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Detection of Human Somatic Cell Structural Gene Mutations by Two-Dimensional Electrophoresis

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ABSTRACT **The feasibility of detecting human somatic structural gene mutations by two dimensional electrophoresis has been investigated. A lymphoblastoid cell line was grown as a mass culture in the presence of ethylnitrosourea, after which cells were regrown as single cell clones. A total of 257 polypeptide spots were analyzed in gels derived from 186 clones. Four structural mutations were detected by visual analysis of the gels. Computer analysis of gels corresponding to the mutant clones was also undertaken. At a spot size threshold of 200 spots to be matched using a computer algorithm, all four mutant polypeptides were detected. These results indicate the usefulness of the two-dimensional approach for mutagenesis studies at the protein level.**

Key words: two-dimensional electrophoresis, ethylnitrosourea, somatic mutations, structural variants.

INTRODUCTION

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), because of its resolving power, allows the simultaneous monitoring of several hundred polypeptide gene products in a single preparation. Applications of the technique in the field of human genetics include the detection of variants due to polymorphism, of alterations associated with genetic disorders, and of mutations that are either spontaneous or induced. We have previously utilized 2-D PAGE to detect genetic variants in plasma, erythrocyte lysates, platelets, and lymphocytes.[1–5] The genetic nature of each variant has been confirmed by a similar observation in either the father or mother of the subject. The proportion of polypeptides on the gel for which heterozygosity for a variant is observed is almost 4%. Similar variant frequencies for plasma and erythrocytes were encountered in separate studies of Japanese individuals in Japan.[6,7]

The reliable detection of genetic variants using 2-D PAGE has encouraged us to pursue the detection of mutations using the same method. An important goal of the studies is to develop an approach for the assessment of mutational risk to humans due to exposure to mutagenic agents.[8] Because mutation is a rare event, a large number of locus tests is required before a mutation frequency can be estimated. As part of our strategy for the study of mutation using 2-D PAGE, we have developed a computer program for automatic polypeptide spot matching in two-dimensional gels.[9,10] While the matching algorithm could be useful for various applications, it has specifically been refined for the purpose of detecting mutations.

In this paper we present the results of a study in which cells in culture were exposed to a mutagenic agent. Clones were grown from single cells, and their proteins were visualized by 2-D PAGE and silver staining, and the preparations were analyzed by eye. Gels in which mutations were observed were also analyzed using our matching algorithm. Our findings support the feasibility of studies aimed at the efficient detection of mutations in humans across a large group of loci.

MATERIALS AND METHODS

Cell culture

A subclone of a human lymphoblastoid cell line (TK-6) with a high plating efficiency was grown as a mass culture.[11] To induce mutations, cells were exposed to 50 μl/ml of ethylnitrosourea (ENU) for 40 minutes, after which they were washed, plated by the limited dilution method, and regrown as single cell clones. The cloning efficiency following exposure to the above mentioned dose of ENU decreased to approximately 10%. After approximately 3 weeks of growth, clones were harvested and routinely subdivided into four aliquots. Two aliquots each containing approximately 2×10^6 pelleted cells without supernatant were used for 2-D PAGE. Two additional aliquots were frozen in vials in a viable state for subsequent propagation and repeated analysis if indicated.

2-D PAGE

Cell pellets were solubilized by addition of 40 μl of a lysis buffer consisting of (per liter) 9 M urea, 40 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5–10), 20 ml of 2-mercaptoethanol, and 0.2 mM of phenylmethylsufonyl fluoride in distilled deionized water. The pH was adjusted to 9.5. In most cases 30-

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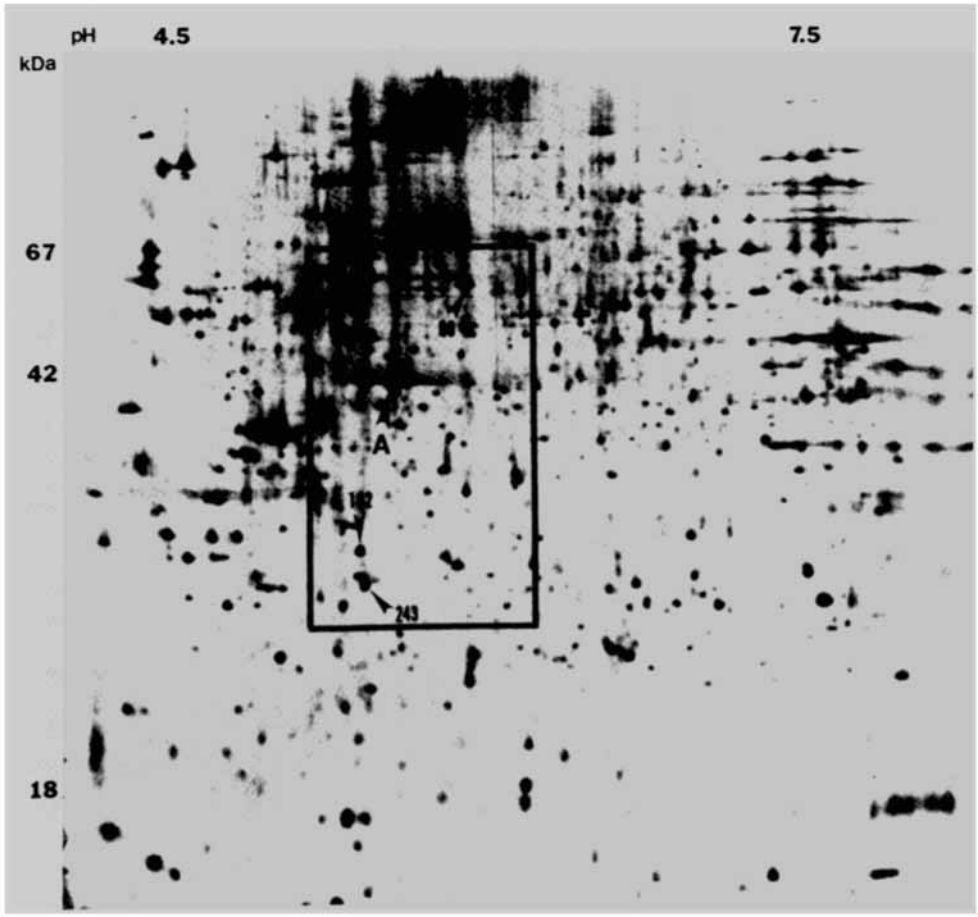

Fig. 1. Two-dimensional pattern of lymphoblastoid TK-6 polypeptides. The pH is indicated on the horizontal axis and the molecular weight (in kilodaltons) on the vertical axis. The rectangle indicates the location of polypeptides for which mutations were detected. The letter A refers to actin.

μl aliquots containing solubilized cells (2×10^6) were immediately applied onto isofocusing gels. First-dimension gels contained 20 ml of ampholytes per liter (pH 3.0–10). Isofocusing was done at 1,200 V for 16 hours and 1,500 V for the last two hours. Twenty gels were run simultaneously. For the second-dimension separation, an acrylamide gradient of 11.5–14.0 g/dl was used having 2.6% cross-linking with bisacrylamide.[8] The polypeptides in the gels were visualized by the silver-staining technique of Merril et al.[12]

Visual Analysis of Gels

A total of 296 polypeptide spots per gel were initially selected for scoring to detect mutant polypeptides characterized by an altered electrophoretic mobility. The spots chosen for analysis were evenly distributed in the gel; spots within 2 cm of the gel margins which were generally excluded from study because of frequent streaking of spots or variable position. Two other criteria were applied for selection: One is that of lack of crowding around the selected spot so that if an electrophoretic variant were to occur, it would be easily detected. Another criterion was that the spot have sufficient intensity such that if a mutation were to occur both the mutated and non-mutated protein gene products would be well above the detection threshold with silver staining. Gels were examined for the appearance of a new spot in the vicinity of a polypeptide spot selected for scoring and for a change in intensity or complete absence of any of the 296 selected spots. To substantiate that a variant in a given clone is a reproducible finding as would be expected for a mutation, a second cell pellet of the same clone was solubilized for 2-D PAGE, and frozen cells were recultured and the cells harvested for 2-D PAGE analysis.

Computer Analysis of Two-Dimensional Gels

Four gels in which a mutation was detected visually were analyzed using a computer matching algorithm. The central one half of the gel (in which all four of the mutants were observed) was digitized and

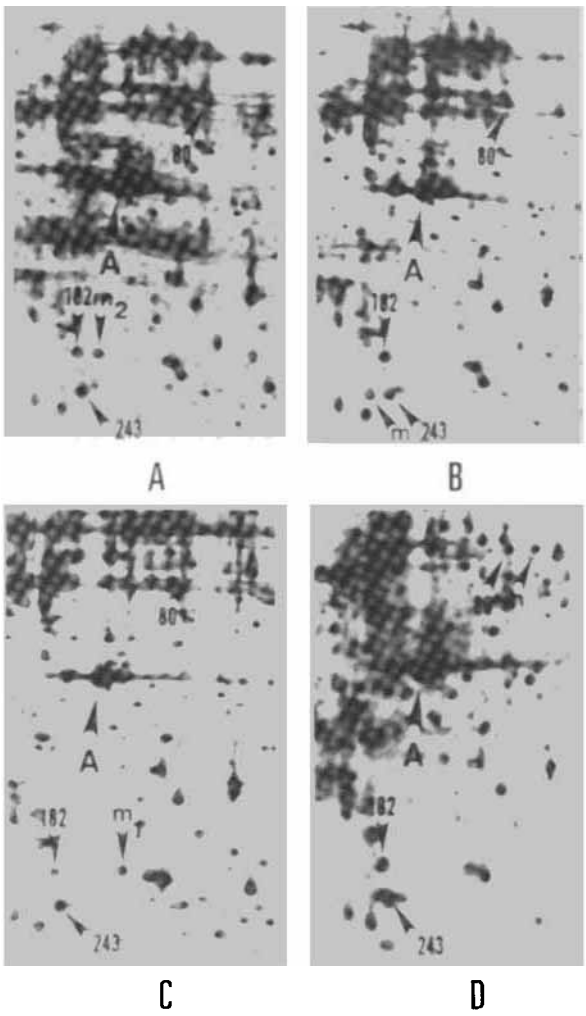

Fig. 2. Close-ups of two-dimensional gels from four clones exhibiting a mutation. The letter M refers to the localion of mutant polypeptides. Two mutations (M1 and M2) were observed for polypeptide 182. Arrows point to the location of actin and of the normal polypeptides for which a mutation was observed, for reference.

spots were detected using the BioImage visage system (Ann Arbor, MI). A 1,024 × 1,024 pixel format was utilized, giving 101 μm per pixel, each pixel having 256 possible gray-scale values. Spot matching was performed using our previously described algorithm.[9] A key parameter for the algorithm is the number of spots initially to consider as being above a threshold for matching; thus, selecting a cutoff of 120 spots with the greatest relative intensity, the algorithm attempts to match all spots that are among the 120 largest spots on each gel. In situations where a spot on gel A is above the threshold but an above-threshold spot that matches it cannot be found on gel B, the algorithm searches through the spots that are below threshold on gel B and in the same relative vicinity of the spot on gel A, to determine if one of these is the correct match. This strategy of having an internal threshold below which the matching algorithm may search if need be, is an important feature of the matcher and helps make the algorithm particularly useful for detecting mutations. Because the spots that are initially above threshold are not necessarily exactly identical on most gels, the actual number of spots matched is larger than the cutoff number.

RESULTS

Visual Analysis of Gels

A total of 192 clones were analyzed by 2-D PAGE. A representative gel is shown in Figure 1. Of the original 296 spots selected for scoring, 29 were found to be unreliable because, as the number of gels analyzed increased, it became clear that they exhibited substantial quantitative variability from gel to gel. Therefore the analysis was focused on a final group of 257 spots. While for most gels, a vast majority of the 257 spots could be readily scored, technical factors not related to the polypeptides themselves, such as inadequate electrophoresis, gaps in the gel, nonuniform staining or tears in the gel prevented the scoring of some or all spots in a subset of gels. As a result, a second gel was prepared for some clones. The criterion was that for gels in which 5% or more of the spots to be analyzed were not scorable, a repeat gel was obtained. For the 192 clones included in this study, approximately one-third required a repeat gel, and 6 were unscorable on both gels. Of a grand total of 47,802 polypeptide spots to be analyzed in the final 186 clones, 725 spots (1.5%) were not scorable, giving an average of 3.9 nonscorable spots per clone preparation and a final total of 47,077 scored polypeptide spots.

Somatic Mutations Detected

Among the 186 clones, four structural mutations were detected, each in a separate clone (Fig. 2). A variant of polypeptide 80 was observed in one clone (Fig. 2D). The variant polypeptide migrated more basically relative to the normal polypeptide. Both the variant and normal polypeptides in the mutant clone were of equal intensity. Polypeptide 182 exhibited two electrophoretically different mutants (Fig. 2A,C). In each case, the occurrence of a mutant was associated with readily detectable decreased intensity of the normal polypeptide. For both mutations related to polypeptide 182, the mutant and normal spot were of equal intensity. One mutant migrated approximately two charge differences more basic than the normal polypeptide, (Fig. 2C), while the other was closer to the normal polypeptide, on the basic side (Fig. 2A). The fourth mutant spot occurred in the vicinity of polypeptide 243 and two other slightly smaller polypeptides being scored (Fig. 2B). The relatedness of the mutant spot to 243 is evidenced by the decreased intensity of polypeptide 243 in the mutant clone. In all instances where a mutant was detected in the original gel, a repeat analysis of the clone from

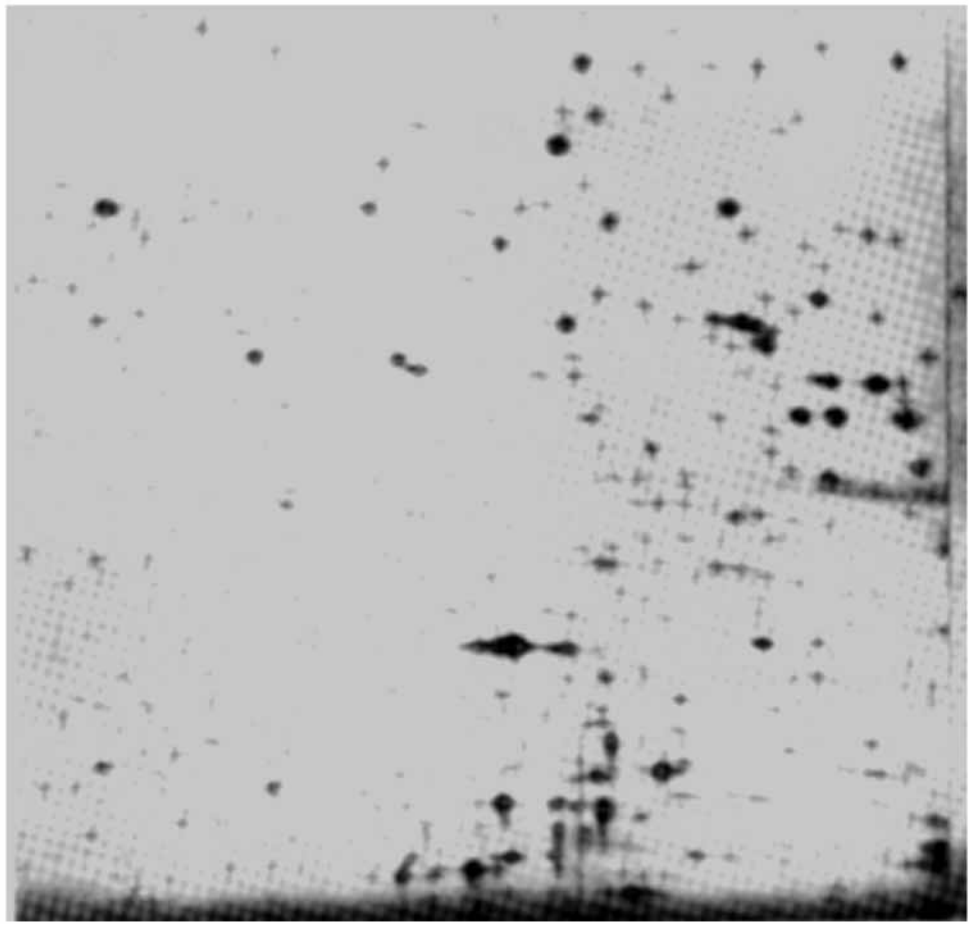

Fig. 3. Two-dimensional gel of a lymphoblastoid clone as displayed on the monitor. Crosses point to the location of spots subjected to computer analysis.

the same harvest as well as from repropagated material yielded the same phenotype with respect to the mutations.

Computer Analysis of Mutant Clones

Because all clones were derived from the same clonal cell line and might have differed from each other as a result of the mutagenic exposure, gels could be compared with each other without the need for a standard gel against which all gels would be compared. Computer analysis of the mutant clones was undertaken in a pairwise manner. Gels were matched in two sets of two gels. Initial matching was performed using an integrated spot intensity threshold yielding 120 spots for matching (Fig. 3). The results are presented in Table 1. Spot matching for each pair of gels was accomplished in less than 2 minutes. Of the four mutants, three were detected as unmatched spots, and one (mutant spot 80) was not (Fig. 4). In addition, the algorithm detected seven other

TABLE I. Results of Computer Matching at the 120-Spot Cutoff Level*

Clone	Mutant spot	No. spots detected	No. spots matched	Mutant detected	No. of other node difference
14	243	355		Yes	
			141		1
58	182 #1	403		Yes	
35	182 #2	458		Yes	
			123		6
20	80	356		No	

*Clones 14 and 58 were compared with each other, and clones 35 and 20 were compared with each other.

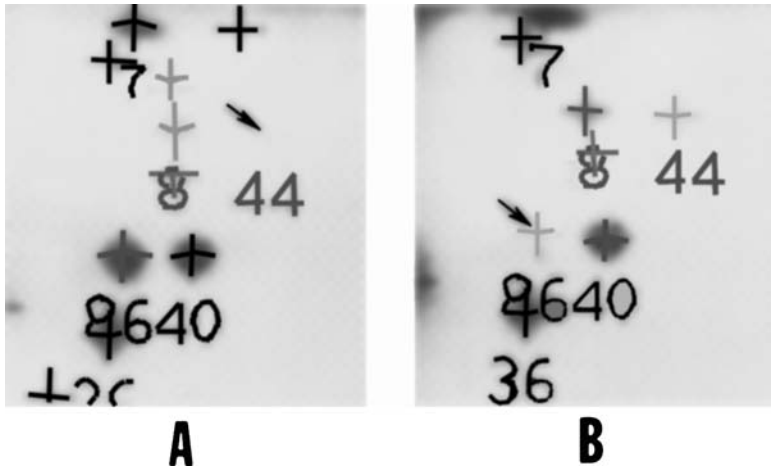

Fig. 4. Close-up sections of two gels each exhibiting a mutant. The spot designated 86 (**A**) is the mutant form of spot 243, which is designated as spot 40 in the computer-matching numbering system. Spot 44 (**B**) is a mutant of spot 182, which is designated as spot 8 in the computer-matching numbering system. In matching two gels, the occurrence of a spot in one gel that has no match in the other is signaled by the assignment of a number to the "orphan spot" and a green cross in the location where the spot is missing in the other gel. In this figure, arrows point to the location of green crosses.

spot differences between gels. Close inspection of the gels revealed that the differences were due to imperfections associated with the spots themselves and with the spot detection algorithm and not due to matching algorithm related errors. Because of the failure of the matching algorithm to detect mutant spot 80, two other strategies were attempted for the detection of this mutant spot. One was to increase the number of spots above the intensity threshold to 200. This approach also failed to detect the mutant spot. Another approach was to rely on contrast (peak height above background) as a measure of spot size instead of integrated intensity, while maintaining the number of spots to be matched at 200. This alternate strategy resulted in the detection of the mutant spot. Matching times increased to 4 minutes for 200 spots. The number of non-mutation-related spot differences increased to 16.

DISCUSSION

Quantitative data on mutation for humans have been hard to obtain, because of the rarity of mutations and the limitations of existing technology. At the somatic cell level, most studies have focused on a relatively few loci for which a convenient selective system for the detection of mutants can be utilized.[13] The data obtained, by its nature, cannot be extrapolated to the whole genome. This point is illustrated by the disparity in the frequency of spontaneous mutations observed in different studies. Therefore, there remain considerable uncertainties concerning the process of gene mutation in somatic cells. At the germinal cell level, the biochemical approach to the study of mutation has relied, for the most part, on one-dimensional electrophoresis (1-D E) to detect alterations in a relatively small number of protein gene products. At the level of exposure of most populations to mutagens, the small number of "good" 1-D E systems requires that for a meaningful study, a large number of individuals be examined.[14]

The 2-D PAGE approach provides two important advantages for both somatic as well as germinal mutation studies: 1) a large number of gene products can be monitored simultaneously; 2) a more random and therefore less biased sampling of the genome is possible. With these advantages in mind, we have undertaken the development of a 2-D PAGE approach for the study of mutations in humans.

The aim of the present study was to determine the extent to which structural mutations can be detected by 2-D PAGE and to evaluate the performance of a matching algorithm developed for the purpose of detecting mutations. The finding that of 296 spots initially selected only 29 could not be reliably scored, points to a high degree of reproducibility of the 2-D PAGE approach. Variations in intensity and position of the 29 polypeptides eliminated from the study could be due to their difficult solubilization, poor staining, post-translational modifications, or to fluctuations in their abundance resulting from metabolic regulation.

The observation of four mutations among 47,077 polypeptide spots scored yields an induced mutation frequency of 4.2×10^{-5} alleles, assuming that each spot is the product of two alleles at the same locus. The mutations observed are most likely induced by ethylnitrosourea since in studies of spontaneous mutations currently in progress using the same cell line, we have not observed any mutations in 330,000 locus tests. In single-locus studies, ENU exposure of cultured human lymphoblastoid cells resulted in a 100-fold increase of mutations to 6-thioguanine resistance and 500-fold increase of mutations to ouabain resis-

tance (Chu, unpublished data). It is noteworthy that in all four instances where mutation was suspected on an initial gel for a clone, repeat analysis of the clone from the same harvest as well as from a repeat culture of the same clone consistently yielded the same variant polypeptide. The fact that two of the four mutations have been found at the same locus is of interest. The significance of this finding with respect to the randomness of mutations can only be assessed as part of a larger study.

While our matching algorithm has been previously tested under conditions simulating the occurrence of mutations,[9] the actual observation of four mutant spots in this study prompted an evaluation of the performance of our computer program with gels exhibiting protein variations which represented real mutations. In the context of an automated approach to gel matching, while a flawless performance resulting in the detection of all spot differences and no errors in matching is the ultimate goal, some compromise should be acceptable as long as the error factor is the same in the control material. In the context of mutation, a working strategy is to minimize the frequency with which mutations fail to be detected, in view of their rarity, even though some false spot differences might be generated in the process. Errors of this kind are remedied by having a human operator make the final verification provided their number is manageable.

With the integrated intensity chosen as the parameter for ranking spots and 120 as the number of spots to match, one mutant was not detected by the matching algorithm. In that situation, of 363 spots initially detected in the gel exhibiting the spot 80 mutant, there were only 94 spots of smaller integrated intensity than the mutant spot. Therefore the detection of the mutant would have required matching of nearly every spot. Such a strategy would have resulted in the generation of numerous spot differences, primarily corresponding to faint spots that are at the limit of detectability.

The ranking of spots on the basis of contrast raised the rank of mutant spot 80 and allowed its detection by the matching algorithm as a cutoff of 200 spots to be matched. It is likely that ranking on the basis of contrast would result in a superior performance than ranking on the basis of integrated intensity. The explanation is that the former approach tends to select spots that are sharper and less streaky.

Even though the vast majority of spots were correctly matched at a cutoff of 200, a number of spots detected on one image failed to be matched on the other image because they were too faint; blurry; closely overlapped a neighbouring spot; or were elongated, "streaky' spots such that the spot detection algorithm identified them as two spots on one gel and as a single spot on the other. In these cases, the action of the matching algorithm was considered correct. The errors resulted from differences at the initial spot detection level. Nevertheless, these errors are tolerable, in contrast to the failure to detect a mutation.

It also follows from the above considerations that the frequency with which mutations or "real" differences between gels fail to be detected by the algorithm can be minimized at the risk of generating false-positive differences. The proportion of one type of error relative to the other is determined by the threshold. In other words, it is related to the fraction of the spots to be matched relative to the total number of spots detected. Further improvements in the performance of the matching algorithm is likely to result from implementing additional criteria for matching, based on quantitative considerations. For example a decrease in the size measure of a spot in a given gel would itself trigger the algorithm to search for the occurrence of a new spot in the neighbouring region of the spot. The results of this investigation provide a basis for a more extensive study of the mutagenic effect, at the protein level, of radiation and chemical carcinogens.

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