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Investigation of Ornithine Decarboxylase Activity and Two-Dimensional Electrophoretic Protein Profile Following Exposure of T24 Bladder Carcinoma Cells to Tumor Promoter and Carcinogen

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Summary: To develop appropriate screening tools for biomarkers of effects of exposure to occupational chemical insult. Changes were investigated in T24 human bladder carcinoma cell ornithine decarboxylase activity and protein profiles by quantitative two-dimensional polyacrylamide gel electrophoresis (2D PAGE), biochemical events potentially altered by an established human bladder carcino-

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gen and tumor promoter. A unique chromatographic approach was used to demonstrate that in vitro exposure of T24 cells for 6 h to varying concentrations of the carcinogen 4-aminobiphenyl elevates enzyme activity 5.3- to 5.9-fold. As a second method to identify potential biomarkers of exposure, two-dimensional gel electrophoresis was used to compare the protein pattern of vehicle control-treated T24 to 4-aminobiphenyl or tumor promoter (12-*o*-tetradecanoylphorbol-13-acetate)-treated cells. Changes in abundance and modification of proteins are determined using the Kepler software package to analyze and compare gels across treatment groups. With this technology, protein markers are identified by significant alterations in spot density (mean ratio of Coomassie Blue intensity; $p < .001$, Student's *t* test) following T24 treatment with the carcinogen or the tumor promoter. Fifteen protein spots from a detectable pool of 542 demonstrate two-fold or greater changes in intensity. The results illustrate the potential of automated two-dimensional gel analysis for classifying different gel patterns, an approach that can be applied to patterns whose differences are obscured by the minor changes in spot intensity that arise between separate cell cultures. In addition to the ornithine decarboxylase assay, 2D PAGE offers much promise to evaluate potential biomarkers for occupational and environmental carcinogens. These results will be used to further develop NIOSH efforts in the molecular epidemiology of occupational bladder carcinogenesis. **Key Words:** biomarker, bladder carcinogenesis, occupational exposure, ODC, 2D PAGE.

Tumors originating from the urothelial tract constitute a significant cancer problem in the United States, with an incidence of over 50,000 new cases annually [1]. Despite advances in chemotherapy, survival rates for bladder cancer have not improved. Bladder cancer, the sixth most common cancer in the United States, accounted for roughly 10,000 deaths in 1990 [2]. Although the etiology of bladder cancer is not certain, a number of modifiable host and environmental/occupational risk factors have been identified [3]. The observed higher incidence in males when compared to females (3:1) has been attributed to increased exposure to selected chemical carcinogens in the workplace [1]. However, few if any methods exist to determine whether a worker has been exposed to an occupational carcinogen at a level sufficient to compromise the integrity of the urothelial tract. Since many bladder cancers are indolent [4], there is a need to develop appropriate biomarkers that reflect early effects initiated by occupational carcinogens. Recognition that clinical cancer is the endpoint of the underlying disease of carcinogenesis [5] suggests that appropriate intervention with individuals determined to be at risk could prevent the development of life-threatening disease.

Identifying specific biochemical markers for the changes resulting from the process of carcinogenesis offers an attractive approach to identifying individuals at risk for bladder cancer. This investigation focuses on the development of biomarkers for effects of exposure to a human bladder carcinogen and tumor promoter. Perera refers to such biomarkers as a preclinical response that is a biological or biochemical change in target cells associated with the occurrence of cancer [6]. In this study we first examine the effect of a human bladder occupational carcinogen on an intermediate endpoint, i.e., a marker of cellular events associated with one stage in the evolution and progression of carcinogenesis [7]. We utilize T24, the transitional human bladder epithelial cell line on which a breadth of characterization data exists, including a

database for ornithine decarboxylase (ODC) [8]. Ornithine decarboxylase, the rate limiting enzyme in polyamine synthesis, catalyzes the conversion of ornithine to putrescine and plays an important role in normal and neoplastic cell proliferation [9]. ODC induction, with subsequent increases in polyamines, has been correlated to tumor cell multiplication [10]. The enzyme has been associated with the tumor promoting activities of a variety of agents [9,11]. Hsieh and Verma characterized T24 cells for ODC induction by 12-*o*-tetradecanoylphorbol-13-acetate (TPA), a well-documented tumor promoter and powerful stimulator of ODC [8]. While significantly high levels of ODC activity have been reported for rodent carcinogenesis models [12–14], inhibition of enzyme activity exerts beneficial effects in animal models of proliferative disease [15]. Interestingly, this ODC inhibitory effect includes chemically induced rat urinary bladder carcinogenesis [16].

We examine here the effect of a documented human bladder carcinogen, 4-aminobiphenyl (ABP), on T24 ODC activity to provide a clue as to its effect on this intermediate endpoint, a potentially contributing factor to carcinogenesis. ABP, the most potent known experimental bladder carcinogen, is a ubiquitous environmental and occupational chemical [17]. As early as 1954 [18] and confirmed more recently [19], it has been reported that individuals exposed to this arylamine showed an increased incidence of bladder cancer that was related to the intensity and duration of the exposure. ABP is present in synthetic fuels [20,21] and has been identified as one of many chemicals in cigarette smoke [22]. By validating the effect of ABP on ODC activity in vitro, the potential human bladder carcinogenicity of other environmental and occupational agents may be similarly assessed.

Changes in ODC activity following occupational chemical exposure, however, may not be highly discriminatory for bladder cancer risk assessment. ODC is highly responsive to changes in drug exposure and the hormonal and nutritional status of an individual [23]. Due in part to a short half-life (10–45 min), a new level of enzyme may be reached rapidly after the application of an appropriate stimulus [24]. Consequently, a combination of markers is necessary to determine a profile capable of accurately predicting the risk of bladder cancer to the chemically exposed individual.

Therefore, as a second technique for identifying biomarkers of exposure to heretofore unknown occupational bladder carcinogens, we use two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Because quantitative differences in cellular gene expression and protein levels probably are responsible for most of the differences between malignant and normal phenotypes [25], 2D PAGE, the most sensitive means available for detecting differences in protein composition, represents an excellent tool to study biomarkers for effects of exposure. It has become a widely used method for separation and analysis of complex protein mixtures, including the qualitative and quantitative analysis of protein expression during cellular proliferation and cancer development. To identify additional markers, we use this approach to detect subtle protein changes that may occur in T24 cells following treatment with bladder carcinogen or tumor promoter. Toward this end, we analyze two-dimensional gels using Kepler, a software system capable of automatically finding specific spots, matching the protein patterns of multiple gels, and comparing these for significant, quantitative differences.

MATERIALS AND METHODS

Cell Line and Treatment

The transitional cell carcinoma cell line T24 [26], derived from a high-grade bladder cancer, was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells are cultured in the presence of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% fetal calf serum and 50 νg/mL gentamycin sulfate (GIBCO BRL Life Technologies, Gaithersburg, MD, USA). T24 cells at approximately 90% confluency are deprived of growth factors by changing to fetal calf serum-deficient DME media. After 48 h, cells are changed into fresh media containing either vehicle control, dimethyl sulfoxide (DMSO, Sigma Chemical, St. Louis, MO, USA), 12-*o*-tetradecanoylphorbol-13-acetate (TPA; Sigma) (0.1–1.0 νM], or 4-aminobiphenyl (ABP; Sigma) (0.01–10 νM). After incubation at 37°C for 6 h the cells are harvested by trypsinization. Cells are washed with phosphate-buffered saline (PBS) and resuspended in cold 50 mM sodium phosphate buffer, pH 7.2. Cells are disrupted by freeze/thawing three times at −196°C (liquid nitrogen) and 37°C. A postmitochondrial supernatant fraction is obtained by centrifugation at 14,000*g* for 20 min.

Ornithine Decarboxylase Assay

Cation exchange thin-layer chromatography is used to separate ornithine from putrescine by ammonium hydroxide elution. Polyamines remain at the origin, while ornithine moves to the solvent front. The enzyme reaction mixture contains 30 νL of 50 mM sodium phosphate buffer, pH 7.2, 0.4 mM pyridoxal phosphate (Sigma), 0.1 mM EDTA (Sigma), 4.0 mM dithiothreitol (Sigma), 0.5 mM L-ornithine (Sigma), 1 νCi (1 νL) L-[2,3-^{3}H]ornithine (Amersham, Arlington Heights, IL, USA), and T24 postmitochondrial supernatant (20–80 νg). After incubating at 37°C for 60 min, 3 νL of mixture is withdrawn and spotted onto Whatman Carboxymethyl Cellulose 300 thin-layer plate (Fisher Scientific, Pittsburgh, PA, USA) predeveloped with distilled water to remove chemical impurities. To serve as markers for enzyme substrate and product, ornithine (40 nmol) and putrescine (40 nmol; Sigma), respectively, are also chromatographed. The plate is developed with 40 mM ammonium hydroxide for 60 min. A zero time reaction point serves as the blank. The markers are stained by spraying with 0.2% ninhydrin (Sigma) in isopropanol followed by warm air drying. The spots corresponding to putrescine and ornithine and the chromatography strip between these are cut out and placed in counting vials containing 5.0 mL Permafluor V liquid scintillation cocktail (Aldrich Chemical, Milwaukee, WI, USA). Radioactivity is determined by scintillation counting on a Beckman LS8100 (Beckman Instruments, Fullerton, CA, USA). Percentage of product formed is calculated by dividing the radioactivity of the putrescine spot by that of the samples. ODC specific activity is expressed as nanomoles putrescine formed per milligram protein per hour.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Sample Preparation

T24 postmitochondrial supernatant proteins are concentrated by high-speed centrifugation (4 h at 400,000*g*) in Beckman TL-100 (Beckman Instruments). The resulting

pellets are solubilized in 2% NP-40 (Sigma), 9 M urea (BioRad, Hercules, CA, USA), 0.5% dithiothreitol (DTT; Sigma), and 2% ampholytes (pH 9–11; Pharmacia, LKB, Piscataway, NJ, USA) to a final protein concentration of 20 *v*g/*v*L. An initial loading series revealed that sample proteins were undegraded, and that 20 *v*L is the appropriate loading volume. Samples (T24 cells treated with DMSO, TPA [0.1–1.0 *v*M], or ABP [10 *v*M]) are analyzed in quadruplicate within one batch of 2-D gels.

Two-Dimensional Electrophoresis

Using the ISO-DALT system (Hoefer Scientific, San Francisco, CA, USA) [27], 20 *v*L of solubilized protein sample is placed on each of 20 first-dimension gels (25 cm × 1.5 mm) containing 4% acrylamide (Crescent Chemical, Hauppauge, NY, USA), 2% NP-40 (Sigma) and 2% ampholyte (pH 4–8, Pharmacia) and electrophoresed for 33,000 to 34,500 volt-hours at room temperature using a progressively increasing voltage protocol. Each tube gel is then placed on a second-dimension DALT slab gel (20 cm × 25 cm) containing a linear acrylamide gradient. Gradient slab gels are poured reproducibly using the Angelique computer-controlled gradient maker (Large Scale Biology, Rockville, MD, USA). This system enables one to reduce run-to-run variability in the polyacrylamide gel concentration, an essential characteristic for protein pattern image analysis. The top 5% of the gel is 11% acrylamide, and the lower 95% of the gel varies from 11 to 18%. First-dimension IEF tube gels are loaded directly onto the slab gels without equilibration and held in place with polyester fabric wedges (Large Scale Biology). Second-dimension slab gels are run for 18 h at 150 V in a DALT tank held at 10°C (Hoeffer Scientific). Molecular weight standards (Bio Rad) are comigrated on the gel margin, while carbamylated creatine kinase (Sigma) charge-train is used for internal charge standards. Following SDS electrophoresis slab gels are stained using a colloidal Coomassie blue G-250 procedure. This involves fixation in 1.5 L of 50% ethanol/2% phosphoric acid for 2 h, transfer to 1.5 L of 34% methanol/ 17% ammonium sulfate/2% phosphoric acid for 1 h and addition of 1 g of Coomassie blue G-250 stain (Serva, Paramus, NJ, USA) for 4 days to reach protein equilibrium intensity.

Software Analysis of Protein Pattern

Scanning and Image Analysis

Each stained gel is digitized in red light at 120*v*m resolution using a Molecular Dynamics (Sunnyvale, CA, USA) laser scanner or an Eikonix 78/99 CCD scanner. Raw digitized gel images are archived on high-density DAT tape. Each 2D gel is processed using the Kepler software system (Large Scale Biology) with procedure PROC008 to yield a spotlist giving position, shape, size, and density information. This procedure makes use of digital filtering, mathematical morphology techniques, and digital masking to remove background. It uses full two-dimensional, least-squares optimization to refine the parameters of a 2D Gaussian shape for each spot. The computed resolution and level of Gaussian convergence of each gel are inspected and archived for quality-control purposes.

Assembly of Data From Multiple Gels

An experimental package is constructed using the Kepler experiment definition database to assemble groups of 2D patterns corresponding to each treatment. Each pattern is matched to a ''master'' pattern, a computer composite of protein patterns from numerous treated T24 gels. It serves as a baseline to which all patterns from different treatment groups are compared to each other based on spot numbers assigned from the master pattern. During initial analysis a subgroup of specific protein spots are tagged on each gel. Subsequently, the software uses an automatic program to match additional spots to the master pattern using these manual landmark data as a basis. After the automatic matching, the results are inspected for spots considered important to the experiment. Spots are judged to be quantitatively different if they meet two criteria: (1) their mean intensities differ by at least a factor of two; and (2) their intensity distributions indicate there is a $<0.1\%$ chance that the difference is due to a random process (as determined using group-wise statistical parameters, e.g., the Student's t test). These two criteria ensure a low number of false positives. Proteins satisfying these criteria are represented as highlighted spots on computer-plotted protein maps and stored as spot populations in a protein database.

Normalization

To eliminate quantitative differences due to gel loading or staining, groups of gels are normalized or scaled together by a linear procedure based on specific spots. Spots selected are those that are detected on almost all gels and with low initial intragroup coefficient of variation (CV), a good (nonelongated) shape, and an integrated density between certain limits. A coordinated decrease or increase in the magnitude of several hundred proteins compared to the master is most likely caused by a difference in total protein applied to the gel. The computer multiplies the spot abundances for each protein pattern by a gel-specific scaling factor to account for loading differences. This allows for valid comparisons of protein expression among groups.

RESULTS AND DISCUSSION

Ornithine Decarboxylase

Studies in tumor promotion models have shown a strong relationship between induction of ODC activity and the tumor-promoting ability of carcinogens [12,28]. Here we measure the effect of a known occupational carcinogen on human bladder transitional cell ODC activity. We use a new method employing cation exchange thin-layer chromatography to determine enzyme activity. To validate the assay the production of putrescine in T24 cells is studied as a function of enzyme reaction time and protein content. Putrescine formation varies linearly as a function of time (0 to 80 min) and amount of cell protein (0 to 100 νg) (data not shown). This experimental approach is substantiated by comparison with a previous result that employed the more conventional CO_2 trapping method. Hsieh and Verma reported that addition of 50 nM TPA to T24 cells in culture results in a dramatic increase at 6 h in soluble ODC activity [8]. We conduct a similar study employing TPA in which we confirm a 6-h peak of maximum ODC using the chromatographic assay (data not shown).

We investigate ODC activity following 6 h in vitro exposure of the bladder carcinoma cells to varying ABP concentrations (0.01 to 10 νM) using TPA as a positive control. Following treatment with 0.1 νM TPA, ODC activity in T24 cells increases 27-fold relative to DMSO (Table 1). Increasing ABP results in an increase in enzyme activity of 5.3- to 5.9-fold (Table 1). This elevation following ABP treatment reflects a presumed T24 cellular hyperproliferation, and may therefore serve as one biomarker for the presence of neoplasia in the human bladder after chemical exposure.

The ODC assay, however, when used alone may not be highly discriminatory for carcinoma risk assessment. Although most studies find ODC and polyamine content activity to be generally elevated in colorectal neoplastic tissue, some variability and overlap between tissues and patients exists [29–31]. Several hormones, drugs, and nutritional status have been demonstrated to affect ODC activity [23]. ODC is therefore highly responsive to changes in the environmental milieu. This is attributed in part to an extremely short half-life (10–45 min), which enables a new level of protein to be reached rapidly after the application of an appropriate stimulus [24]. Consequently, a combination of biomarkers is necessary to determine a risk profile capable of accurately predicting bladder cancer in an individual following occupational chemical exposure.

Two-Dimensional Protein Profile

We use two-dimensional polyacrylamide gel electrophoresis to detect subtle protein changes, potentially additional markers, that may occur in T24 cells following treatment with a bladder carcinogen or tumor promoter. The strength of the 2D electrophoretic technique lies in its ability to resolve literally thousands of cellular proteins based first on their content of acidic and basic amino acids (isoelectric focusing) and second by molecular weight (SDS electrophoresis). In combination, these two separation techniques produce a two-dimensional protein pattern uniquely characteristic for a given cell line. Individual proteins within the pattern can be analyzed for alterations in abundance, charge, and molecular weight.

TABLE 1. *Effect of TPA and ABP on ornithine decarboxylase specific activity in T24 cells*

Treatment	Concentration (νM)	ODC specific activity (nmol/mg h^{-1})[a]	Relative increase[b]
DMSO[c]	—	0.31	1
TPA	0.10	8.24	26.8
ABP	0.01	1.70	5.5
ABP	1.0	1.84	5.9
ABP	10	1.61	5.3

[a] Cation exchange thin-layer chromatography is used to separate ornithine from putrescine. The enzyme reaction mixture contains radiolabeled ornithine and treated T24 postmitochondrial supernatant. Percentage of product formed is calculated as dpm putrescine/dpm sample. Specific activity is expressed as nmol putrescine formed/mg protein h^{-1}.

[b] Enzyme activity after chemical treatment relative to activity after DMSO treatment.

[c] Vehicle control.

We study the two-dimensional gels using the Kepler computerized system to automatically match multiple gel patterns. Computer analysis of two-dimensional gels allows one to search for changes that are both consistent and statistically significant. However, analysis of the pattern of cultured cells is complicated by intraclonal variability in the synthesis of different proteins [32]. That is, the intensity of a large fraction of the spots in parallel cultures of the same cells may be measurably different. These differences are likely a result of accumulation of minor, but uncontrollable variations between different cultures. To control for this we pool the results of four gels per each treatment group and use computer analysis to search for spots that are different to the 99.9% significance level and where the magnitude of the change (twofold or greater) makes them unlikely to be statistical artifacts.

We compare the two-dimensional gel patterns of T24 cells treated with either TPA (0.1 or 1.0 νM) or ABP (10 νM) to vehicle-treated cells. A master T24 cell profile illustrates a universally representative pattern for T24 proteins evident under all treatment conditions (Figure 1). It provides spot numbers for the proteins (not shown) and serves as a template to match single gel patterns. Depending on the treatment 164 to 382 protein spots match the master cell profile (Table 2).

Small quantitative changes in a wide range of proteins may be detected in treated T24 cells after determining whether good gel to gel reproducibility occurs within a treatment group. Approximately 140 to 320 protein spots per treatment group are measured with a relatively low within group coefficient of variation ($<$15%) (Table 2). Evidence of reproducibility within groups is also noted by bar graphs showing relative values for specific protein spots present on all gels (Figures 3 and 5).

After individual gels are matched to the master, quantitative data for each matched spot are examined. A change in spot density may reflect alterations in protein abundance and suggests altered regulation of the genome or changes in protein turnover rates. A shift in p*I* reflects charge modifications (or changes in normally expressed microheterogeneities) and suggests post-translational protein alterations have occurred.

Using this approach we identify specific protein spots that may be useful starting points as additional biomarkers associated with human bladder cancer. Following TPA treatment (0.1 or 1.0 νM), 5 spot densities change at least twofold. Figure 2 demonstrates the position of these proteins within the master protein profile. Bar graphs of these proteins provide confirmation of the relative differences following treatment (Figure 3). Three of the proteins (502, 505, and 537) show a decrease in abundance after TPA treatment, and two proteins (52 and 276) show an increase in spot density or abundance ($p < .001$) (Figure 3, Table 3). A change in spot density reflects alterations in protein abundance and suggests that changes in protein turnover rates or altered regulation of the genome has occurred. Shifts in p*I* were not noted.

After T-24 cells are treated with ABP (10 νM), 10 spot densities display a mean change of at least twofold. Figure 4 demonstrates the relative position of these proteins within the master profile. Three spots increase (302, 456, and 504), and seven proteins decrease (19, 41, 55, 61, 198, 244, and 422) after treatment ($p < .001$) (Figure 5, Table 4). Shifts in p*I* were not observed. Since the exposure period is only 6 h long, the seven proteins that decrease appear to be rapidly turning over, and may represent regulatory molecules with a critical role in ABP-induced carcinogenicity.

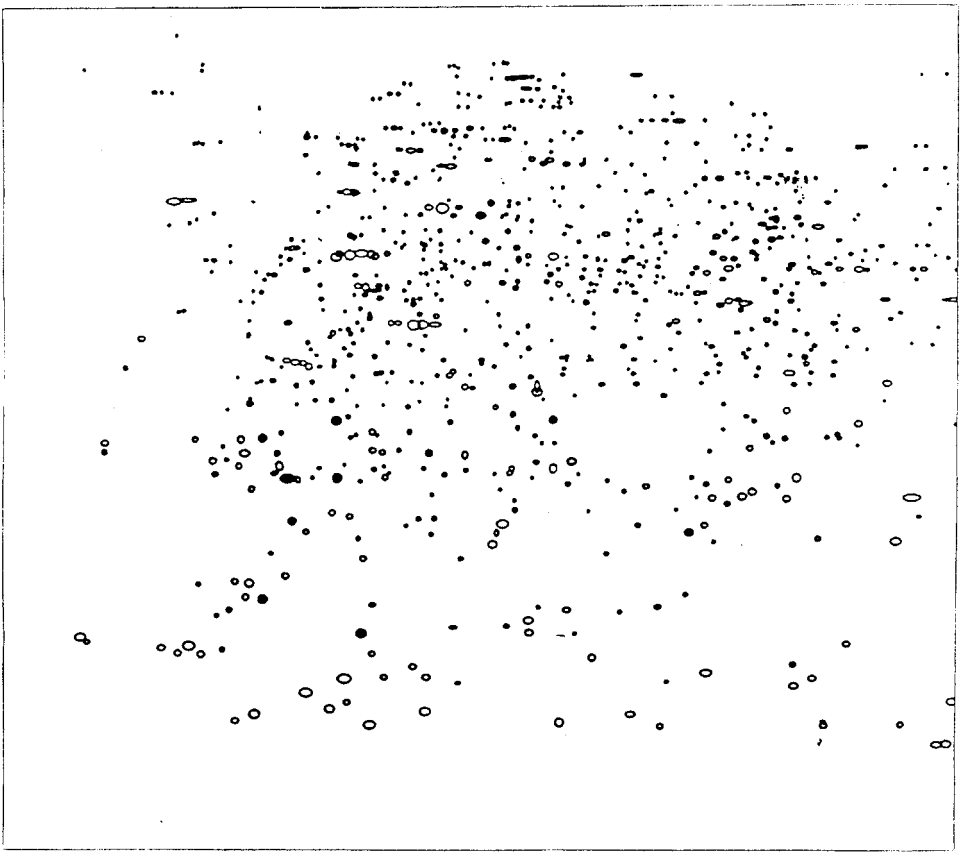

FIG. 1. Master T24 cell protein 2D PAGE profile. Acidic proteins are to the left and high molecular mass proteins are at the top. Filled ellipses indicate those proteins that are both reliably detected (present in all gels) and demonstrate a significant ($p < .001$) difference from a treatment group.

TABLE 2. *T24 protein spots matched to master gel and with coefficient of variation (<15%)*

Treatment	Concentration (νM)	Protein spots matched[a] (No.)	Protein spots with CV < 15%[b] (No.)
DMSO	—	364	283
TPA	0.10	164	138
TPA	1.0	382	320
ABP	10	238	148

[a] Number of spots matched to master gel per treatment group.
[b] Number of spots with coefficient of variation <15% within group.

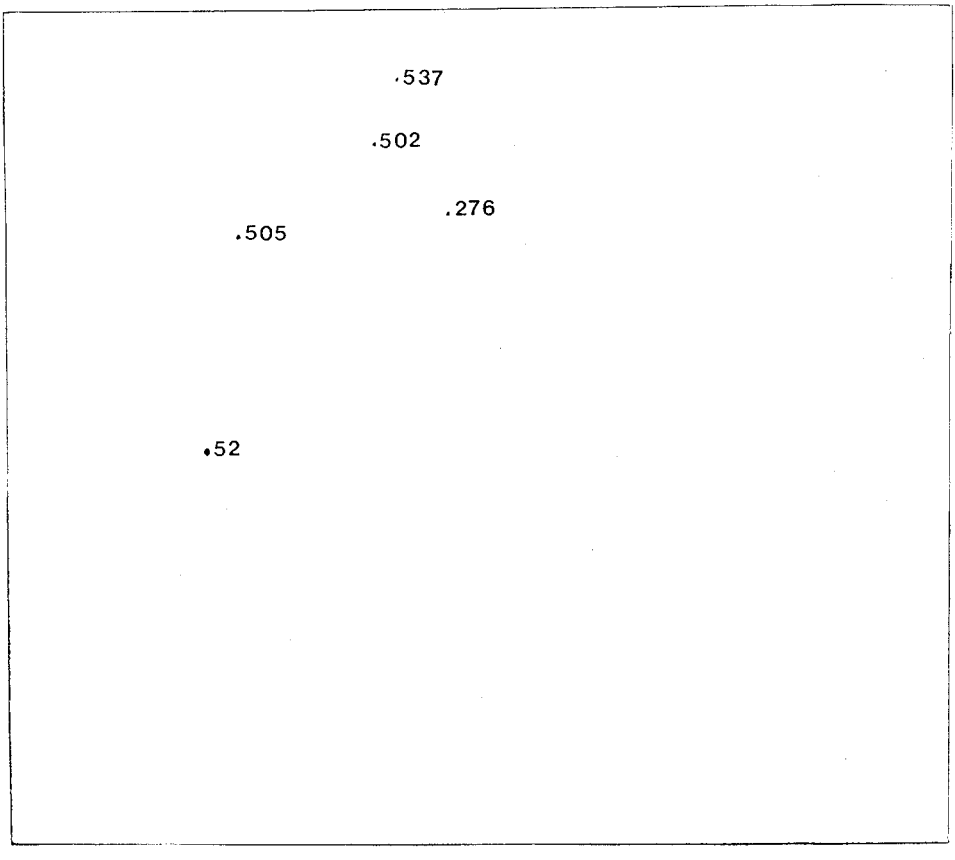

FIG. 2. The same pattern as in Figure 1 except showing only those proteins and their associated spot numbers that are both reliably detected (present in all gels) and demonstrate a significant ($p < .001$ vs. control) twofold or greater difference following TPA (0.1 and 1.0 νM) treatment.

The ability of 2D-PAGE to simultaneously determine changes in a large number of proteins makes possible the selection of specific proteins that may deserve further examination and identification. The assigning of specific spots to known proteins will further enhance the ability to make mechanistic interpretations of the observed spot changes and provide greater insight into the mechanisms associated with occupational carcinogen/promoter exposure.

Measurements of phenotypic profiles may be useful in the identification of individuals at increased risk of bladder cancer due to chemical exposure. This capability could have clinical or public health benefits. Specific biomarkers could serve as indicators of recent and biologically important exposures. Critical in this regard is the need to distinguish homeostatic or other noncarcinogenic changes from those that are predictive of a cancer risk. Once validated for disease, the biomarkers could also be used to target individuals in a preclinical state who could possibly benefit from aggressive intervention or chemoprevention [33].

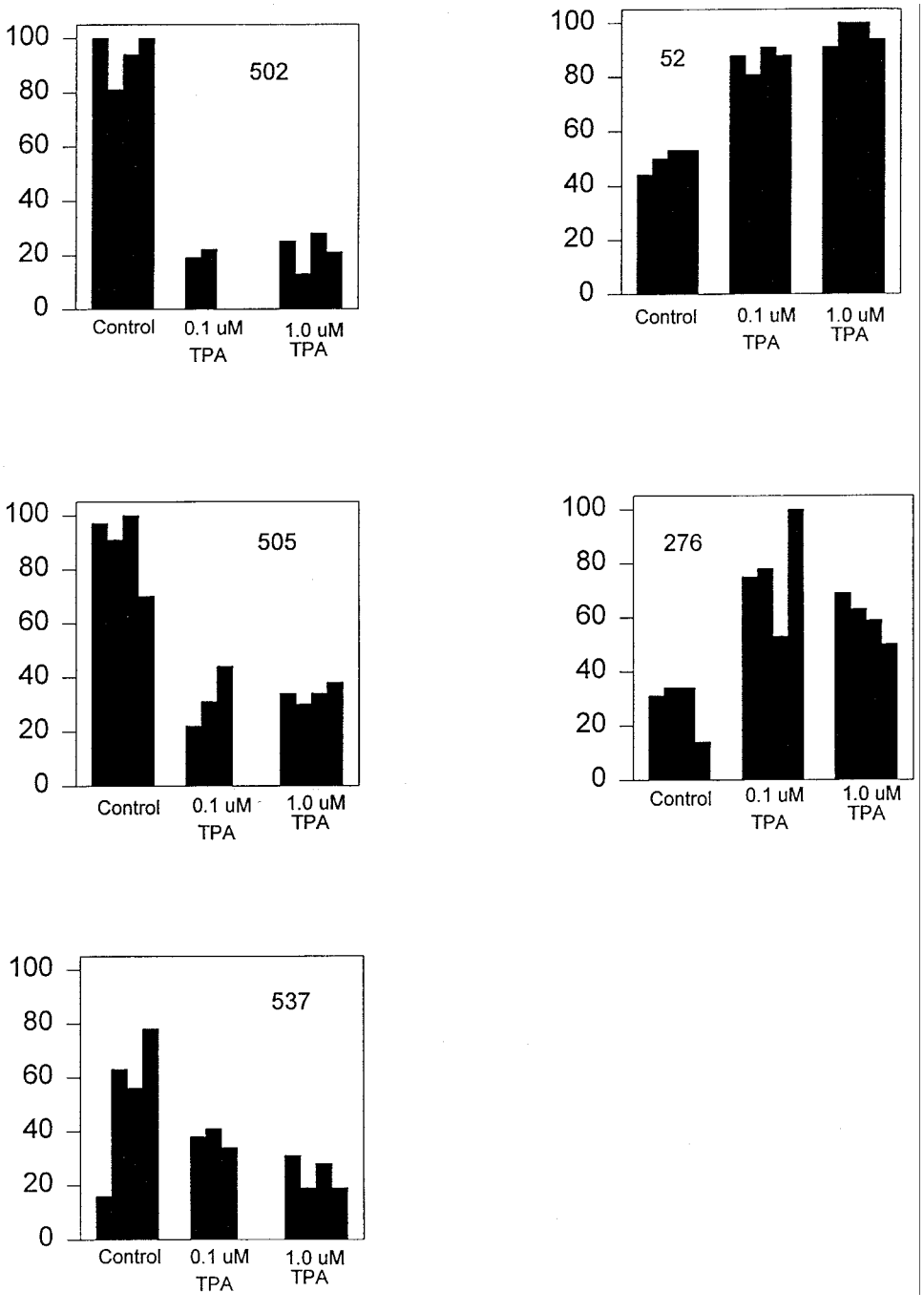

FIG. 3. T24 bladder carcinoma proteins demonstrating an alteration in abundance following in vitro TPA exposure ($p < .001$; twofold or greater). Each panel shows data for one protein spot (spot number in the upper corner). Single bars represent the relative abundance for one 2D gel ($n = 4$ gels per treatment group), and each set of bars represents replicate measurements.

TABLE 3. *T24 proteins demonstrating significant alteration[a] in abundance following in vitro TPA exposure*

Protein no.	DMSO	TPA (0.1 *v*M)	Relative decrease	Relative increase	TPA (1.0 *v*M)	Relative decrease	Relative increase
502	94 (4)[b]	10 (6)	9.4	—	22 (3)	4.3	—
505	90 (7)[b]	24 (9)	3.8	—	34 (2)	2.6	—
537	53 (13)	28 (10)	2.0	—	24 (3)	2.2	—
52	50 (2)	87 (2)	—	2.0	96 (2)	—	2.0
276	28 (5)	77 (10)	—	2.8	60 (4)	—	2.1

[a] $p < .001$ vs. control (Student's *t* test).
[b] Percent relative abundance by 2D gel analysis on the Kepler system, $n = 4$ gels/sample, mean (±SEM).

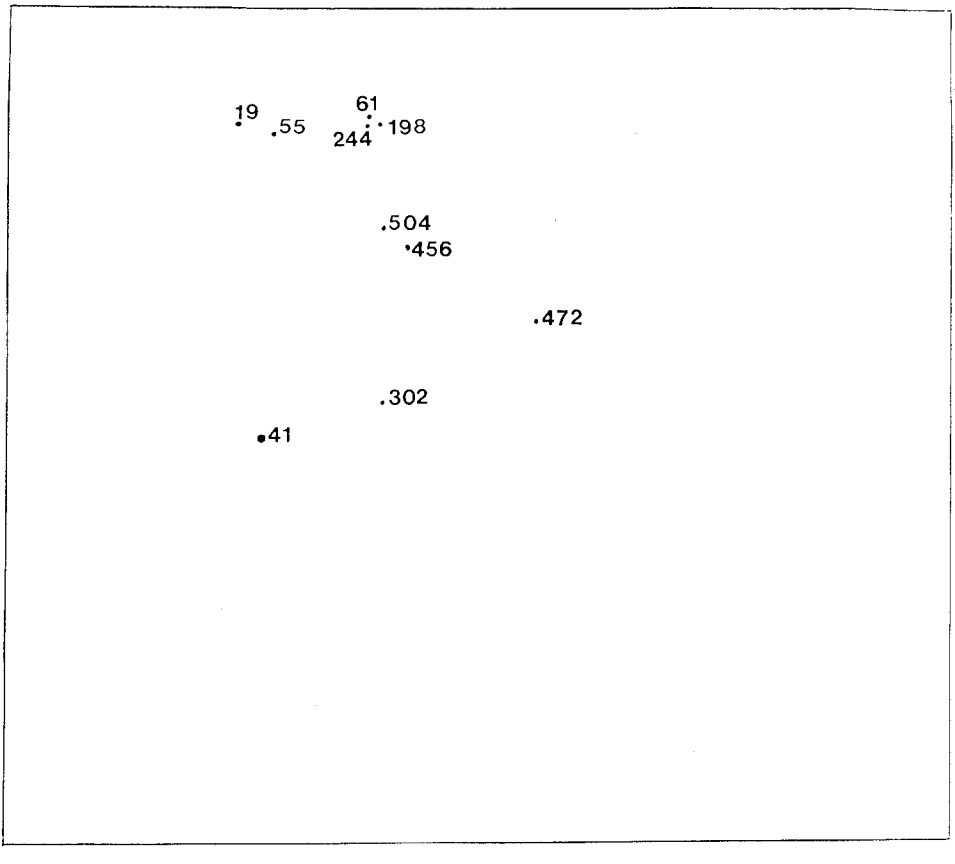

FIG. 4. The same pattern as in Figure 1 except showing only those proteins and their associated spot numbers that are both reliably detected (present in all gels) and demonstrate a significant ($p < .001$ vs. control) twofold or greater difference following ABP (10 *v*M) treatment.

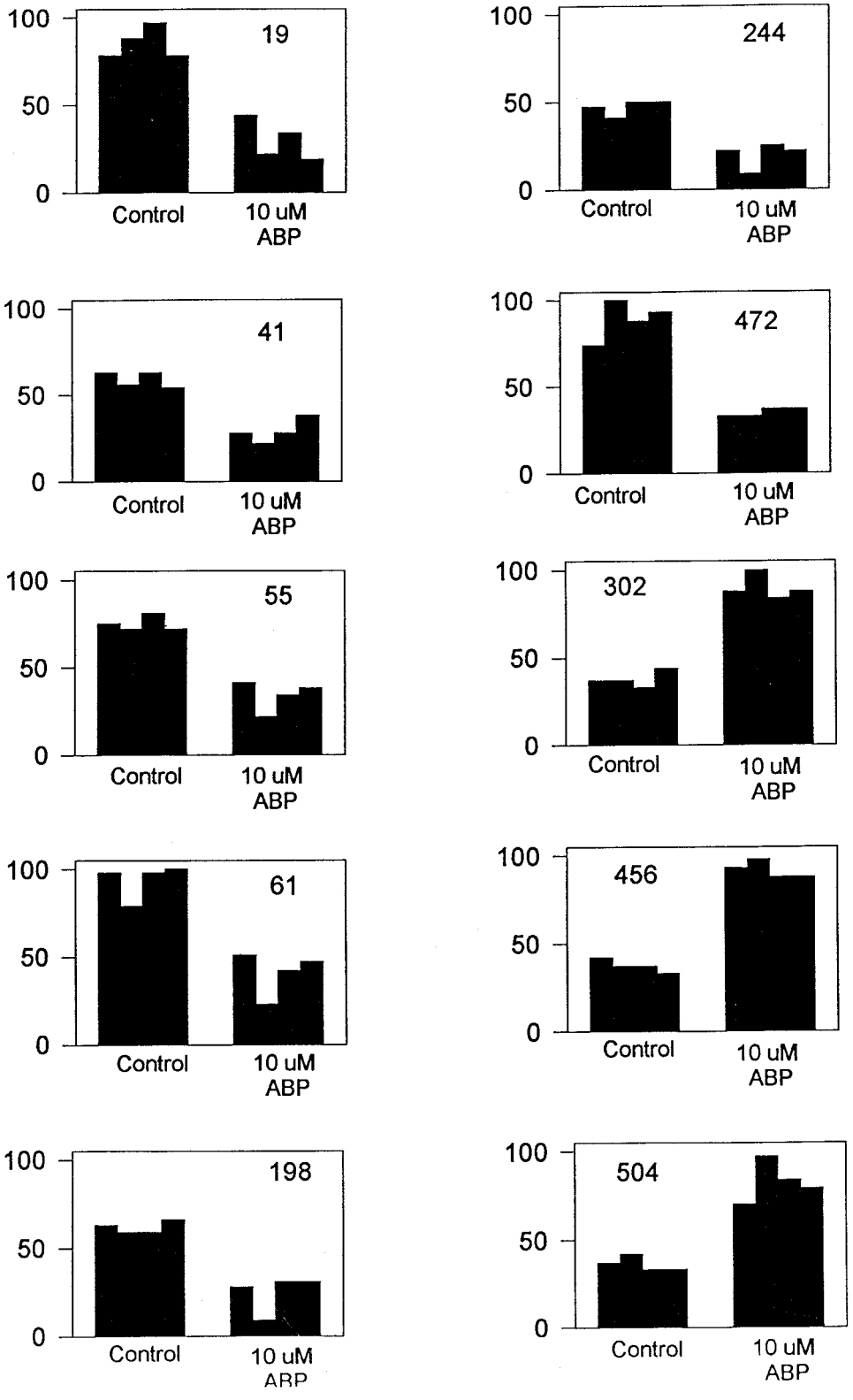

FIG. 5. T24 bladder carcinoma proteins demonstrating an alteration in abundance following in vitro ABP exposure ($p < .001$; twofold or greater). Each panel shows data for one protein spot (spot number in the upper corner). Single bars represent the relative abundance for one 2D gel ($n = 4$ gels per treatment group), and each set of bars represents replicate measurements.

TABLE 4. *T24 proteins demonstrating significant alteration[a] in abundance following in vitro ABP exposure*

Protein no.	DMSO	ABP (10 *v*M)	Relative decrease	Relative increase
19	85 (5)[b]	30 (6)	2.9	—
41	59 (2)	29 (3)	2.0	—
55	75 (2)	34 (4)	2.2	—
61	94 (5)	41 (6)	2.3	—
198	62 (2)	25 (5)	2.5	—
244	47 (2)	20 (4)	2.4	—
472	89 (5)	35 (1)	2.5	—
302	38 (2)	90 (4)	—	2.4
456	37 (2)	92 (2)	—	2.5
504	36 (2)	83 (5)	—	2.3

[a] $p < .001$ vs. control (Student's *t* test).
[b] Percent abundance by 2D gel analysis on the Kepler system, $n = 4$ gels/sample, mean (±SEM).

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