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Two-dimensional gel electrophoresis analysis of Syrian hamster embryo cells: Morphological transformation is not cell type specific

Studies were conducted to investigate the protein phenotype of normal and morphologically transformed Syrian hamster embryo (SHE) cells. Based upon two-dimensional gel protein phenotype analysis, we conclude that (i) SHE cells are a mixture of multiple cell types including mesenchymal and epithelial cells and (ii) several cell types present in the SHE cell population can be morphologically transformed by a variety of genotoxic and non-genotoxic carcinogens.

1 Introduction

Syrian hamster embryo (SHE) cells are commonly used for both the study of chemical carcinogenesis *in vitro* and as a screening tool for assessing the carcinogenic potential of chemicals [1–33]. The potential of chemicals to morphologically transform SHE cells has been shown to correlate approximately 90% with a chemical's carcinogenic activity *in vivo* [1–33]. Transformation in SHE cells is comprised of a defined set of stages following carcinogen exposure including: (i) normal, primary cells; (ii) morphologically transformed cells, which display altered cellular morphology and colony growth characteristics but still have a finite life span; (iii) immortalized cells which have infinite growth potential; and (iv) tumorigenic/tumor-derived cells where an immortal cell has acquired the tumorigenic phenotype characterized by growth in soft agar, tumor suppressor gene inactivation, marker gene expression and chromosome aberrations [4, 5, 9, 34–45]. Cells representative of each of these stages of transformation can be isolated and studied to provide a detailed picture of the cellular phenotype at that stage of transformation.

SHE cells are a complex mixture of cells since they are isolated from whole hamster embryos [9, 10]. This mixture of cell types offers both advantages and disadvantages for *in vitro* transformation studies. Several advantages are that SHE cells are both able to metabolize most carcinogens without the aid of liver microsomes and detect tissue-specific carcinogens [24, 46, 47]. A disadvantage is that multiple cell types are targets for transformation, thereby making the transformed cell somewhat difficult to study since it does not originate from a clonal population of cells. Recently, Cizdziel *et al.* [45] found chondrocytic-specific collagen expression in two cloned isolates of transformed SHE cells, and concluded that chondrocytes are unusually susceptible to transformation since they comprise only a small proportion of cells in the nontransformed SHE cell population. The experiments described in the current report were undertaken to examine the possibility of cell-type specific susceptibility to transformation in more detail. In particular, we have examined clones of SHE cells before and after

carcinogen-induced morphological transformation. We report that SHE cells are indeed a complex mixture of cell types including cells with both epithelial and mesenchymal cell markers. In addition, there is apparently no clonal selection for carcinogen-induced transformation of a particular cell type.

2 Materials and methods

2.1 Cell culture

SHE cells were isolated and cultured as described previously [9, 10]. For experimental manipulation, cells were cultured in LeBoeuf's modified Dulbecco's modified Eagle's medium (LMDMEM; Quality Biologicals) containing 10% fetal calf serum (10% FCS) (HyClone) in an incubator (Forma Scientific) at 10% carbon dioxide atmosphere and 37°C.

2.2 Morphological transformation

Primary SHE cells were morphologically transformed with three genotoxic carcinogens, namely benzo[a]pyrene (BP), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), and lead acetate (LA), and two nongenotoxic carcinogens – reserpine (RP) and phenobarbital (PB), using previously published procedures [9, 10]. All chemicals used in this analysis were the highest quality available from the Aldrich Chemical Company. Chemically treated, morphologically transformed colonies, chemically treated yet normal colonies, and untreated control SHE cell colonies were ring-cloned and placed in T25 tissue culture flasks and expanded until the cultures were confluent. These cells were then transferred to a T75 tissue culture flask, expanded until they were 70% confluent and harvested for two-dimensional (2-D) gel electrophoresis. Cells were harvested for 2-D gel electrophoresis by washing the cells three times with phosphate-buffered saline, scraping the cells off the flask surface into phosphate-buffered saline, collecting the cells by centrifugation, removing the phosphate-buffered saline, and lysing the cells in 20 µL of 2-D gel electrophoresis lysis buffer. 2-D lysis buffer is composed of 2% Nonidet P-40, 9 M urea, 0.5% dithiothreitol, and 2% pH 9–11 carrier ampholytes. Ten µL of the cell lysate were run per gel.

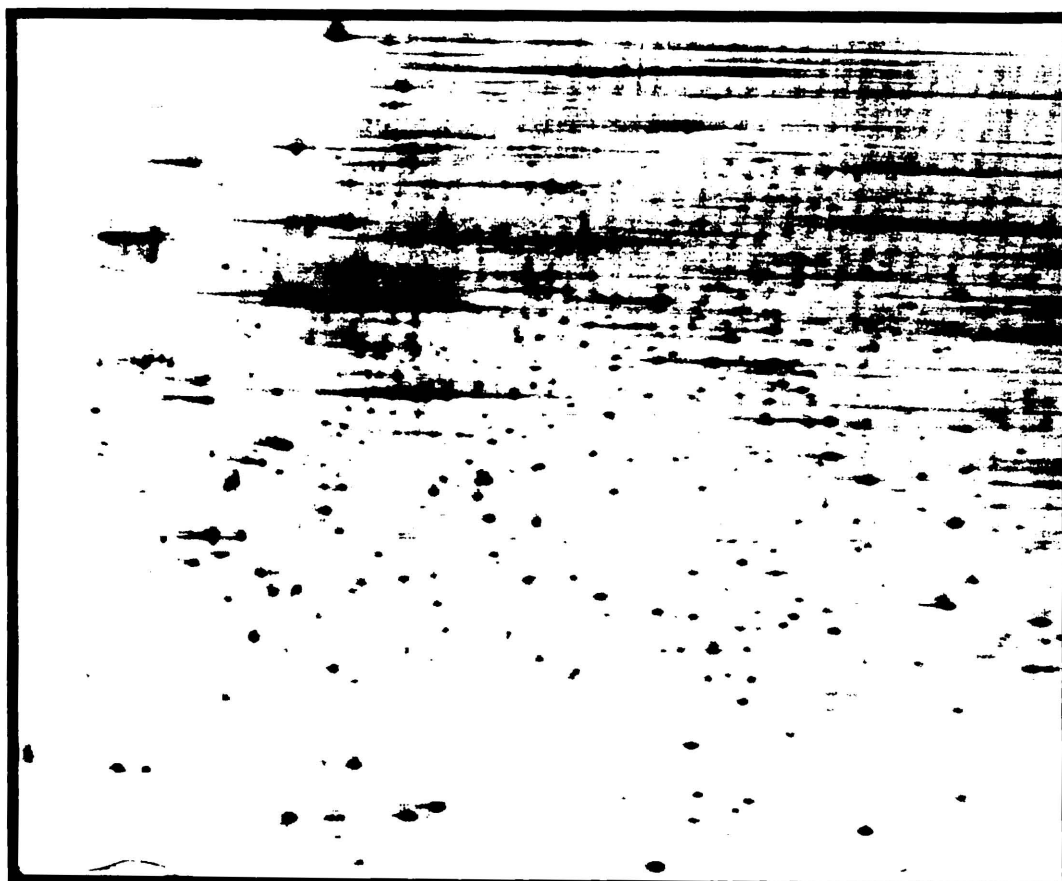
2.3 2-D gel electrophoresis and data analysis

Sample proteins were resolved by 2-D electrophoresis (LSB) using the 20 × 25 cm ISO-DALT 2-D gel system

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Abbreviations: BP, benzo[a]pyrene; 2-D, two-dimensional; LA, lead acetate; MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; PB, phenobarbital; RP, reserpine; SDS, sodium dodecyl sulfate; SHE, Syrian hamster embryo

A)



B)

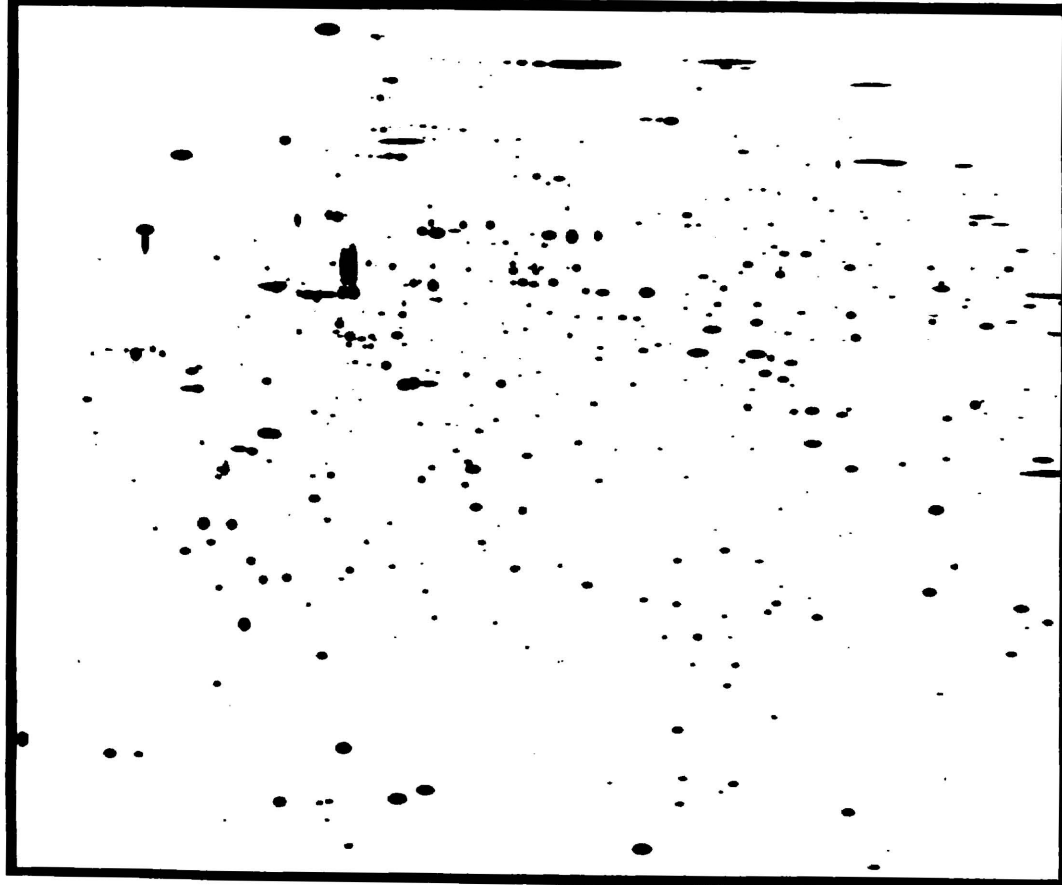


Figure 1. Prototypical 2-D gel pattern for SHE cells. (A) Picture of actual 2-D gel of sample LA9T. (B) Synthetic image of (A) showing 1296 identifiable spots.

operating with 20 gels per batch. First-dimensional isoelectric focusing gels were prepared using a single standardized batch of carrier ampholytes (BDH, 4-8A) and run for 33 000 to 34 500 Vh. An Angelique computer-controlled gradient casting system was used to prepare second-dimensional sodium dodecyl sulfate (SDS) gradient slab gels in which the top 5% of the gels was 11%T acrylamide and the lower 95% of the gel varied linearly from 11% to 18%T. Each gel was identified by a filter paper label polymerized into the gel. First-dimensional tube gels were loaded directly onto the slab gels without equilibration and were held in place by polyester fabric wedges to avoid the use of hot agarose. All samples were analyzed twice.

Following SDS-electrophoresis, the slab gels were stained for protein using a colloidal Coomassie Brilliant Blue G-250 procedure in covered plastic boxes with 10 gels per box. This procedure involved fixation in 1.5 liters of 50% ethanol/2% phosphoric acid/48% water (v/v/v) for 2 h, followed by three 30 min washes in 2 L of cold tap water. Following the washes, the gels were transferred to a 1.5 L solution of 34% methanol/17% ammonium sulfate/2% phosphoric acid/47% water (v/v/v) for 1 h. Finally, 1 g of powdered Coomassie Blue G-250 was added to 1.5 L of 34% methanol/17% ammonium sulfate/2% phosphoric acid/47% water (v/v/v). Gels were stained for 4 days to achieve equilibrium intensity.

The stained protein pattern on each slab gel was digitized in red light at 120 micron resolution with an Eikonix 78/99 charge coupled device (CCD) scanner. Digitized gel images were processed with the Kepler software system by proce-

ducing PROC008, which includes background and streak subtraction, erosion/dilation spot cutout, and 94 cycles of 2-D Gaussian fitting. Groups of gels corresponding to the experimental groups of clones were assembled, and all were matched to a standard pattern for SHE cells. This pattern, copied initially from that of a representative clone, was supplemented with spots observed only in other clones to develop a comprehensive master pattern. Individual gels were scaled together using a linear fit to the abundances of matched spots, in order to compensate for differing total protein loads. Group-wise statistical comparisons were made using Student's *t*-test and the results were displayed in montage format using the KPL42 module. Gels from six different batches (representing two runs of every sample) were merged to form the master pattern.

The search for coregulated spots made use of the Pearson product-moment correlation coefficient calculated between a specific spot and all the other spots in the experiment. Graphical results were prepared in Postscript and printed on an Apple Laserwriter, or reproduced from the workstation screen using a Seikosha videoprinter. Digitized gel images and reduced data are archived permanently on 1.2 Gbyte DAT tape cartridges.

3 Results and discussion

3.1 Experimental design

Experiments in this study were designed as follows. SHE cells were treated with either nothing or with one of five car-

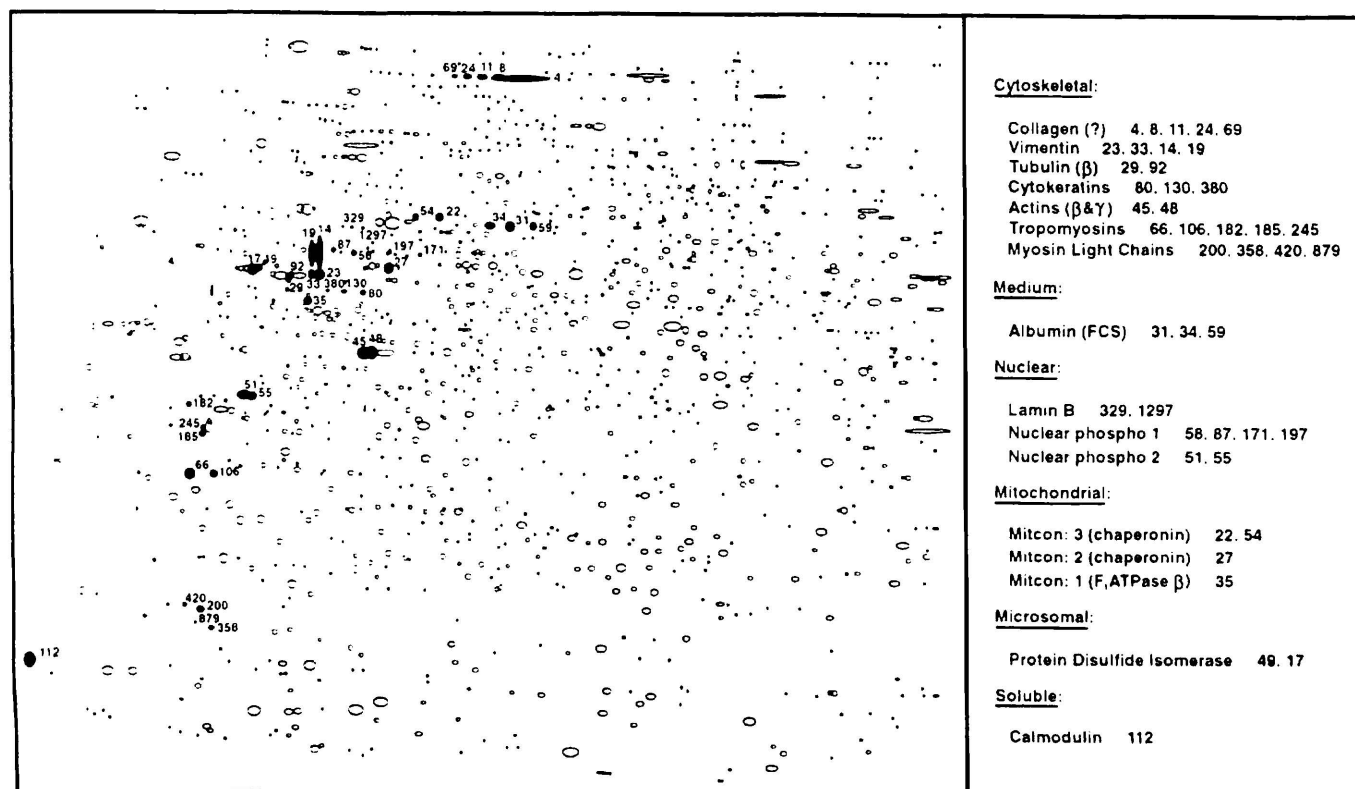


Figure 2. 2-D gel protein map of SHE cells. Included in the map is the identification of specific protein locations based upon similar migration patterns of homologous proteins in rat, mouse and human cells. Migration is determined by isoelectric point and molecular weight.

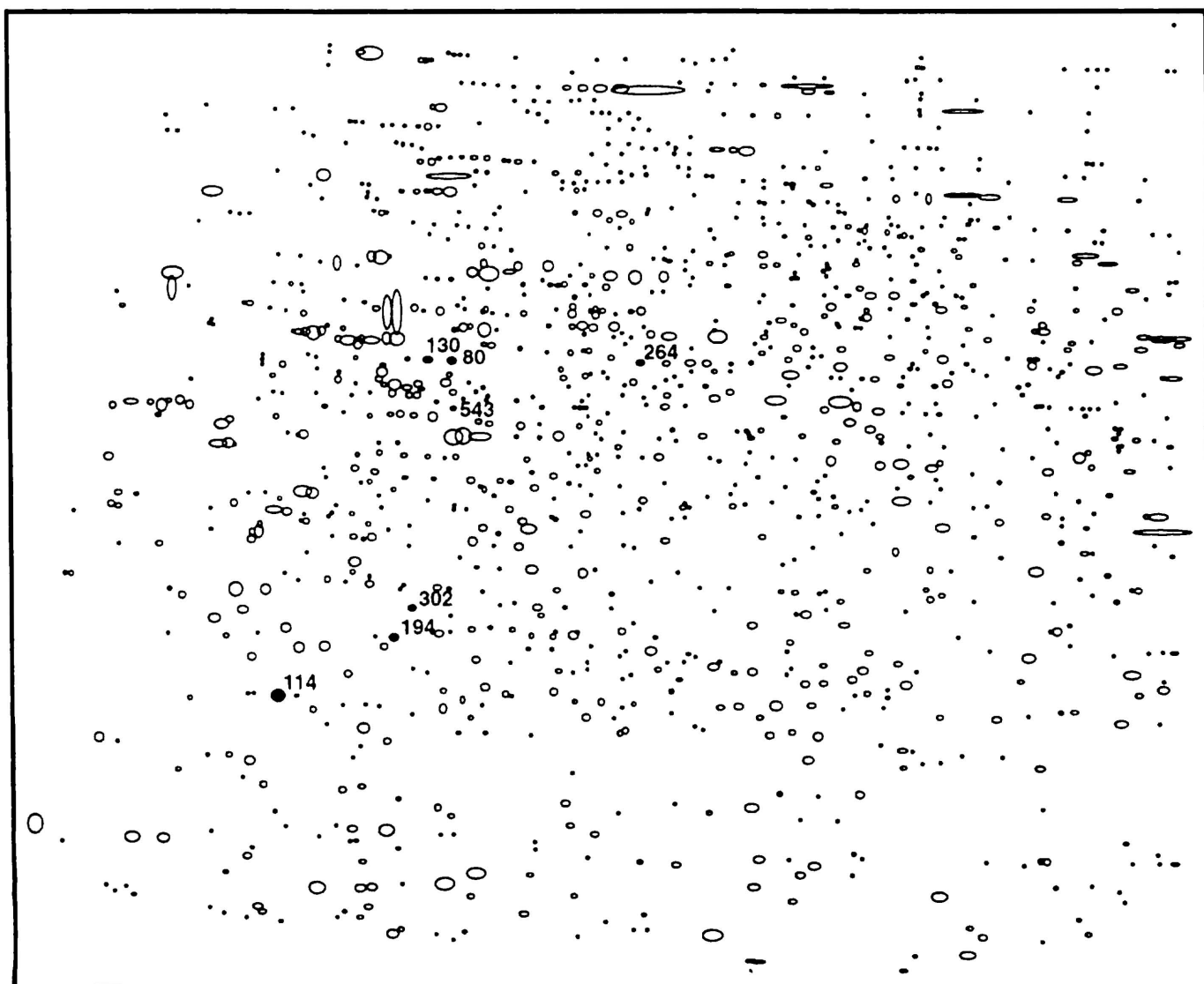
cinogens BP, MNNG, LA, PB and reserpine RP. Ten untreated, individually cloned SHE cell colonies (NTC 1–10) served as individual controls while each of the five carcinogen-treated groups consisted of three carcinogen-treated yet non-morphologically transformed colonies and seven morphologically transformed colonies. The nomenclature for each clone consists of three parts: treatment group, clone number and "N", for treated but nontransformed, or "T", for morphologically transformed clone. For example, BP1N refers to BP treated, clone 1 that is nontransformed while BP4T refers to BP-treated, clone 4 that is morphologically transformed. The cells from individual clones were prepared for 2-D gel electrophoresis and the proteins from those cells were separated by 2-D gel electrophoresis and quantitated as described in Section 2.3. An example of such a separation for sample LA9T is shown in Fig. 1A. In this figure, the proteins were well separated with approximately 1296 spots available for quantitation and analysis. Figure

1B illustrates the digitized image of the gel shown in Fig. 1A. The digitized image was a good representation of the original stained gel image, and was therefore used for quantitative analysis.

3.2 2-D SHE cell landmark map

Figure 2A and 2B represent a tentative map of SHE cell proteins. Individual protein identification is based on the protein's relative migration compared to the same protein in human, mouse and rat cells. The agreement in migration, *pI*, and molecular weight of these proteins with their counterparts in the human, mouse and rat systems was excellent (N. L. Anderson, unpublished observation). This map represents the first protein profile for SHE cells. Experiments are presently underway to confirm the tentative protein spot identifications and to expand the map to include more reference proteins.

A



3.3 Analysis of morphologically transformed clones, chemically treated nontransformed clones and normal clones

Experiments were undertaken to compare the 2-D protein profile of normal, chemically treated nontransformed and chemically treated morphologically-transformed SHE cell clones. This analysis did not identify either novel expression of proteins or changes in the level of proteins that could be correlated with transformation. Transformation-associated protein changes were not identified, apparently due to cell-type specific protein expression, which makes statistical analysis of the 2-D protein spots of such a large number of clones and proteins difficult. Clone-specific expression of proteins arises from the fact that SHE cells are composed of multiple cell types since entire embryos are cultured [9, 10]. This view is further substantiated by the finding of both cytokeratin and vimentin, proteins which

are expressed in epithelial and mesenchymal cells, respectively, in the total SHE cell protein extract (Fig. 2A and 2B). Transformation experiments using clonal SHE cell populations are currently in progress to address the question of transformation-specific protein expression changes.

The observed cell-type specific protein differences did allow us to address the question of whether a specific cell type, based on its protein phenotype, was selectively transformed by a particular carcinogen. This analysis was performed on groups of proteins whose expression is tightly coordinated. The reasoning behind this approach is that coordinately expressed proteins usually represent proteins that have a similar developmental control of expression [48-51]. Coordinated regulation of protein expression is often used to characterize a particular cell type [48-51]. In fact, protein spots 80 and 130 are tentatively identified as cytokeratins, a well-known marker for epithelial cells [48]. As

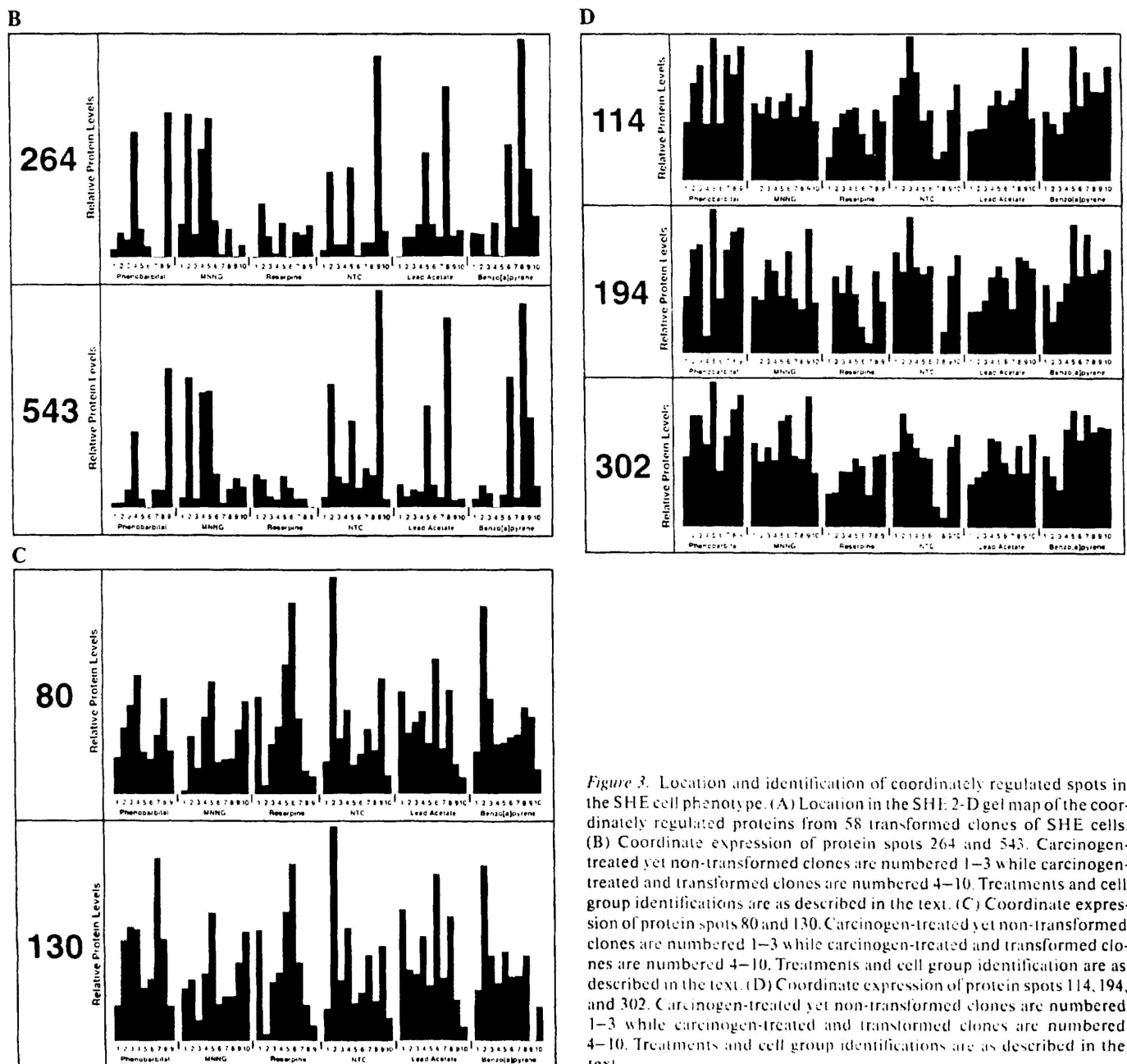


Figure 3. Location and identification of coordinately regulated spots in the SHE cell phenotype. (A) Location in the SHE 2-D gel map of the coordinately regulated proteins from 58 transformed clones of SHE cells. (B) Coordinate expression of protein spots 264 and 543. Carcinogen-treated yet non-transformed clones are numbered 1-3 while carcinogen-treated and transformed clones are numbered 4-10. Treatments and cell group identifications are as described in the text. (C) Coordinate expression of protein spots 80 and 130. Carcinogen-treated yet non-transformed clones are numbered 1-3 while carcinogen-treated and transformed clones are numbered 4-10. Treatments and cell group identification are as described in the text. (D) Coordinate expression of protein spots 114, 194, and 302. Carcinogen-treated yet non-transformed clones are numbered 1-3 while carcinogen-treated and transformed clones are numbered 4-10. Treatments and cell group identification are as described in the text.

Fig. 3 A–D illustrate, a series of tightly coordinated proteins, whose level of expression varied from cell clone to cell clone, was observed. The relative expression of these proteins was independent of carcinogen treatment, with high-level and low-level cell clone expressors found in all treatment groups. Except for the 80/130 protein spot pair, the other coordinately regulated protein spots were not simple isoforms of the same protein since individual proteins of a coordinated set differed greatly in both molecular weight and isoelectric point. These results suggest that multiple unique clones are present in the SHE cell population and no unique clone is selected during transformation. Coordinated expression of these proteins is contrasted with the non-coordinated expression of four other proteins in the same cell type (Fig. 4).

These results are in contrast to recent results from Cizdziel *et al.* [45] who conclude that chondrocytes are selectively sensitive to transformation by carcinogens. In our comparison of 58 different SHE cell clones, transformed with five different transforming agents (including both genotoxic and non-genotoxic carcinogens), we can find no difference in the clonal cell type between untreated control clones, carcinogen-treated non-transformed clones and carcinogen-treated transformed clones. Our results indicate that many cell types present in the complex SHE cell mixture can be morphologically transformed by carcinogens. This view is further substantiated by the finding that there are both high- and low-level expressors of cytokeratins, an epithelial cell-specific intermediate filament. Therefore, epithelial cells comprise one class of cells which are morphologically transformed while low cytokeratin expressors

comprise a second class. This result supports the idea that transformation of SHE cells is a valuable carcinogen screening assay since a cell-type specific carcinogen will have a potential target in the SHE cell population. The availability of cell types that represent a variety of tissues for transformation could explain why SHE cells respond in a predictive way to a wide variety of carcinogens.

4 Concluding remarks

Two-dimensional gel electrophoresis of SHE cells was used to identify distinct types of cells in this complex cellular mixture based on differential protein expression. This study demonstrates that multiple cell types can be transformed by a variety of carcinogens. Future work will concentrate on both the identification of specific cell types in the SHE cell population and transformation-specific protein changes in the specific cell types.

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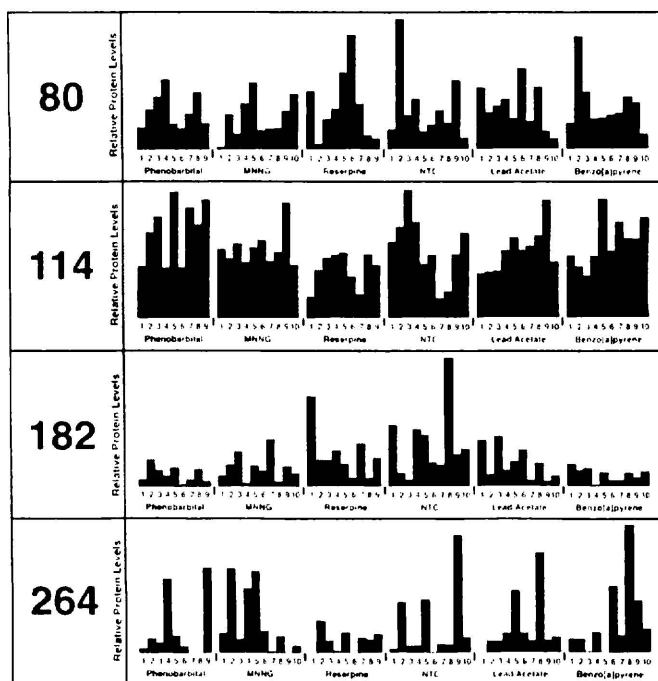


Figure 4. Expression of four non-coordinately expressed proteins from the SHE cell 2-D gel protein phenotype. Protein spots 80, 114, 182 and 264 refer to unique proteins randomly picked from the SHE phenotypic pattern. Carcinogen-treated yet non-transformed clones are numbered 1–3 while carcinogen-treated and transformed clones are numbered 4–10. Treatments and cell group identifications are as described in the text. Figure description is as in Fig. 3.

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