates the number of spots with CVs between the values marked on either side. Most proteins show CVs less than 10 or 15 %, and may be considered reliably measured by the 2-D system.

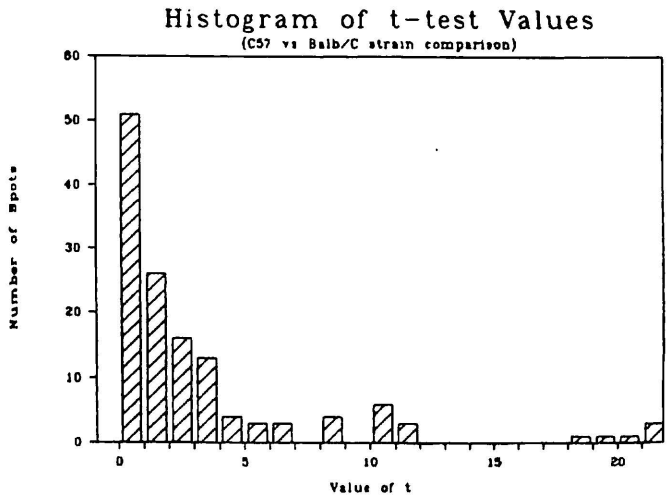

Figure 5. A histogram showing the distribution of values of the approximate *t* statistic for the spots analyzed in experiment Mouse 3. The *t*-test provides a measure of the likelihood that two sets of measurements (here the abundances of a spot in C57BL/6 and BALB/c gels) are derived from a single statistical distribution; *i. e.*, that there is really no difference between the strains as regards level of the protein in question. In this case, the system is conservatively assigned 6 degrees of freedom (*versus* 12 if the variances were equal), and hence *t*-test values of 2.45, 3.71 and 5.96 correspond to significance levels of $P<0.05$, 0.01 and 0.001, respectively. A majority of spots have $t<3.71$, and are not considered to show a significant strain difference in this experiment. A number of spots show values of $t>5.96$, indicating significant levels of strain difference.

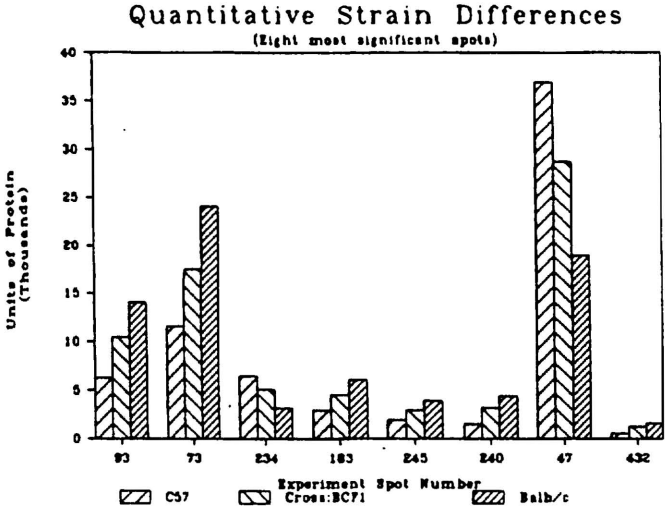

Figure 6. Average amounts of eight proteins in each of the three mouse strains examined. The eight proteins shown were selected as showing the most significant C57BL/6-BALB/c differences, as indicated by the largest values of the t statistic, while still showing detectable levels in both strains. The quantitative variation in these spots thus cannot be due to the existence of positional-variant alleles in the two strains. In each of these cases, the level of protein in the BCF_1 cross is close to the average between parental levels. The experiment spot numbers refer to spots indicated in Fig. 8.

criterion of absence in one parental strain (abundance < 700 pixel-grey values) and presence (abundance > 2100) in the other for selection of likely positional variants, we found 9 of the 22 variant proteins (at $P<0.001$) to be likely positional variants. Likewise 12 of the 32 proteins significantly different at $P< 0.01$ appeared to be positional variants. Since each positional variant is likely to consist of two alleles at different gel positions, these figures probably correspond to half the stated numbers of positional variant loci; *i. e.* 4 or 5 and 6, respectively. This result suggests that the present approach may detect five times as many variants as a simple search for positional variants.

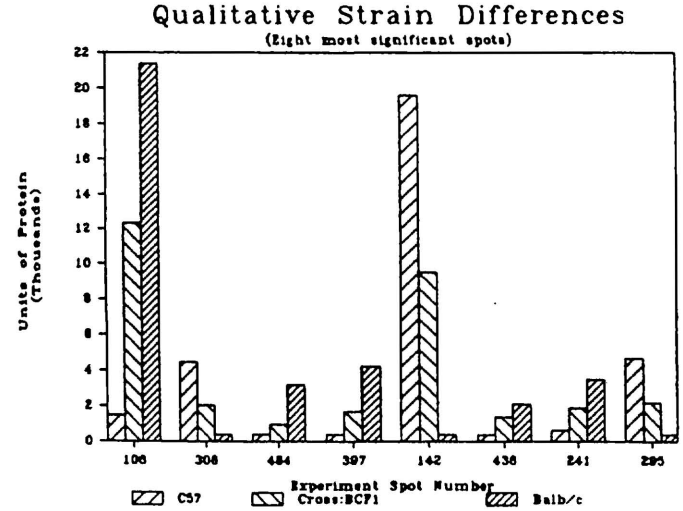

Figure 7. Data analogous to that of Fig. 6, but for proteins that appear to be missing in one of the parental strains and thus are likely to represent qualitative changes. These spots are likely to represent one of two alleles differing in position on the gel. Spots 106 and 142 constitute a pair of such candidate alleles separated on the gel by a small horizontal (p*I*) shift appropriate for a single charged amino acid substitution. Since it is impossible to demonstrate the total absence of a protein spot, we show, for spots that were not detected, the maximum amount of protein that could have been present without being detected at the appropriate gel position.

Using this collection of data, it is possible to ask whether protein abundance is determined directly by simple gene dosage, or whether it is actively regulated to maintain a constant amount of protein. When the proteins showing a parental strain difference significant at $P< 0.01$ were selected, the relative amount of protein in the heterozygote is distributed as shown in Fig. 9. This distribution is strongly concentrated around a value intermediate between the parental levels. Almost none of the proteins showed a heterozygote level significantly outside the range of parental values.

4 Discussion

4.1 Reproducibility

It is clear from the results of Section 3.1 that measurements of high accuracy, competitive with many enzymatic and immunological assay systems, can be obtained by analysis of CB-stained 2-D gels. Since reproducibility appears to be best for spots that do not overlap others, data quality is strongly influenced by gel resolution. In this regard, we have found expanded focusing pH ranges better than wide ranges, and the larger (20 × 25 cm) gel system generally superior to the smaller (18 × 18 cm) system.

4.2 Amount of data, improved data scaling, and comparison of gels

Given adequate quantitative accuracy, the chief feature of the 2-D gel plus computer analysis system is volume of data obtained. In the present investigation, 4070 measurements of specific protein abundance were made. This number of measurements would represent a large amount of effort if undertaken with any conventional procedure. In addition, the measurement of many proteins in each sample makes it possible to normalize the values from different samples in a very straightforward way: the sum of values for a large number of proteins can be set to the same constant for all gels. This approach presupposes that the total level of protein for the selected set is constant in all samples, and provided that the selected set is large enough and representative of the overall tissue composition, such an assumption is likely to be valid. The individual measurements are, therefore, on a scale relative to total tissue composition, and any reasonable errors in sample loading can be removed. The penalties associated with sample volume inaccuracies in conventional protein analysis methods are therefore much decreased.

4.3 Genetics of quantitative differences

The results presented demonstrate clearly that a large number of quantitative protein differences can be detected between common laboratory mouse strains. Of the 135 proteins selected on criteria of positional reproducibility [21] and lack of overlapping neighbors, almost 15 % showed significant abundance differences. Some of these spots appear to be absent from one parent strain, indicating either that this parent is homozygous for a null allele for the gene, or else that the allele present is found elsewhere on the gel. One particularly obvious case of "positional" alleles is presented by spots 106 and 142 (Fig. 8). The total amount of 106 + 142 in all three strains is similar. Spots 308 and 397 may represent a similar system of

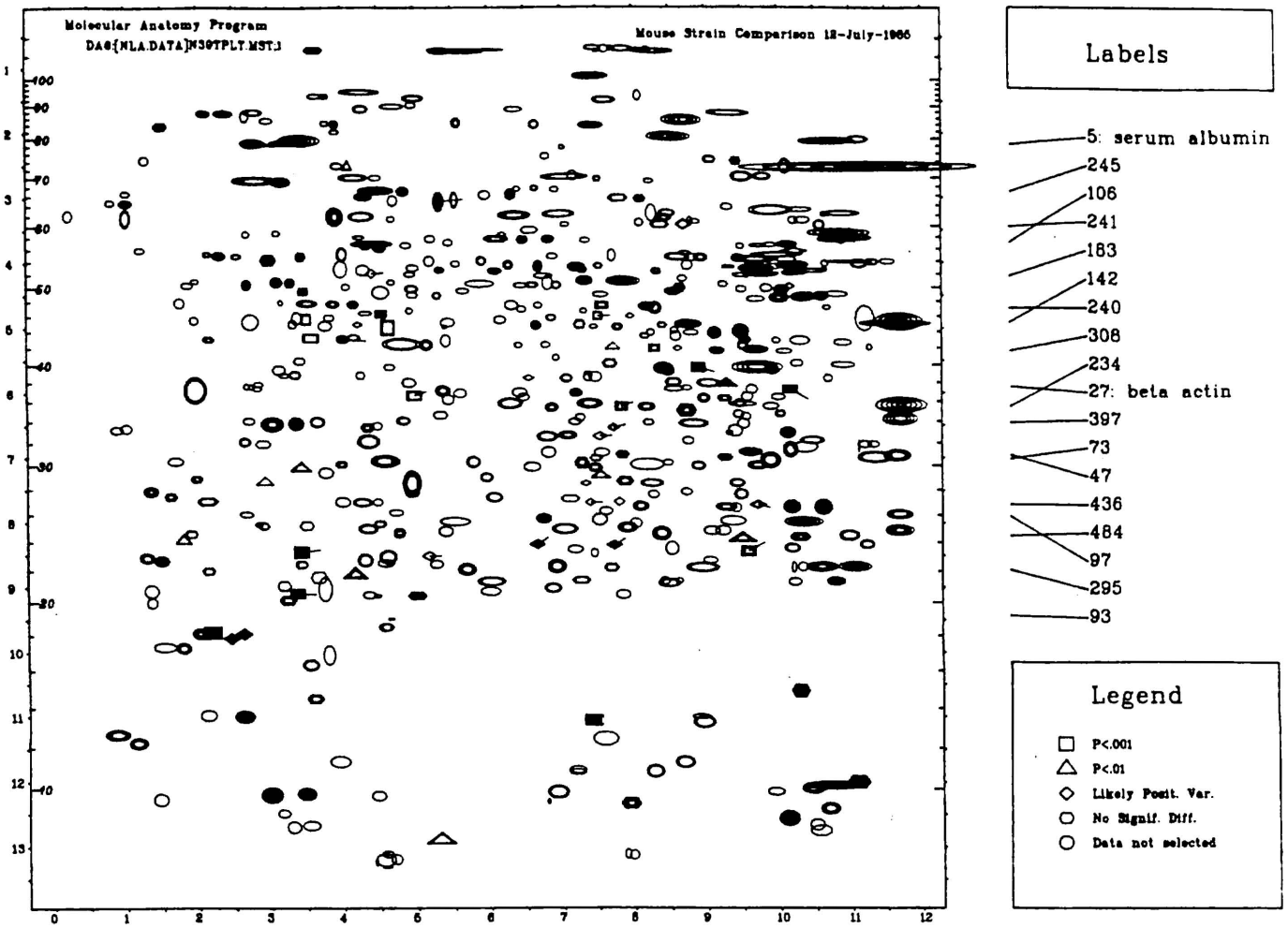

Figure 8. A schematic plot of the protein pattern derived from the master gel of the mouse strain comparison (experiment Mouse 3). Spots are plotted as symbols to indicate whether they were different between strains at $P<0.001$ significance (triangles), likely to represent one member of a pair of positional alleles (diamonds, missing in one strain), found to show no statistically significant interstrain difference (hexagons), or not included in the statistical analysis because of possible confusion with other spots or other ambiguity (ellipses). The number of contours plotted for a spot is proportional to spot abundance (each contour represents 4000 pixel-grey value units). Labels along the right-hand margin indicate spot numbers in the numbering system of this experiment: using a straightedge (or the edge of a sheet of paper) the reader can follow the line emanating from a label to the short line segment extending from the spot indicated. This is the best way we have found for indicating specific spots without cluttering or obscuring parts of the pattern. The positions of beta actin and mouse serum albumin are indicated, and a molecular mass scale derived from a series of 18 human plasma and other well-characterized proteins [24] is included.

alleles, with the difference that the two are slightly separated in both isoelectric point and sodium dodecyl sulfate (SDS) molecular mass, rather than isoelectric point alone.

Most of the significant quantitative differences are observed in proteins present at some level in both parental strains. Since these strains are each expected to be homozygous at essentially all loci, then the protein levels measured must represent the full diploid (two dose) amounts produced. The strain differences thus represent, in these cases, a reflection of true regulatory differences having a genetic basis. Most proteins in this class show quantitative strain differences of 1.5 to 2-fold and rarely more (Fig. 6). Such variation in the amounts of these proteins may easily be tolerated by liver cells. Alternatively, it could be that the spots in the parental strains are different, though electrophoretically silent, structural alleles whose different functional efficiencies require their production at different levels. In either case, the observed intermediate expression levels in heterozygous mice provides fairly strong evidence for some 'linear'-type model of gene regulation. It is generally consistent with the simplest model in which these proteins are expressed constitutively and not regulated to achieve a preset universal mouse level. A study involving a large number of individual backcross animals (generated by backcrossing the BCF_1 heterozygotes with one of the pure parental strains) will be required to analyze the precise genetic nature of the quantitative and qualitative polymorphisms; such an investigation is now under way in our laboratory.

4.4 Applications in mutation research, toxicology and pharmacology

The ability to measure small quantitative changes in the abundance of proteins in mammalian tissues is of critical importance in the effort to detect radiation-induced genetic damage. It is believed that deletions are the most common result of radiation damage to DNA [23], and thus the most frequent molecular phenotypic effect should be the loss or truncation of one of the two copies of a gene. If synthesis from the

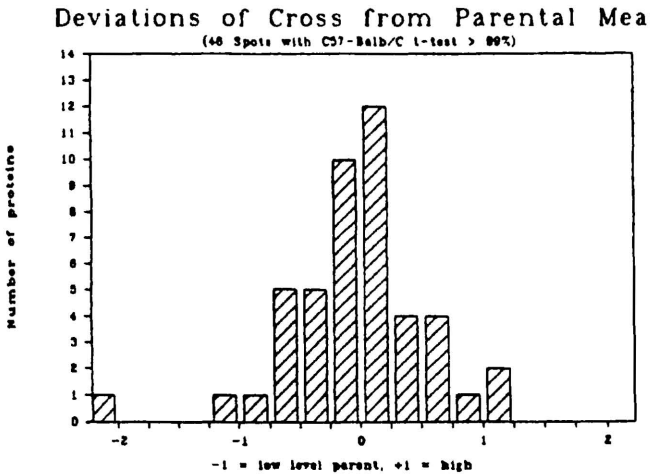

Figure 9. A histogram showing the amount of each protein in the BCF_1 heterozygote animals relative to the amounts in the two parental strains for the 40 spots showing most significant strain differences. The scale of abundance is such that the amount of protein present in the parent expressing the lower level is at position -1, and the amount expressed in the other (high-level) parent is at position +1. An ideal heterozygote animal, in which one copy of each of the two parental alleles is expressed at the original (parental) level, would thus produce an intermediate amount of protein and be placed at position 0. The experimental distribution is indeed clustered at a level equal to the average of parental levels (position 0), but some proteins show marked departures from this simple linear combination. Nevertheless, no proteins show heterozygote levels significantly outside the parental range.

other (intact) copy is not increased to compensate for the halved gene dosage, then a decrease in level of the appropriate protein by 50 % would be observed as a result of the deletion. It is therefore crucial to mutation detection studies that a 50 % change be detectable, and that the statistical likelihood of observing such a decrease due to chance fluctuation be small (perhaps once in ten gels on which 100 spots are quantitated). The results obtained here demonstrate that a 50 % change is definitely detectable for a large number of proteins; such a change exceeds three standard deviations for the more than 100 proteins having CVs less than 15 % in experiment Mouse 9b, and a 40 % change (a more useful detection limit) is equivalent to 2.7 SD. Although individual measurements (one gel per sample) could still give an unacceptable false positive rate due to purely random variation, the use of two (or more) gels per sample would reduce this problem to a negligible level. Considering a set of spots that are as well-measured as those in Mouse 9b (maximum CV 15 %) and for which accurate means and standard deviations are available from a large series of control gels, a 40 % or larger decrease would be observed by chance at a frequency of 0.0039 (one tail of a Gaussian distribution beyond 2.667 SD) in single gels, and in a series of 100 spots observed on each of ten gels (ten exposed animals), about four false positive events would be detected. This rate is too high, given the expected frequency of real variants (below 0.0001). If two gels of each sample were run and the results averaged, this rate would fall to 0.000072 ($2.667 \times \sqrt{2}$ SD), giving good protection against false positive mutant detection; only about one non-mutant individual in 100 would have to be reinvestigated. Using this approach, we should be able to reliably detect protein abundance decreases of 40 % or more due to radiation-induced loss of one gene copy.

Accurate quantitative data of this type can also be very useful in the classification of organisms and cell types [21]. If, as suggested by our results, almost 15 % of the proteins show quantitative differences between inbred mouse strains, it may be possible to extend the methods of numerical taxonomy to a much finer level of classification than has previously been possible due to paucity of differentiating characters. Since it is widely believed that regulatory effects may play a larger role in evolutionary change than structural gene mutations, it may be that taxonomies based on quantitative differences are in some sense superior to those based on conventional isozyme analyses.

In pharmacology and toxicology, the ability to measure small quantitative changes in tissue protein composition caused by drug administration will lead to a significantly better picture of the mechanisms of toxic and therapeutic effects on cells and tissues of intact animals. By correlating the pattern of protein change with information on the subcellular location of the affected proteins, as well as their properties and functions (where known), it will be possible to map out the major aspects of the effects of a drug from a single small experiment. That this level of technology can be applied to whole animal test systems already in place for drug testing means that all previous histological and survival data can be correlated with protein patterns to further enhance their explanatory and predictive potential. Model experiments to demonstrate these possibilities are under way and will be described elsewhere.

4.5 Statistics and 2-D analysis

A critical point brought home by the study of reproducibility is the necessity for repetition in experimental designs. The analysis of variance applied to 2-D gel data clearly shows that the number of differences detected is a function of the number of individuals compared. Although this point is a trivial one in statistics, it is rarely encountered in 2-D work. The effort of running many samples and the lack of generally available methods for spot quantitation perhaps account for this deemphasis of statistical approaches. Nevertheless, the application of protein mapping for quantitative work will require a substantial expansion in the number of gels that must be run. Methods and devices that makes gel running easier and more reproducible (*i. e.* automatic) are therefore of great importance.

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