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THE CURRENT STATUS OF TWO-DIMENSIONAL ELECTROPHORESIS IN GERM CELL MUTATION RESEARCH

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INTRODUCTION

O'Farrell (1975) demonstrated that isoelectric focusing followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis can be used to resolve hundreds of proteins from a single sample into a pattern of well-defined polypeptide spots. The possible application of this two-dimensional electrophoresis (2DE) technique to the detection of heritable mutations was recognized and demonstrated by Klose (1975, 1977) using mouse tissues. The studies done by Klose demonstrated that actual implementation of 2DE for large genetic studies, however, required rigorous pattern reproducibility and methods of extracting numerical data from the patterns. The development of equipment for multiple parallel 2DE analyses (Anderson and Anderson, 1978a,b) and of computer software for the analysis of the resulting 2DE patterns (Anderson et al., 1981; Taylor et al., 1981) during the 10 years since O'Farrell's first description of 2DE have provided the necessary tools for the application of 2DE to genetic studies.

The long range goal of all mutation studies is to assess the effect of mutagens on the human population. Genetic heterogeneity and multiple uncontrolled environmental factors may influence the detection of mutation events in human samples at the level of protein

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expression by introducing a significant level of background noise. Therefore, studies to define the criteria for mutation detection on the basis of 2DE separation of proteins are best carried out in a model system where genetic differences are minimized and environmental factors can be controlled. Data from these model experiments can then be used to form stategies for application of the 2DE method of mutation detection to human samples.

We are currently using inbred strains of mice to study the mutation detection capability of 2DE. The levels of sample and pattern reproducibility required for detection of significant changes in protein expression are being defined; the detection of mutations induced by different classes of mutagen (e.g., those causing large versus small alterations in DNA) is being assessed; and the population of proteins (and therefore genes) monitored by 2DE analysis is being characterized.

EXPERIMENTAL APPROACH

Our studies involve experiments of two types: (a) analysis of tissue from the F_1 offspring of mutagenized sires and untreated dams and (b) analysis of tissue from heterozygous carriers of well-defined mutations, such as recessive lethals (Roderick, 1983) or chromosomal deletions in the region of the albino locus (Gluecksohn-Waelsch, 1979; Russell et al., 1982). Studies of the first type demonstrate the efficiency of the 2DE approach for detecting unknown mutational events, while those of the second type serve to characterize known mutational events identified by methods other than electrophoresis at the level of protein expression.

Liver tissue is used for these studies for several reasons. The mouse liver 2DE pattern (Figure 1) is composed of hundreds of well-resolved protein spots that have a wide range of isoelectric points and molecular weights. Unlike plasma protein patterns (Anderson and Anderson, 1977) or urine protein patterns (Anderson et al., 1979), the liver protein pattern shows little

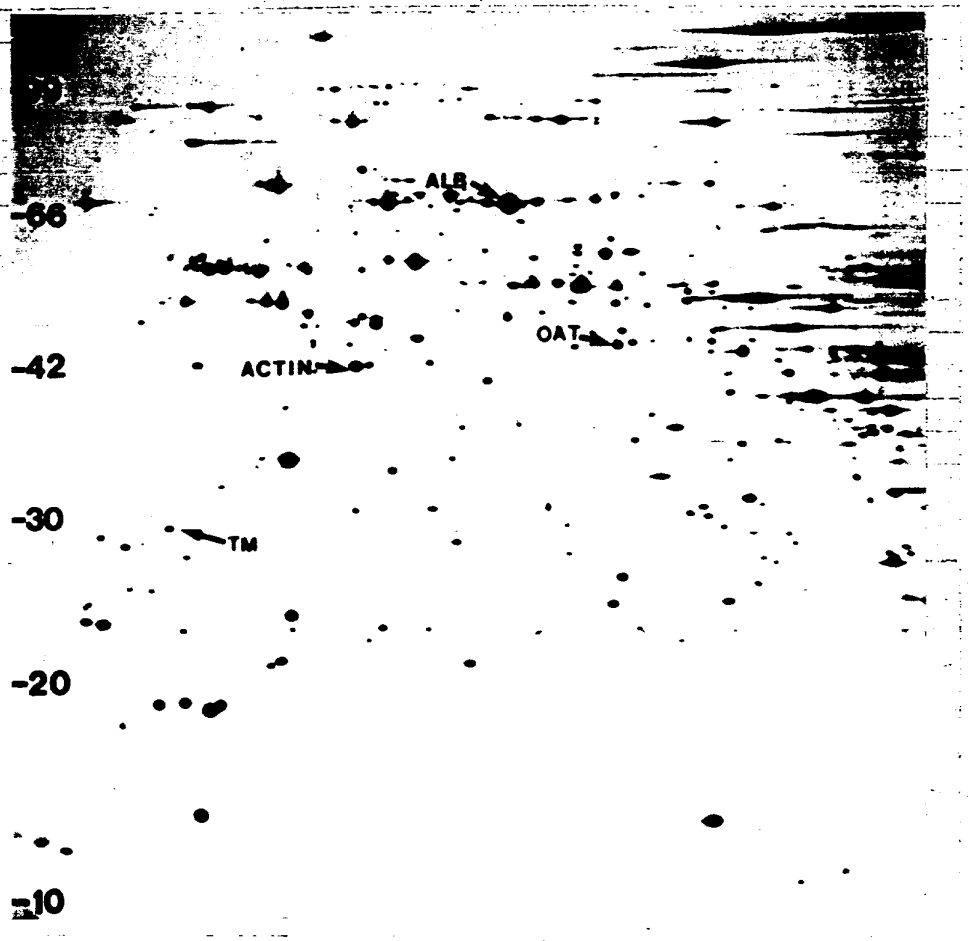

Figure 1. Two-dimensional electrophoresis pattern of $CB6F_1$ mouse liver proteins. Numbers along left side refer to approximate molecular weights ($\times 10^{-3}$). Tm, tropomyosin; Alb, albumin; Act, actin, OAT, ornithine aminotransferase. Pattern is oriented with acidic side to the left, basic side to the right.

evidence of products that result from post-translation modification. In addition to providing an ideal 2DE pattern, liver is a soft tissue that can be easily and reproducibly homogenized, producing a highly reproducible 2DE pattern. Samples can be taken by partial hepatectomy so the individual animals may later be used in heritability studies.

Liver tissue is homogenized directly into a solution containing 9M urea, 5% 2-mercaptoethanol, 4% Nonidet P40, and 2% ampholytes (pH 9-11) to eliminate protein modification by endogenous proteases. The proteins soluble in this solution are then separated by 2DE (Anderson and Anderson, 1978a,b) and stained with Coomassie Blue R250 (Giometti et al., 1987). The stained 2DE patterns are then digitized (Giometti et al., 1987) and spot files are generated for each pattern (Anderson et al., 1981). The individual spot files for each mouse sampled are matched to a common mouse liver protein pattern, and the patterns are then searched for (a) new proteins, (b) missing proteins, and/or (c) proteins significantly (i.e., 30-50%) increased or decreased in integrated density. Detection of an alteration in protein expression in any of these categories is assumed to be the result of a mutational event and is confirmed as hereditary by test crossing the carrier.

RESULTS

Detection of new proteins

An interactive search of digitized 2DE patterns for unmatched spots reveals proteins expressed in a test animal that are not found in control individuals. If such a protein is a rare event (i.e., occurs in only one individual within a set of several hundred), the alteration in protein expression is considered to be a presumptive mutation and is verified as heritable by analysis of offspring from test crosses. New proteins were detected in four out of 320 offspring sired by male C57BL/6JANL mice exposed to 150 mg/kg of N-ethyl-N-nitrosourea (ENU) (Giometti et al., 1987). All four of these protein variants appeared as new proteins with a corresponding quantitative decrease in the expression of a normally occurring neighboring protein. An example of such a case is shown in Figure 2. Such variant proteins are believed to be isoelectric point variants of the neighboring protein, a result of a base substitution mutation at the DNA level.

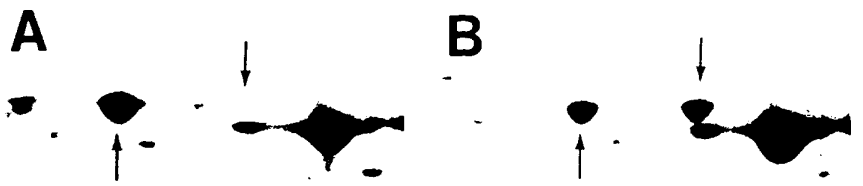

Figure 2. Portion of 2DE pattern of liver proteins from (A) Control $CB6F_1$ mouse and (B) $CB6F_1$ offspring of a sire exposed to ENU. Arrow pointing up indicates a normally occurring mitochondrial protein; arrow pointing down indicates a new protein. Note decrease in abundance of the normal protein with the appearance of the variant protein.

Detection of missing proteins

The spot files can be searched automatically to identify samples from which protein spots are missing. For example, one offspring from an ENU-exposed sire produced a liver pattern that lacked spot number 150 (Figure 3B). A protein not seen in control offspring appears directly to the right (indicated with an arrow). Spot 150 is expressed abundantly in C57BL/6JANL mice but is barely detectable in BALB/cJANL mice. Thus, the 2DE pattern shown is believed to indicate an ENU-induced base substitution occurred in the gene coding for protein 150 resulting in the appearance of an isoelectric point variant named ENU6.

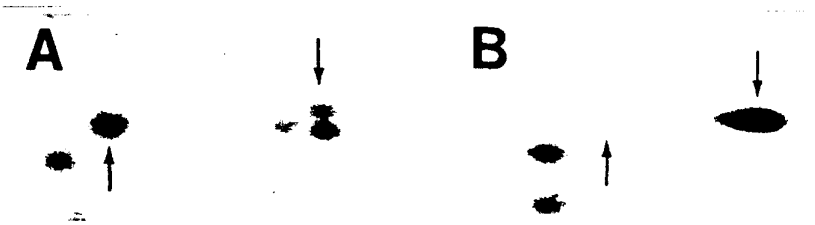

Figure 3. Portion of 2DE pattern of liver proteins from (A) Control $CB6F_1$ mouse and $CB6F_1$ offspring of a sire exposed to ENU. Arrow pointing up indicates the position of spot 150 missing from (B); arrow pointing down indicates position of an ENU-induced variant protein.

Detection of quantitative alterations in protein expression

A search for quantitative outliers within a designated set of protein spots across a set of 2DE patterns is used to identify proteins that are altered significantly in abundance. In the pattern shown in Figure 2B, a decrease of 40% in the expression of a neighboring protein was found to occur along with the appearance of a new protein (Giometti et al., 1987). In contrast to the detection of new or missing proteins where all of the protein spots in each pattern are screened, detection of quantitative outliers is restricted to a subset of proteins that have coefficients of variation (CV) of 15% or less. This restriction is necessary to avoid an overwhelming number of false positive responses to the outlier query. The number of mouse liver proteins with CV values of 15% or less has been increased from approximately 50 (Giometti et al., 1987) to 95 by sampling mice at 13 rather than 7 weeks of age (Giometti et al., 1989). Further optimization of sampling and electrophoresis parameters are anticipated to further increase the number of proteins with the required quantitative reproducibility, thus increasing the number of genetic loci that can be monitored.

Analysis of liver expression in individuals known to carry defined mutations

The results summarized thus far represent the detection of mutations in first generation heterozygotes sired by mutagenized males. To correlate 2DE detection capabilities with other test systems, studies are underway to examine protein expression in heterozygous or homozygous carriers of well-characterized heritable mutations induced by either chemical agents or ionizing radiation. In one study, liver protein expression in heterozygous carriers of eight different recessive lethal mutations (induced by exposure of sires to either triethylene melamine, x-rays, or gamma rays [Roderick, 1983]) was analyzed. Although detection of strain-related differences among this group of individuals

indicated the sensitivity of the 2DE system to quantitative differences in the samples, no protein differences were found that correlated with the mutations (Giometti et al., 1988). Preliminary data from the analysis of homozygous and heterozygous carriers of chromosomal deletions around the albino locus indicate that although significant protein differences are seen in the liver patterns of the homozygotes, the heterozygotes have liver protein expression comparable to wild-type individuals (Giometti et al., 1989). Therefore, had only heterozygotes been examined, no liver protein alterations that correlated with the mutation would have been found in this system either.

DISCUSSION

Published data on mouse germ cell mutation rates from both the specific locus test (Russell et al., 1979) and the one-dimensional electrophoresis test (Johnson and Lewis, 1981) show that the frequency of ENU-induced mutations is approximately 5×10^{-4} per locus per generation. Although it is difficult to identify the number of loci actually represented in a 2DE pattern of mouse liver proteins, these estimates indicate that appearance of an alteration in protein expression should be assumed to be a rare event, thus requiring relatively large numbers of 2DE analyses (hundreds rather than tens) to be done. Data from our studies have shown that the sample and 2DE pattern reproducibility now achievable is adequate for the detection of new proteins, loss of proteins, and changes in abundance of 40% or more. Mutations assumed to be the result of base substitution events by the chemical nature of the mutagen (e.g., ENU) induce the appearance of new proteins in 2DE patterns with a per mouse frequency comparable to that found with the one-dimensional electrophoresis technique. Johnson and Lewis (1981) reported 9 ENU-induced mutation events among 1072 offspring analyzed and our study detected 5 events among 320 offspring analyzed. The absence of protein alterations in heterozygous carriers of known large mutation events such as chromosomal deletions,

however, has been encountered. Perhaps the deletions do not involve structural genes for the liver proteins seen in the 2DE patterns used for analysis, but rather affect regulatory genes. More systems with characterized deletion-type mutations must be analyzed to better understand the capability of the 2DE approach for the detection of mutations other than base substitutions.

The requirement of tight quantitative reproducibility for the detection of differences in abundance of 40% or greater has been well documented by the studies done thus far. For mouse mutagenesis studies, the number of proteins with the necessary reproducibility (i.e., CV of 15% or less) has been increased by sampling animals well after they have reached sexual maturity (age 10-13 weeks) rather than those in puberty (age 6-7 weeks). Other parameters such as diet and circadian rhythm may also be investigated as to their effects on liver protein expression, and, if those effects are found to be significant, additional changes in sampling protocols can be made. The amount of quantitative variability in human samples has not as yet been assessed, although the rate of genetically controlled positional variation among a variety of human sample types has been reported as 1-2%. One expects that the degree of genetically regulated quantitative variability in the outbred human population will be greater than 1-2%, and will vary greatly depending on the sample analyzed. A survey of CV values for several samples (e.g., serum, lymphocytes, platelets, fibroblasts) from a human population would provide valuable data relevant to the applicability of 2DE for detection of mutations in humans.

The application of 2DE to the field of germ cell mutagenesis provides yet another tool for the investigation of mechanisms and outcome in biological systems exposed to toxic agents that are suspected of causing heritable effects. With the capability to examine events that occur in whole animals at the level of molecular damage but using a broad range of molecular markers rather than a narrow list of known loci, the 2DE approach to mutation studies promises to teach us new things about the process of mutagenesis.

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