

DIFFERENCES BETWEEN 2-D ELECTROPHORETIC PROTEIN PATTERNS OF LIVERS OF MALE AND FEMALE MICE

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RESUME

Le travail présenté ici est issu d'un programme de recherche où le système "foie de souris" est utilisé pour estimer l'effet *in vivo* de produits mutagènes et de diverses substances toxiques. Les échantillons de foie sont prélevés par chirurgie et les protéines étudiées par électrophorèse bidimensionnelle associée à un système d'analyse des gels assisté par ordinateur. Un des aspects importants d'une telle étude systématique est l'identification des différences entre les diagrammes protéiques des animaux mâles et femelles.

Nous présentons ici les premiers résultats de différences quantitatives associées à un échantillon de 88 spots choisis pour la qualité de leur résolution sur le diagramme. 75 mâles, 40 femelles et 7 femelles gestantes ont été étudiés (10736 protéines quantifiées). Environ 35% des protéines révèlent des différences statistiquement significatives ($P < 0.001$) entre mâles et femelles; dans la plupart des cas l'amplitude de variation est de l'ordre de 25%. Plusieurs différences entre femelles gestantes et non gestantes ont été identifiées dans d'autres groupes de protéines, mais aucun des 88 spots du groupe étudié ici ne présente de différences hautement significatives sur ce critère.

INTRODUCTION

The analysis of two-dimensional electrophoretic protein patterns with a suitable computer image analysis system can produce accurate quantitative measurements of hundreds of proteins. We have recently shown that Coomassie Blue-stained 2-D gels of mouse liver proteins, produced by the ISO-DALT electrophoresis system (1,2) and analyzed by the TYCHO data reduction system (3), can provide measurements of at least 100 proteins with coefficients of variation less than 15% (4). Using this technology, we can search for genetic polymorphisms and mutations, toxic effects of chemicals and treatments, and other possible regulatory effects at a new level --that of protein abundance. The ability to see subtle quantitative effects, by exposing a new level of complexity, also brings new requirements for comprehensive control of the biological systems. To our surprise, the variation among individual mice of the same strain, age, and sex is remarkably small for many proteins; inter-individual CVs are often less than 15%. It is therefore possible to intercompare two or more large groups of animals to discover quite small differences in protein expression. This paper describes a preliminary comparison aimed at discovering what fraction of proteins show some difference between sexes.

PROTEIN MAPPING AND COMPUTER ANALYSIS

A large number of BCF₁ animals (prepared by crossing male Balb/C with female C57BL/6 mice) has been analyzed in an experiment to search for mutations produced by treatment of the fathers with ethylnitrosourea or x-rays. A series of 122 of the F₁ animals has been considered here for purposes of analyzing sex differences: we assume that the rare mutation events to be detected have no appreciable effect on these sex differences. This group consisted of 75 males, 40 females, and 7 pregnant females. The apical end of the left lobe of the liver was removed surgically and solubilized directly in 9 M urea, 2% NP-40, 2% Ampholytes, and 2% mercaptoethanol. Samples were centrifuged at 450,000 x g for 5 minutes and stored frozen at -70°C. Two-dimensional electrophoresis was performed with 25 cm isoelectric focusing gels, the center portions of which were run on 18 x 18 cm second-dimension gradient slab gels prepared by a computer-controlled casting device ("Angeline"). Biolyte ampholytes were used for focusing. All gels were stained with Serva Blue R and digitized with an Eikonix 785 scanner. The TYCHO/PEPSI/GR42 software system was used to remove background, find and quantitate spots, match all gels to a common master pattern and stretch them into physical registration. A set of 88 spots was selected as well matched on each and every gel of the set; these spots form the data set analyzed here. This criterion excludes all qualitative differences (which would be missing in one sex) and concentrates only on quantitative effects. A statistical t-test (5) was used to determine the significance of intergroup differences. We used a value for the number of degrees of freedom appropriate for the smaller of the two groups compared, thereby taking a conservative approach to the selection of significant differences. Lotus 1-2-3 was used for the preparation of some graphics. Assignment of proteins to subcellular fractions was performed as described elsewhere (6).

RESULTS

Typical patterns of male, female and pregnant female BCF₁ mice are shown in Figure 1. Several sex-associated differences are immediately apparent to the eye, but in general the patterns appear quite similar. Likewise, at least one protein shows a change due to pregnancy: a pair of spots (squares labeled 1) appears to shift to more acid pI. However, a statistical analysis of the differences shows that many more proteins are regulated (at least partially) by sex differences than one can detect visually. A comparison between the 75 male and 40 female patterns considered here indicates that about 35% of the proteins differ in abundance between males and females at a very high level of significance (Table I). No proteins are affected by pregnancy at this significance level, a fact at least partly due to the smaller number of animals (and consequent lower statistical power) associated with the pregnant group.

Table I : Number of Proteins Showing Difference at Given Significance Level

Comparison	P<.001	P<.01	P<.05	P>.05	Total
Male vs. Female	30	13	11	34	88
Pregnant vs. Non-pregnant	0	3	9	76	88

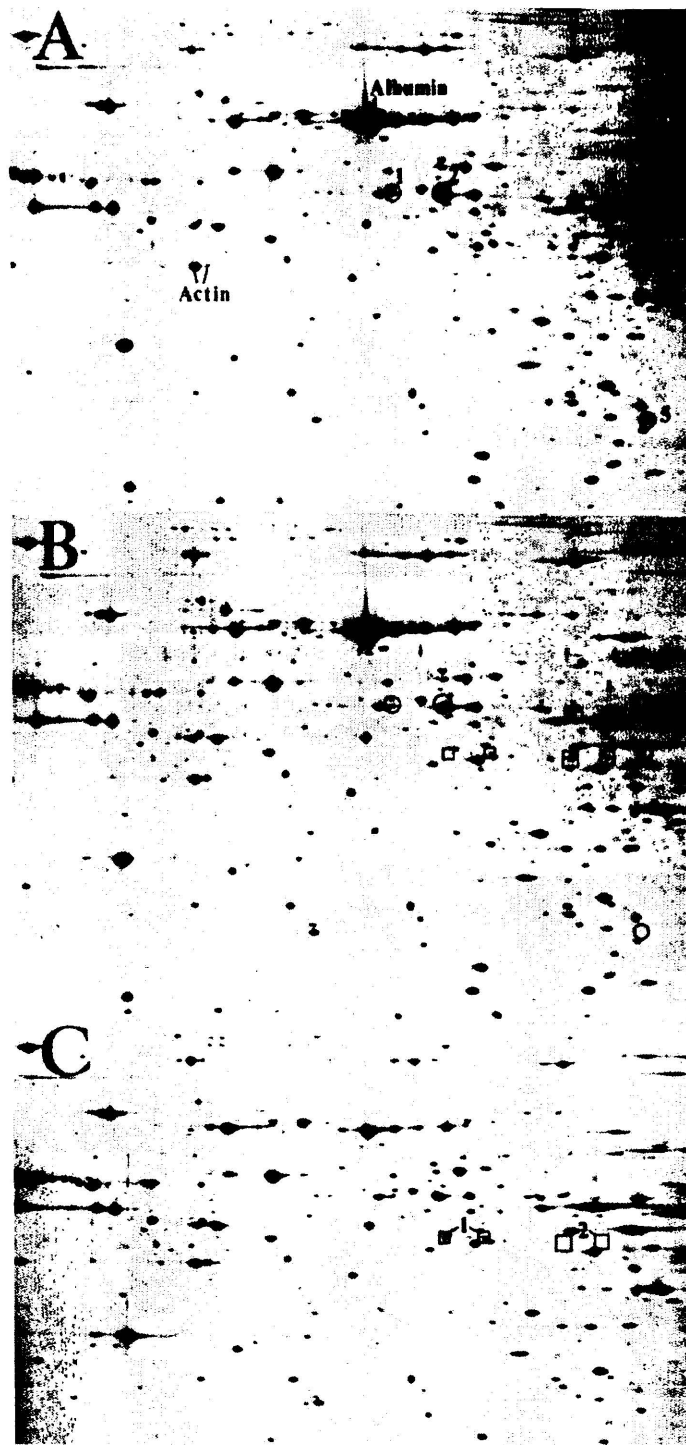


Figure 1. Coomassie Blue-stained two-dimensional protein patterns of typical male (A), female (B), and pregnant female (C) liver. Serum albumin and non-muscle actins are labelled for reference. Circles indicate major differences detected by eye between male and female, while squares indicate visually detected pregnancy differences. Quantitative differences discussed in the text are not easily visible to the eye.

Of the 30 proteins differing between sexes at high significance, 19 are expressed at a higher level in the males and 11 in females. Since one might expect sex-associated differences in proteins involved in steroid hormone metabolism (many of which are located in the smooth endoplasmic reticulum or mitochondria), we examined the subcellular location of these different proteins. As shown in Table II, 10 of the 19 proteins found in greater abundance in males are associated with microsomes or mitochondria, while only 2 of the 11 proteins found in greater amounts in females are located in these compartments. In contrast, 6 of the proteins increased in each sex are present in the soluble phase. Altogether, 24 of the 30 proteins were assigned to classically prepared subcellular fractions (6).

Table II : Subcellular Location of Proteins
More Abundant in One Sex.

Cell Fraction	Greater in males	Greater in females	Total
Soluble Phase	6	6	12
Microsomes	6	1	7
Mitochondria	4	1	5

Most of the highly significant sex-associated differences are quite small (Fig. 2). More than half (17/30) differ between males and females by less than 25%. The shape of the distribution suggests that given larger numbers of animals in the two groups, and hence higher statistical power, even more small differences could be detected.

Ratio of Abundances (Male/Female)

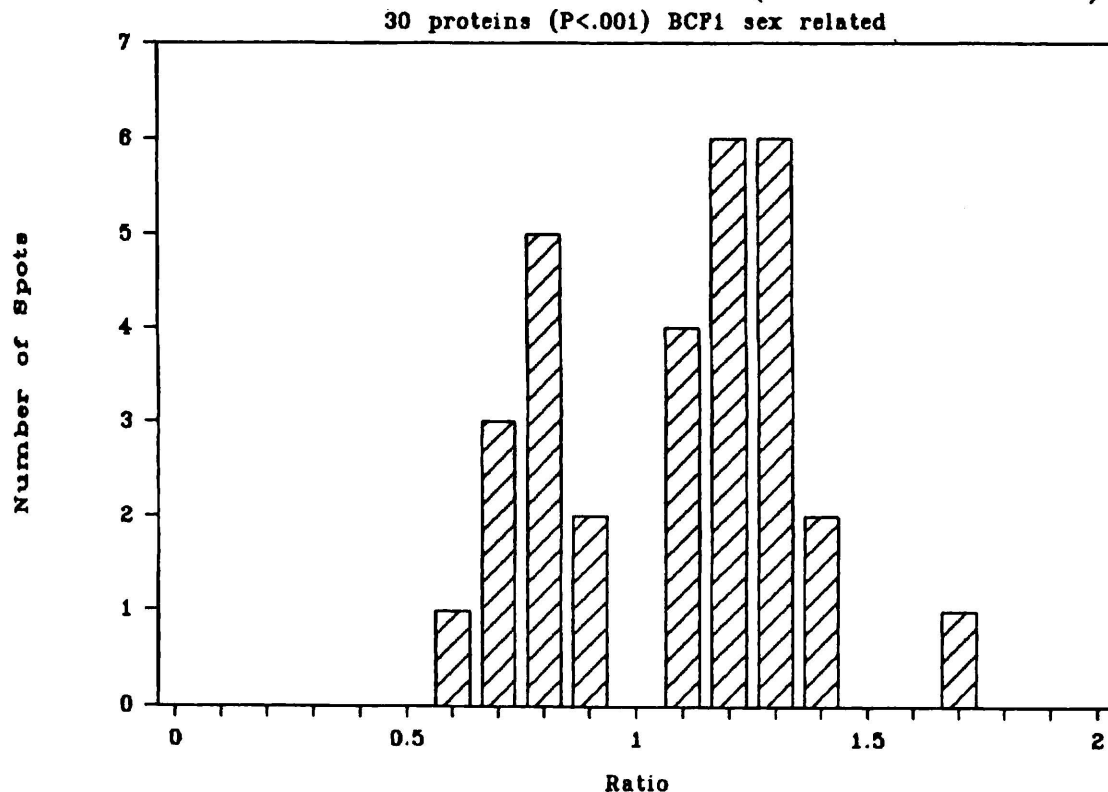


Figure 2. Histogram showing the number of spots having a given abundance ratio in comparison between male and female liver. Most proteins show differences of less than 25%.

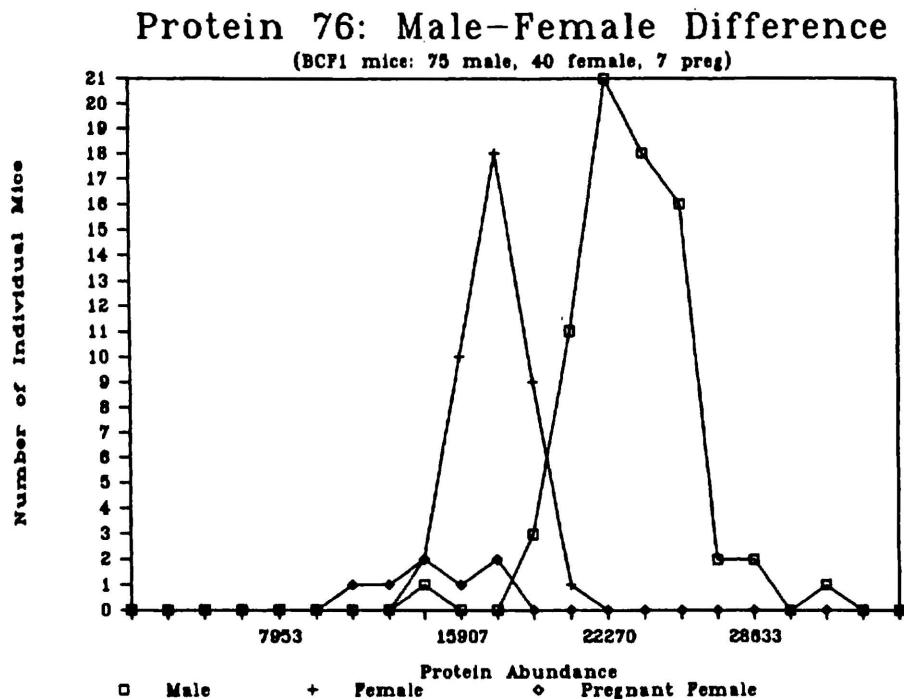


Figure 3. Histogram showing number of mice in the different groups having a given amount of protein #76. In this case a sex associated abundance difference of about 30% is obvious.

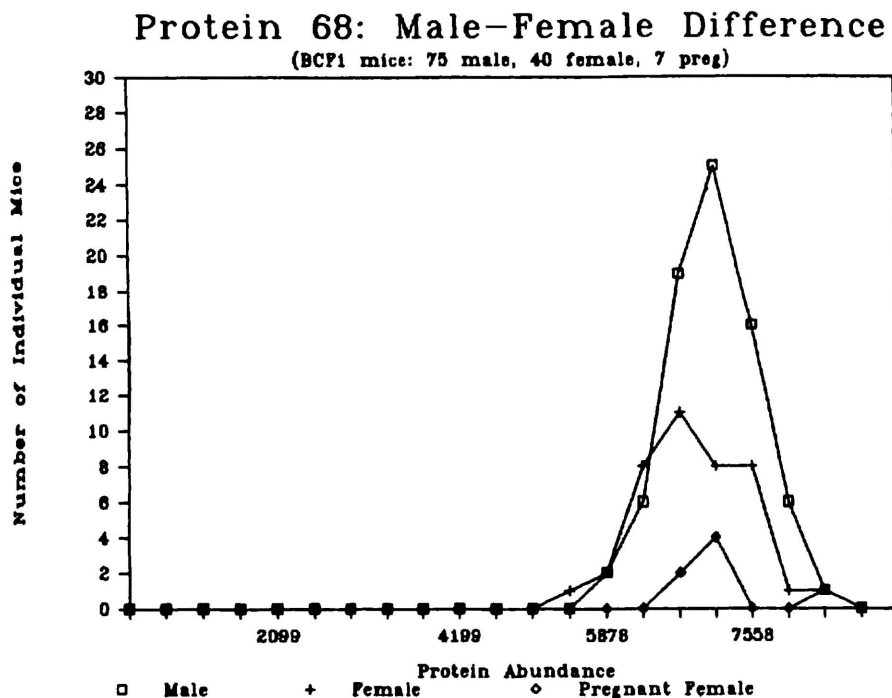


Figure 4. Histogram similar to that in figure 3, but showing abundance data for spot #68. In this case, a much smaller sex-associated difference is observed.

Although some differences are immediately apparent in the abundance histograms (Fig. 3), others are detected only through use of a statistical test. Protein 68 (Fig. 4) shows only a 9% difference in abundance between sexes, but this is very significant because the coefficient of variation is so small (7 and 9% in males and females, respectively).

Although not measured unambiguously on all 122 gels, and hence not included in the original panel of 88 spots, the gamma actin spot shows highly significant ($P < .001$) sex-associated difference (Fig. 5). Beta actin, the more abundant cellular actin form, does not show a significant sex difference ($0.01 < P < 0.05$).

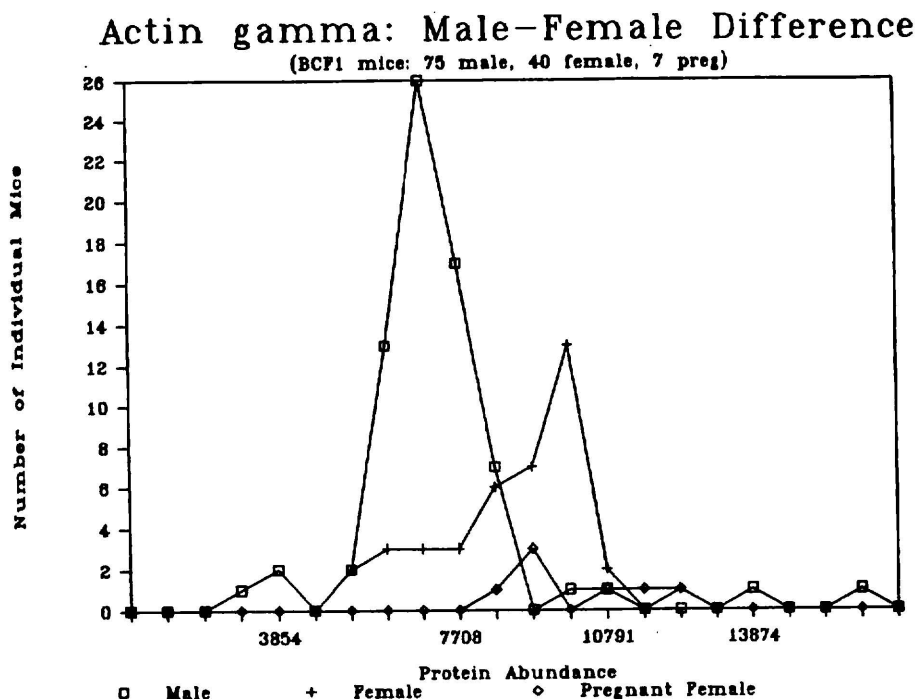


Figure 5. Histogram showing the abundance of the gamma form of non-muscle actin. Expression of this protein is clearly higher in females.

CONCLUSION

The results presented here illustrate the power of computerized 2-D electrophoresis in generating large quantitative data sets. Such data sets are shown to provide a means to detect subtle differences in the molecular phenotype of two groups of animals, in this case males and females. While we have concentrated on a subset of 88 proteins here, 300-500 should be suitable for analysis after additional work. Nevertheless, we found 35% of liver proteins to be regulated, at least partly, by sex. This contrasts with the <2% (2/146) found to differ between male and female rat hypothalamus (7).

The approach can also be applied to the detection of drug effects, mutation, and a host of environmental influences. Ultimately, by relating the resulting body of data with biochemical information on the proteins observed, a comprehensive model of the biochemistry of liver might be obtained.

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