Detection of Heritable Mutations as Quantitative Changes in Protein Expression*

(Received for publication, March 3, 1987)

Carol S. Giometti, M. Anne Gemmell, Sharron L. Nance, Sandra L. Tollaksen, and John Taylor

From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439-4833

A computerized search for the appearance of heritable mutations (as indicated by changes in protein expression) was conducted on three sets of mice, whose sires had been either untreated, exposed to 3 gray units of gamma radiation, or treated with 150 mg/kg ethylnitrosourea. Proteins from the livers of approximately 800 mice were separated by two-dimensional electrophoresis, and abundances were measured by using image analysis techniques. Heritable mutations were detected by the appearance of new proteins or by the quantitative decrease in abundance of normally occurring proteins. Measurements of the variability of the protein abundance indicate that at least 48 proteins are consistent enough to be used in searches when mutations are expected to result in a 50% reduction in the normal amount of protein. New proteins were found in four offspring from ethylnitrosourea-treated mice, and in each case a nearby spot was found to be significantly diminished. These mutations were confirmed in subsequent generations. A computer-assisted search detected three of these mutations on the basis of the abundance decrease alone. These results indicate that two-dimensional electrophoresis can be used to detect mutations reflected as quantitative changes in protein expression, provided that the proteins to be monitored are quantitatively stable when samples from different individuals are compared.

Exposure to a mutagen can cause both point mutations and small chromosomal deletions. The combination of a gamete carrying a point mutation with a gamete carrying the unaltered gene could result in an offspring that expresses an altered protein together with the normal protein at 50% of its normal abundance. A gamete in which a structural gene has been deleted could, if combined with a gamete carrying the normal gene, result in an offspring that expresses the corresponding gene product at 50% of its normal abundance. Therefore, detection of quantitative alterations in protein expression could, theoretically, be used to measure genetic changes that can be tested for heritability and to provide data for estimation of mutation rates.

Two-dimensional electrophoresis has been successfully used to detect qualitative protein changes indicative of point mutations (1, 2) and gene deletions (3) induced by toxic chemicals (1, 2) or ionizing radiation (3). Detection of quantitative protein changes that reflect either point mutations or gene deletions, however, has been hampered by the inability to obtain quantitative measurements from the large number of two-dimensional electrophoresis patterns required for mutation screening (4). Anderson et al. (5) have shown that two-dimensional electrophoresis, coupled with computerized data analysis, can detect a 50% reduction in protein amount, provided that the background quantitative variations are small. However, the contribution of individual sample variability, both experimental and biological, to the overall quantitative data dispersion is currently unknown. The magnitude of such variability may well determine the feasibility of ultimately using two-dimensional electrophoresis protein separations to screen human samples for the occurrence of induced mutations following exposure to known or suspected mutagens.

We report here the results of a mutagenesis study in which heritable mutations, represented as altered protein expression, were detected by computer-assisted screening of two-dimensional electrophoresis protein patterns. This study was designed to assess the quantitative capabilities of two-dimensional electrophoresis and to evaluate the possible application of this technique to mutation studies in humans. To minimize quantitative variability due to genetic heterogeneity and thus concentrate on quantitative variability introduced by sampling and nongenetic biological factors (e.g., age, diet), we chose to use inbred strains of mice for our initial study. Thus, the results presented here represent the simplest case for the application of two-dimensional electrophoresis to screening for mutations that cause quantitative protein changes and serve as a foundation for human studies in which genetic heterogeneity will contribute additional quantitative variability (5–7).

This study included 797 offspring from untreated male mice or male mice treated with either ethylnitrosourea or gamma radiation. Ethylnitrosourea-induced mutations, previously shown to cause the appearance of new protein spots in two-dimensional electrophoresis patterns of mouse liver proteins with a corresponding decrease in the intensity of an adjacent spot (2), allowed the detection of rare quantitative protein alterations in two-dimensional electrophoresis patterns to be validated. The ability to detect radiation-induced mutations could then be realistically assessed.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

The results of this study demonstrate that quantitative two-dimensional electrophoresis can be used to detect muta-

* This work was supported by the United States Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-ENG-38. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed: Division of Biological and Medical Research, Argonne National Laboratory, 9700 S. Cass Ave., Argonne, IL 60439-4833.

1 Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–5, Table 1, and additional references) are presented in miniprint at the end of this paper. The abbreviations used are: CV,
tions that cause an altered gene resulting in the expression of a variant protein together with a 50% reduction in the abundance of the normal protein. The detection of such mutations as quantitative changes in protein expression is, however, limited by the background quantitative variation in each protein monitored. The detection of three out of four ethyl-nitrosourea-induced mutations based on quantitative changes in normal liver proteins demonstrates this limitation and sets the present detection threshold of the two-dimensional electrophoresis system.

The use of two-dimensional electrophoresis to detect mutations that cause the total loss of one gene copy must still be validated. For this experiment, in order to simplify the mutation search protocol, the assumption was made that the loss of one gene copy would result in a 50% reduction in the synthesis of the amount of the corresponding protein. The possibility exists, however, that intracellular regulatory mechanisms may cause compensatory synthesis of proteins in order to maintain normal concentrations. Given the constraint of quantitative reproducibility defined by our present data, such compensatory mechanisms must be investigated, since quantitative changes of less than 50% that could be significant indicators of mutation might otherwise be ignored. The analysis of protein expression in tissues from heterozygous carriers of known gene inversion or deletion mutations (available as mouse stocks) or in cultured cell lines with induced gene deletions should demonstrate whether or not such mutations are detectable by two-dimensional electrophoresis.

The absence of significant changes in liver protein expression among 369 offspring from irradiated males may be a reflection of (a) the influence of cellular compensatory mechanisms that masked gene deletions or (b) the limited number of proteins that had the quantitative stability required for detection of a 50% decrease in protein abundance. Assuming that monitoring the 45 protein spots with coefficient of variation values of no more than 15% would have permitted detection of a 50% reduction in expression and that each of the 45 protein spots represents an independent gene locus, and given a specific locus mutation rate of 2.7 X 10^{-7} per gray unit per locus as representative of the response to single doses of gamma radiation (8), the expected mutation yield in this study would have been about one event in the 369 gametes screened following exposure to 3 gray units. Thus, zero events is well within the limits of expectation for the number of individuals screened. If more protein spots with low levels of normal variability could be monitored, the probability of detecting a quantitative protein variant in a sample of 400 individuals would obviously become more feasible.

In the mouse model system, optimization of the number of protein spots in a two-dimensional electrophoresis pattern that can be monitored for quantitative changes may only require stricter control of quantitative variability introduced by both technical inconsistencies and nongenetic biological factors. Anderson et al. (5) have demonstrated that minor differences in sample loading, electrophoresis, staining and destaining, and computer imaging actually introduce very little quantitative variation into the two-dimensional electrophoresis patterns of mouse liver proteins. Such variation could be limited further by the analysis of each sample on multiple coefficient of variation. ENU, ethyl-nitrosourea; 2DE, two-dimensional electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Reprint Document No. 87 M 687, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Heritable Mutations Detected as Quantitative Protein Changes

Acknowledgments—We thank D. Grazze for his invaluable guidance in the areas of mouse genetics and radiation biology throughout the course of this experiment and for his assistance with the statistical analysis of the data. We also acknowledge N. Anderson, L. Anderson, and F. Giere for their assistance with the experimental design and treatment of the mice, and G. Spicer for his assistance scanning two-dimensional electrophoresis gels.

REFERENCES

1. Klose, J. (1975) Humangenetik 26, 231–243
2. Marshall, R. R., Raj, A. S., Grant, F. J. & Heddle, J. A. (1983) Can. J. Genet. Cytol. 25, 457–466
3. Baier, L. J., Hanash, S. M. & Erickson, R. F. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2123–2126
4. Neal, E. R., Flanagan, B. C., Skolnick, M. M., Hanash, S. M. & Steinberg, S. (1984) in Two-dimensional Gel Electrophoresis of Proteins (Celis, J. E. & Bravo, R., eds) pp. 259–306, Academic Press, Inc., New York
5. Anderson, N. L., Nance, S. L., Tollaksen, S. L., Giere, F. A. & Anderson, N. G. (1985) Electrophoresis 6, 592–599

EXPERIMENTAL PROCEDURES

Treatment of animals—Male C57Bl/6J mice were injected intraperitoneally with 200 rad of X rays at 2 weeks of age, with sampling times done at the same time of day. Liver tissue was chosen to minimize variability in protein recovery, because the tissue could be homogenized quickly and reproducibly. Liver homogenization was done by gently placing a piece of liver tissue in 20 volumes of solution containing 8 M urea, 50 mM Tris-phosphate, 20 mM ascorbate (pH 7.5), and 0.1 M NaC03 (0.04 mole/l). The homogenates were centrifuged for 60 min at approximately 45,000 g in a 41100 microfuge and the supernatants were stored at −70°C until analysis.

RESULTS

Detection of a significant quantitative difference in a single protein spot among the 500-700 spots in each patient required a high degree of pattern reproducibility, both positional and quantitative. Figure 3 shows the 259 protein spots of interest from a single protein homogenate that was used as the master pattern for the metagenomics experiment. The sample was analyzed by two-dimensional gel electrophoresis. The total number of protein spots was determined by scanning the gel and counting spots that were larger than two pixels in size and were detected consistently in at least 90% of the spots. Each protein spot was then analyzed for protein expression and for spot identity. A total of 65 spots were analyzed and correlated for spot identity and protein expression. A total of 65 spots were analyzed and correlated for spot identity and protein expression.

Two-dimensional electrophoresis—Protein electrophoresis was modified by allowing samples to be in gels for 15 min, using the same batch of ampholytes throughout the experiment, and rigorously controlling electrophoresis times and gel concentrations. Two-dimensional gel electrophoresis was used to separate the 65 proteins in the liver homogenate and then stained with silver nitrate after electrophoresis as described by Jovanovitch et al. (1) using Gelsite pI 8.0, 8.1, and 8.2 in 0.05% SDS. The two-dimensional separation was performed as described by Jovanovitch et al. (1) with modifications described by Anderson and Anderson.

Gels were stained with Gelsite pI 8.0, 8.1, and 8.2 in 0.05% SDS and visualized in 0.05% SDS.

Data analysis—The volumes of protein spots in each gels were scaled to correct for differences in the amount of protein loaded onto each gel. The 2D protein spots chosen for use in the scaling were reproducibly found in mouse liver protein patterns, they covered range of molecular weights, electrophoretic properties, and integrated densities (optical densities), and they were used as an internal standard to normalize the protein expression data. The volumes of the protein spots were calculated on the basis of the spot density at the maximum (optical density) and the spot density at the minimum (optical density). Scoring factors for individual patterns varied between 0.75 and 1.0. All scoring factors for each of the same protein spots from each patient were calculated with reference to the same set of reference patterns. The standard volume of each protein spot was calculated on the basis of the spot density at the maximum (optical density) and the spot density at the minimum (optical density). The standard volume of each protein spot was calculated with reference to the same set of reference patterns. Statistical differences in the volumes of the protein spots were then calculated with reference to the same set of reference patterns. Statistical differences in the volumes of the protein spots were then calculated with reference to the same set of reference patterns.

Undefined spots—The computer highlighted all protein spots in the object patterns that had no substantial overlap in the mass spectrometry. The display was then examined individually to determine whether the highlighted spots were reproducible and had a reproducible volume. The volumes of the protein spots were then calculated with reference to the same set of reference patterns. Statistical differences in the volumes of the protein spots were then calculated with reference to the same set of reference patterns.

Quantitative outcome—A search for protein spots with significant quantitative differences in spot volume was done in the same way for each patient. For each patient, the volume of the protein spot was calculated with reference to the same set of reference patterns. Statistical differences in the volumes of the protein spots were then calculated with reference to the same set of reference patterns.
Heritable Mutations Detected as Quantitative Protein Changes

Fig. 1
Plots of relative sample concentration (C/Pratx) vs. spot volume (V, expressed as integrated density units / mm²) for several different concentrations of Pratx. The samples were applied to a nitrocellulose membrane and probed with an antibody against Pratx. The concentration, expressed as integrated density units (IPM), was plotted against the spot volume (V) of the Pratx spots. Approximately 10 ng/ml of specific protein spots were used in four replicate gels. All gels were stained with a two parameter fit to the data. Curve A, used only to demonstrate that the data span the range of protein concentrations, and V upset free parameters. The data were analyzed by a linear regression to obtain a line for the Pratx concentration vs. spot volume (V) of the Pratx spots. The results are shown as a line in Fig. 1B, and the Pratx spots are indicated by spot numbers. The values are indicated on the x-axis of Fig. 1B.

Fig. 2
The summary of the coefficients of variation (%) for matched protein spots. The coefficient of variance was calculated for each matched spot in Fig. 1B. Each matched spot was assigned a number with the number of spots within each range of CV, and the Sauts shows the CV. A "E" indicates the average CV of the data set, and E indicates the percentage of protein spots that had CV values of less than 5%.

Fig. 3
Summary of the coefficients of variation (%) for matched protein spots. The coefficient of variance was calculated for each matched spot in Fig. 1B. Each matched spot was assigned a number with the number of spots within each range of CV, and the Sauts shows the CV. A "E" indicates the average CV of the data set, and E indicates the percentage of protein spots that had CV values of less than 5%.

Table 1
Table of spot volume values for matched protein variants.

| Spec. Mean spot vol^a | Spot vol^b | E reduction | Log of ratio^c |
|-----------------------|------------|-------------|---------------|
| 5 26464 16698 37 6.2 3.82 |
| 99 5064 4294 39 17.3 0.57 |
| 79 6681 5100 54 22.4 1.12 |
| 121 2153 1638 44 14.9 3.15 |

^aMean spot volume: calculated for data set being searched, e.g., male offspring from SBE-treated mice, and expressed in integrated density units.

^bSpot volume with occurrence of SBE variant: a single measurement.

^cLog of ratio: indicator of the significance of quantitative outliers on SBE decrease in protein amount; see Materials and Methods.

The results from the offspring of mice treated with SBE demonstrated that our method was sensitive to decreases of 25% in SBE protein amount for proteins with good quantitative scalability (95% of the proteins). The results from proteins with more background variability (CV greater than 15%). As shown, 80% of proteins in the mouse liver pattern had CV values of less than 15%, suggesting that radiation-induced gene deletions should be detectable in at least those proteins. Although approximately 20 protein indicators for quantitative outliers in contrast to the offspring of the SBE variant were identified by interactive examination of the data, none of these alterations were confirmed when the samples were reanalyzed on replicate gels. Thus, we were able to identify radiation-induced gene deletions by quantitation from the irradiated mice. None of the quantitative outliers (C创业) in the male and female offspring of SBE-treated mice were validated on interactive examination, as none of the control samples were examined.

REFERENCES

1. Anderson, B. & Andrews, H. (1978) Annu. Rev. Genet. 12, 231-260
2. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007-4014
3. Anderson, B. & Andrews, H. (1979) Annu. Rev. Genet. 13, 179-195
4. Anderson, B. L. & Andrews, H. (1981) Annu. Rev. Genet. 15, 21-34
5. Andrews, B. L., Taylor, J., Breslow, R. E., Czok, G. & Andrews, H. G. (1981) Genet. Res. 39, 187-192
6. Taylor, J., Andrews, B. L. & Andrews, H. G. (1981) in Electrophoresis of RNA, (Allen, K. & Arno, F., eds.) pp. 393-409, V. C. New York
7. Taylor, J., Andrews, B. L., Andrews, H. G., Czok, G., Gier, R. E. & Andrews, H. G. (1981) in Electrophoresis of RNA, (Allen, K. & Arno, F., eds.) pp. 393-409, V. C. New York
8. Andrews, B. L., Czok, G. & Andrews, H. G. (1981) in Electrophoresis of RNA, (Allen, K. & Arno, F., eds.) pp. 393-409, V. C. New York
9. Andrews, B. L., Czok, G. & Andrews, H. G. (1981) in Electrophoresis of RNA, (Allen, K. & Arno, F., eds.) pp. 393-409, V. C. New York