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Detection and Characterization of Low Abundance Cellular Proteins That Specifically Increase Upon Loss of the Metastatic Phenotype

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Abstract Human epidermoid carcinoma (HEp-3) cells are highly tumorigenic and metastatic in vivo, but their metastatic phenotype is progressively and uniquely lost upon serial passage in vitro The nonmetastatic phenotype is fully reversible to the highly metastatic state when HEp-3 cells are passaged back in vivo

To study the complex process of metastasis and its possible negative regulation by specific gene products, the expression of specific proteins between the highly metastatic and nonmetastatic HEp-3 cells was investigated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and subsequent computer assisted analyses Concomitant with the in vitro loss of metastatic potential of HEp-3 cells is the elevated expression of a subset of low abundance proteins detectable in 2D-PAGE but not apparent in high resolution one dimensional PAGE When the HEp-3 cells revert to the metastatic state, the expression of these proteins declines The increased cellular abundance of four distinct proteins directly correlates with the loss of the metastatic phenotype: two of the four proteins are associated with isolated cellular membranes (36kD, pI 5 7, 22kDa, pI 5 6), one protein fractionates with the cytoplasm (65kD, pI 6 2), and one protein is enriched in the nuclei fraction (32kD, pI 5 8) These data indicate that computer-assisted analysis of highly sensitive, large-format, 2D-PAGE can be used to identify specific proteins in subcellular compartments that are candidates for negative regulators of the metastatic process

Key words: 2D-PAGE, human tumors, HEp-3, subcellular fractionation, negative regulators

HEp-3 is a cell line derived from a highly aggressive human epidermoid carcinoma isolated from a regional lymph node of a patient who succumbed to metastatic disease originating in the buccal cavity [Dagg et al., 1954]. This cell line displays three distinct phenotypes: tumorigenic-highly metastatic (T^+M^+), tumorigenic–nonmetastatic (T^+M^-), and nontumorigenic (T^-M^-). The HEp-3 cells are highly tumorigenic and metastatic when passaged in vivo [Ossowski and Reich, 1980b]. When initiated from primary tumors and passaged in cell culture, the metastatic phenotype of HEp-3 is only maintained for a limited period of time, 4–14 passages [Ossowski and Reich, 1980b] or 8–45 days, during which time metastatic potential drops dramatically as assayed by periodic in vivo analyses of the cultured cells. Tumorigenicity also declines with in vitro passage although much more slowly [Ossowski and Reich, 1980b]. Cells that have lost metastatic potential but retained the ability to form primary tumors can be passaged in vivo, resulting in a reversion to the highly metastatic phenotype [Ossowski and Reich, 1980b]. The phenotypic changes that take place appear to be a direct response to conditions in the physiological environment, since HEp-3 populations are not heterogeneous with respect to tumorigenicity or metastatic potential, as determined by clonal analysis [Ossowski and Reich, 1983]. Although reversal of the metastatic phenotype does not appear to be a common occurance in vivo the reversable nature of HEp-3 metastatic phenotype provides a model

Abbreviations CAM, chorioallantoic membrane, HB, homogenization buffer, LDH, lactate dehydrogenase, LT, lung tumor, HEp-3, human epidermoid cell line, 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis, huPA, human urokinase plasminogen activator, PA, plasminogen activator, T^+M^+, tumorigenic—metastatic, T^+M^-, tumorigenic—nonmetastatic, T^-M^-, nontumorigenic—nonmetastatic

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system to examine the cellular and molecular events that regulate metastasis.

The process of metastasis is a complex cascade of events, only a few of which can be mimicked in vitro. The in vivo system most commonly used has been the murine animal model [Fidler, 1978]. Metastasis assays are lengthy in this system (3–6 weeks), and the range of studies is limited by the immune response of the host or by the extensive care requirements of athymic mice. In light of these difficulties, the chick embryo has been used as an in vivo model to study HEp-3 metastasis due to its convenience and rapidity: HEp-3 cells implanted onto the chorioallantoic membrane (CAM) of the chick embryo form primary tumors and metastasize spontaneously within 7 days [Gordon and Quigley, 1986; Ossowski and Reich, 1980; Ossowski and Reich, 1980b]. The use of an avian host to reveal the metastatic phenotype of HEp-3 cells is validated by the analysis of this cell line in a mammalian model. HEp-3 cells form tumors and metastasize spontaneously in the athymic mouse system precisely, as they do in the chick embryo model [Ossowski et al., 1987]. The loss of metastatic ability and eventually the gradual loss of tumorigenic potential with increasing time in culture, as well as the reversion of nonmetastatic to metastatic phenotype during in vivo passage, are all reproducible in the rodent system [Ossowski and Reich, 1980b; Ossowski et al., 1987].

The precise mechanisms of HEp-3 metastasis are not defined. However the ability to isolate three distinct phenotypes allows for the study of specific changes that occur in HEp-3 cells as the different phenotypes are expressed. 2D-PAGE has been chosen to correlate protein content of the cells with changes in phenotype. Analysis of the level of protein expression using 2D-PAGE has been used to determine the expression of previously characterized proteins as a function of changes in phenotype, such as alterations in the level of tropomyosin, depending on the metastatic potential of the cell [Takenaga et al., 1988]. This approach also has been used to describe proteins with newly recognized functions such as gelsolin, whose expression is downregulated with transformed phenotype (Vandekerckhove et al., 1990). 2D-PAGE is thus a very sensitive technique that can detect and resolve proteins that exist as 10^{-4}–10^{-5}% of total cellular protein [O'Farrel, 1975; Robertson et al., 1987]. For these reasons, large-format 2D-PAGE was chosen to monitor proteins whose abundance increases with the loss of the metastatic phenotype in HEp-3 cells. This approach may reveal new proteins or known proteins with newly recognized functions important in the regulation of metastatic behavior.

Loss of suppressor function has been implicated in a wide variety of cancers. The retinoblastoma protein and its association with a variety of cellular and viral proteins is most well characterized [Cavanee et al., 1983; Huang et al., 1988]. Proteins such as p53 [Baker et al., 1990; Diller et al., 1990; Finlay et al., 1989], nm23 [Bevilacqua et al., 1989; Steeg et al., 1988a,b], WDNM [Dear et al., 1988, 1989], MCC [Kinzlee et al., 1991], and DCC [Fearon et al., 1990] have also been implicated as suppressors of malignancy. The mechanistic functions of these protein products in the tumorigenic process are less well understood but are bound to be diverse, since their identity ranges from the cell adhesion family of proteins to the nucleoside diphosphate kinase family.

An understanding of the regulation and execution of the complex metastatic process will undoubtedly involve both oncogenes and suppressor genes, their protein products, and the interrelationships that take place. The present study establishes an approach to identify low abundance but potentially important gene products that function in metastasis regulation.

MATERIALS AND METHODS

Cell Lines

HEp-3 cell monolayer cultures were obtained by physical dissociation of finely trimmed HEp-3 tumors that had been serially passaged on the CAMs of 10-day-old chick embryos. Cells maintained in culture less than 30 days were used as highly metastatic cells; cells maintained in culture for 50–70 days were used as tumorigenic–nonmetastatic cells; and cells in culture for greater than 125 days were used as nontumorigenic cells.

All cultures were grown in a humidified 5% CO_2 atmosphere at 37°C in DME supplemented with 10% FBS and sodium pyruvate (Sigma Chemical, St. Louis, MO). Cells were passaged three times per week for 2–3 weeks at 2×10^6 cells per 100-mm plate and then one to two times per week at 1×10^6 cells per 100-mm plate thereafter.

Chick Embryo Assay

Fertilized COFAL-negative eggs were supplied by Spafas (Norwich, CT) and incubated at 37°C with 60% humidity. The eggs were incubated for 10 days and then candled to locate the position of blood vessels and an appropriate position to cut a window through the shell distant from the blood vessels. The procedure for preparing the eggs for tumor cell inoculation was a modification of that previously described [Burnet and Barnard, 1933]. Using a crafts drill, a hole was cut in the shell over the air sac. A second hole was cut through the shell over, but without damaging, the CAM. By applying negative pressure to the first hole, the CAM drops and allows for an adequate-sized window (0.5 × 0.5 cm) to be drilled in the shell over the dropped CAM. HEp-3 cells (2×10^5) were inoculated in 25 μl of serum-free DME, directly onto the CAM. The window was covered with sterile tape; incubation of the embryo was continued until day 17 or 18. At this time, the HEp-3 tumor that formed on the CAM (referred to as the primary tumor) was carefully dissected away from chick tissue and weighed. The tumor could be used to initiate a primary culture or used to inoculate newly prepared 10-day embryos for further in vivo passage.

In order to assay for metastasis, a lung from the test embryo carrying the primary tumor was harvested, finely minced, and transferred to a newly prepared CAM of a 10-day embryo and allowed to grow for an additional 7–8 days. This transfer assay allows for the analysis of disseminated HEp-3 cells in the lung by permitting metastatic cells present in the original lung to increase in number during the additional embryo incubation. The resulting lung tumor outgrowth was harvested and weighed. A suspension of the mince was examined in a phase-contrast microscope for the presence of intact HEp-3 cells, which are easily distinguished from the much smaller cells of chick tissue. The lung tumor suspension was then subjected to a determination of human urokinase plasminogen activator (huPA) activity in a standard chromogenic PA assay, since HEp-3 cells, and not chick cells, produce huPA. The level of huPA in the lung tumor has been shown to correlate directly with the number of viable HEp-3 cells present in the original lung tissue [Gordon and Quigley, 1986; Ossowski and Reich, 1980].

Plasminogen Activator Assay

Embryonic lungs and/or lung tumors were extracted in 25 mM Tris and 0.5% Triton × 100, pH 7.5, and homogenized with a tissue grinder (Biospec Products, Bontlesville, OK). Insoluble material was pelleted by centrifugation at 10,000*g* for 10 min. The protein concentration in the tissue homogenates were determined by the BCA method of protein quantitation (Pierce Chemical, Rochford, IL). The samples were diluted to 0.1 mg/ml in extraction buffer. PA assays [Friberger, 1975] were performed in 96-well flat bottomed culture plates (Nunc, Roskilde, Denmark). The assay consists of 10 μg of homogenate protein diluted in the Tris–Triton extraction buffer, incubated with 3.0 μg of human plasminogen per well (140 μl total volume) for 2 h at 37°C. Following incubation, 15 μg of S2251 chromogenic substrate (KabiVitrum, Stockholm, Sweden) (30 μl) was added to each well, and absorbance was recorded at 405 nm at 0, 15, 30, and 60 min. The activity, linear with time over 60 min, was recorded and compared to that of a urokinase standard.

Scanning Electron Microscopy

HEp-3 cells were grown to 75–80% confluence on glass coverslips. The cells were washed twice with PBS and fixed in 3% buffered glutaraldehyde. The cells were fixed a second time in 1% unbuffered osmium tetroxide. The sample was dehydrated in increasing concentrations of ethanol; the ethanol was then replaced with increasing concentrations of amyl acetate before critical point drying in CO_2. The coverslips were mounted on aluminum stubs with colloidal silver adhesive, sputter-coated with 20 nm of platinum, and examined with a scanning electron microscope at an accelerating voltage of 10 kV.

Radioactive Labeling of HEp-3 Cells

Cells were grown in 100-mm tissue culture plates (Becton Dickinson, Lincoln Park, NJ) in DME supplemented with 10% FBS. When the cultures were 70–80% confluent ($4–5 \times 10^6$ cells), they were incubated with cysteine- and methionine-deficient DME supplemented with 1.0% FBS. The cultures were labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine (sp. act. 1,000 and 1,300 Ci/mmol, respectively) at 50 μCi/ml (Amersham Corp., Arlington Heights, IL). Following 16 h of incubation, the cultures

were washed three times with ice-cold PBS containing 1 mM EDTA and then lysed.

Preparation of Cell Lysates for SDS–PAGE

To prepare total cell lysates, monolayers grown in 100-mm tissue culture plates were rinsed three times with ice-cold PBS containing 1 mM EDTA. Lysis buffer (0.3% SDS, 1.0% βME, 50 mM Tris–pH 8.0) was heated to 100°C and added directly to the 100-mm plates. The cell lysate was scraped into a microcentrifuge tube and chilled in an ice bath. DNase 1 (0.1 mg/ml) and RNase A (25 mg/ml) in 0.5 M Tris pH 7.0 and 50 mM $MgCl_2$ were added to the lysate and mixed well for 2 min at 0°C. Cellular debris was removed by centrifugation at 10,000*g* for 10 min. The supernatant was aliquoted, quick frozen in liquid nitrogen, and stored at −70°C.

Subcellular Fractionation

Monolayer cultures were washed with PBS containing 1 mM EDTA three times before being mechanically scraped off the tissue culture plate in PBS. Intact cells were pelleted by centrifugation at 1,200*g* for 10 min. The PBS wash was discarded, and the cell pellet was resuspended in 2-ml homogenization buffer (HB) (0.25 M sucrose, 0.1 M Tris–HCl, 1 mM EDTA, pH 7.4) per 100-mm plate. The cells were incubated in this hypotonic buffer for 5 min on ice. The suspended cells were then subjected to dounce homogenization with 50–70 strokes or until 90–100% of the cells appeared broken by phase microscopy. Nuclei were pelleted at 2,000*g* for 10 min. The supernatant was transferred to a clean tube and the nuclear pellet was resuspended in ×10 volume of HB. Both the supernatant and the resuspended nuclei were centrifuged for 10 min at 2,300*g*. The pellet from both tubes were combined as the nuclear fraction. Supernatants from both tubes were combined and centrifuged at 100,000*g* for 60 min to pellet the total cellular membranes. The pellets were rinsed with HB, combined, and resuspended in HB as the membrane fraction. The 100,000*g* supernatants were combined as the soluble cytoplasmic fraction and concentrated 10- to 20-fold in an Amicon centriprep 10 (Amicon Div., Danvers, MA). Each fraction was aliquoted, quick frozen in liquid nitrogen, and stored at −70°C.

Efficiency of fractionation was determined by phase microscopy, as well as by monitoring three subcellular markers. An aliquot of each subcellular fraction was subjected to protein quantitation by the BCA assay (Pierce Chemical, Rockford, IL). The PA enzymatic assays were performed on aliquots of sample adjusted to a protein concentration of 0.1 mg/ml. Plasminogen activator is associated with the membranes of HEp-3 cells and thus serves as a marker for the presence of cell membranes in each of the three fractions. An aliquot from each fraction was subjected to the chromogenic PA assay as described above. Lactate dehydrogenase, a soluble cytoplasmic enzyme [Cabaud and Wroblewski, 1958; Holbrook et al., 1975], was used to indicate the presence of cytoplasmic proteins in each of the subcellular fractions. An aliquot of each fraction was subjected to the lactate dehydrogenase diagnostics kit (Sigma Chemical, St. Louis, MO). In order to monitor the presence of nuclei (DNA) in each fraction, a companion culture was radiolabeled with ^{3}H-thymidine for 36 h. This culture was fractionated in parallel with the experimental cells. The level of nuclear DNA (TCA precipitable ^{3}H-thymidine cpm) was then determined for an aliquot of each subcellular fraction.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed in our laboratory using the large format (22 cm × 22 cm) Millipore Investigator system (Millipore, Bedford, MA) and at the Cold Spring Harbor 2D Gel laboratory [Garrels, J., 1979, 1989; Garrels and Franza, 1989].

Cell lysates or subcellular fractions were loaded onto isoelectric focusing (IEF) tube gels (9.5M urea, 2% NP-40, 4.1% acrylamide, 2.3% ampholytes-pH 4–8) with 150,000–500,000 dpm of TCA precipitable ^{35}S-methionine-cysteine radioactivity, corresponding to 5–15 μg of total protein per gel. The IEF gels were electrophoresed for 18,000 V-h. The gel was then extruded from the tube into an SDS equilibration buffer (0.3 M Tris base, 0.075M Tris–HCl, 3% SDS, 50 mM DTT, 0.01% bromophenol blue). The tube gels were equilibrated for 2 min, then layered in direct contact onto a 10% polyacrylamide SDS slab gel [Laemmli, 1970]. Second dimension gels were maintained at 15°C and electrophoresed for 5.5 h at 20 W per gel. Gels were fixed in 50% methanol and 10% acetic acid, soaked in Amplify (Amersham Corp., Arlington Heights, IL) and subjected to autoradiography. Autoradiographs were scanned into the Quest computer analysis system (Cold Spring Harbor). During analysis, each spot that was detected by the computer was

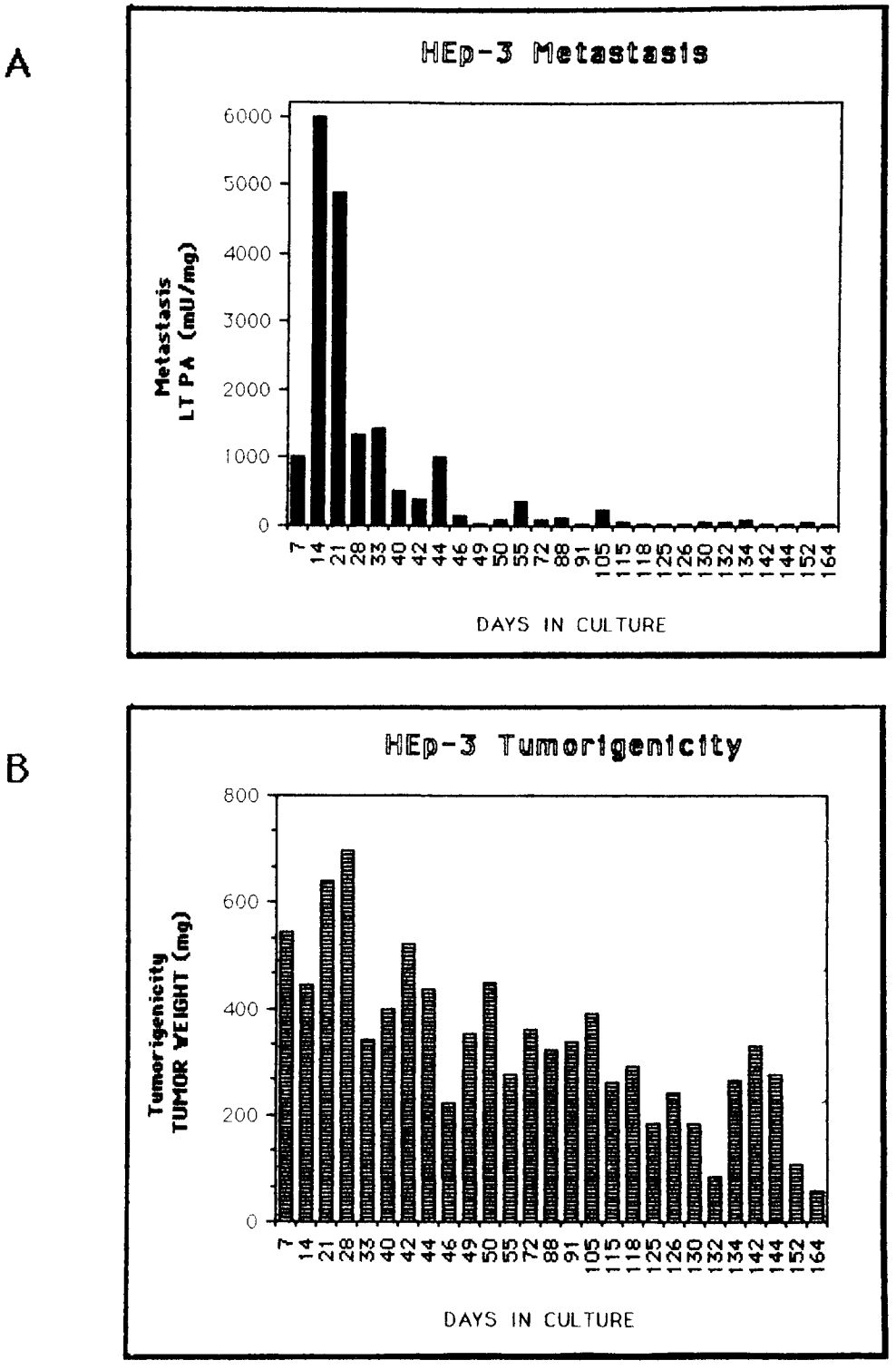

Fig. 1. Metastatic potential and tumorigenicity of HEp-3 cells dissected from a primary tumor and carried in culture continuously. On the days indicated, 2×10^5 cells were tested in the in ovo CAM assay. Seven days following HEp-3 cell inoculation onto the CAM of a 10-day-old embryo the primary tumor was excised, trimmed, and weighed. The lungs from each embryo were aseptically minced and transferred to a newly prepared CAM of a 10-day embryo and allowed to incubate for an additional 7 days. The resulting lung tumor (LT) was excised, homogenized, and analyzed for the level of human urokinase-type plasminogen activator (LT PA) activity to monitor the presence of disseminated HEp-3 cells (**A**). The level of LT PA activity (1,000–6,000 mU/mg) is a reflection of metastatic potential. The lungs from mock inoculated embryos results in a tissue extract with less than 100 mU/mg of PA activity. **B:** The primary tumors were weighed as a measurement of tumor forming potential. Mock inoculations of serum-free DME onto CAMs results in an excised control tissue weight of approximately 60 mg.

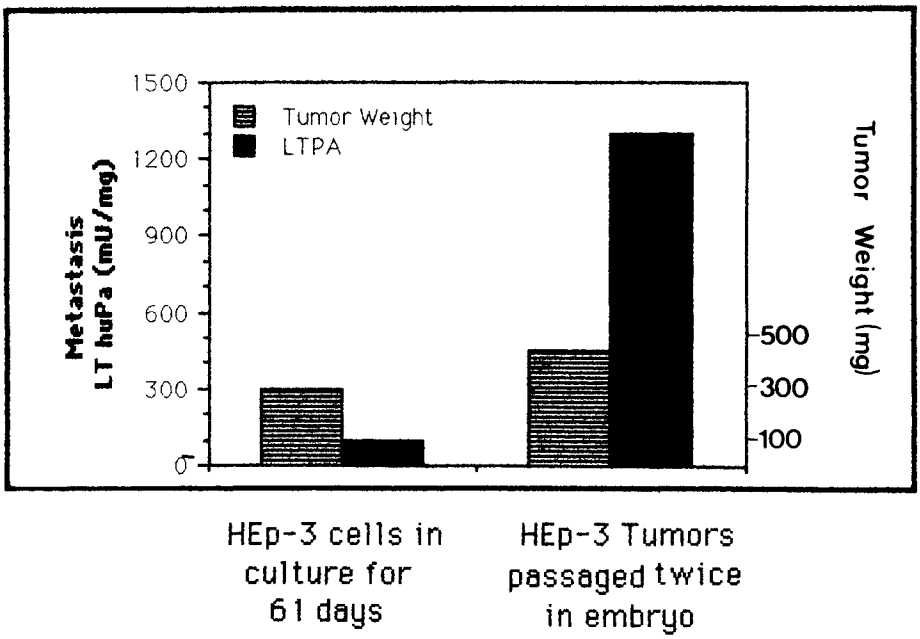

Fig. 2. Reversion from nonmetastatic to metastatic phenotype of HEp-3 cells carried in vivo HEp-3 cells were carried in culture for 61 days, at which time 5×10^5 cells were tested in vivo for tumorigenic (left striped bar) and metastatic (left solid bar) potential as described for Figure 1 The primary tumor was harvested aseptically, minced and inoculated onto another newly prepared CAM Following 7 days of development, the passaged CAM tumor was aseptically harvested and weighed to measure tumorigenicity (right striped bar) One lung from this embryo was transferred to a newly prepared CAM, allowed to develop for seven days and then subjected to human urokinase plasminogen activator activity (LT huPA) determination as a measurement of metastatic potential (right solid bar)

sequentially numbered and given x and y coordinates relative to its isoelectric point and molecular weight. Each spot was quantified as parts per million (ppm) of the total protein radioactivity loaded onto the gel. Each spot could then be matched to the corresponding spot in another gel by using the x and y coordinates and by comparing its position relative to the constellation of protein spots surrounding it [Garrels, 1979, 1989]. Comparing the ppm values of specific protein spots between a large number of gels allowed for the determination of proteins whose intensity changes consistently with changes in cellular phenotype. We analyzed for only those proteins in total cell lysates and in subcellular fractions which exhibited a greater than twofold increase in intensity with the loss of the metastatic phenotype.

RESULTS

HEp-3 cells display three distinct phenotypes depending on mode and length of propagation. Cell cultures initiated from primary HEp-3 tumors grown on the chorioallantoic membrane of chick embryos maintain a tumorigenic, highly metastatic (T^+M^+) phenotype for approximately 7–21 days in culture. Metastatic potential is measured by determining the level of human urokinase plasminogen activator (huPA) activity in the lung tumor outgrowths due to the presence of metastatic HEp-3 cells. The level of huPA activity in lung tumors has previously been demonstrated to be a quantitative measurement of disseminated human tumor cells [Gordon and Quigley, 1986; Ossowski and Reich, 1980b]. A lung tumor plasminogen activator (LT PA) value of $>1{,}000$ mU/mg of lung tumor protein represents the presence of abundant metastatic HEp-3 cells (Fig. 1). As the HEp-3 cells are carried in culture for >3 weeks, their metastatic potential drops dramatically until the cells are nonmetastatic (<100 mU/mg) but remain fully tumorigenic (T^+M^-), yielding primary tumor weights of 300–600 mg. A decline in tumorigenicity (from 300 to 100 mg primary tumor weights) is more gradual than the decline in metastatic potential, but the cells eventually become nontumorigenic (T^-M^-) (Fig. 1B). These data confirm the original observations of HEp-3 phenotype [Ossowski and Reich, 1980b; Os-

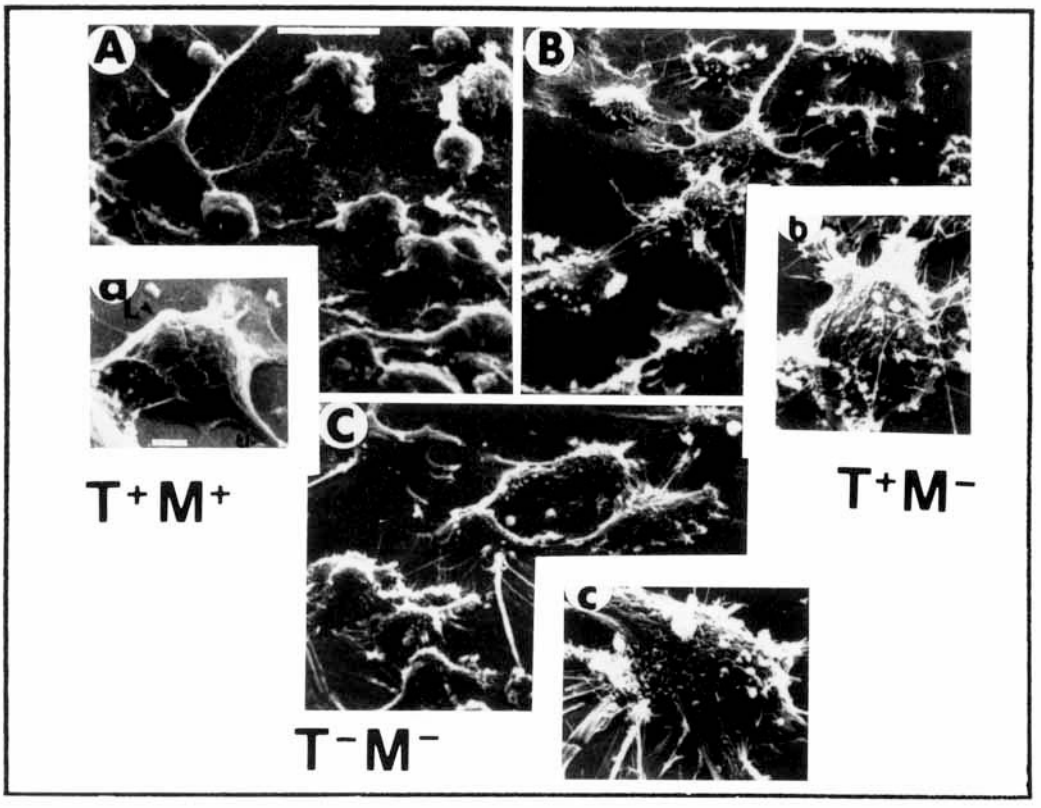

Fig. 3. Scanning electron micrographs of metastatic and nonmetastatic HEp-3 cells. HEp-3 cells were grown to 50–60% confluency on glass coverslips, fixed, and subjected to critical point drying prior to scanning electron microscopy. **A,a:** Cells grown in culture for 20 days (T^+M^+). **B,b:** Cells grown in culture for 85 days (T^+M^-). **C,c:** Cells grown in culture for 160 days (T^-M^-). The leading lamellipodium (L) and the tapered uropod (U) of a single cell is labeled in a. Bar A (upper center), 25 μm also applies to B and C; bar a (lower center), 5 μm also applies to b and c.

sowski and Reich, 1983] and provide a more detailed time course during the loss of metastatic function in HEp-3 cultures.

Nonmetastatic cells (T^+M^-) fully regain the metastatic phenotype (T^+M^+) when the cultured cells are implanted onto embryos, and serial passaged in vivo for at least two passages (Fig. 2). When the in vivo passaged cells are placed back into continuous culture, they maintain and then lose their metastatic potential with similar kinetics as described in Figure 1 for the original HEp-3 cells (data not shown).

Subtle but distinct morphological changes occur with HEp-3 cells as the phenotype changes with time in culture. Scanning electron microscopy was used to detect cellular alterations visually that occur with the malignant phenotypic changes described above (Fig. 3).Metastatic cells display a shape and directionality generally considered to apply to highly motile cells [Bellairs et al., 1982], that being a broad, flat leading lamellipodium with a tapered uropod ending in retractile fibers (Fig. 3a, insert). The nonmetastatic cells (T^+M^- and T^-M^-) fail to display an apparent directionality; in spite of an increased number of surface projections, they lack a marked lamellipodium or uropod (Fig. 3b,c, inserts). These data suggest that there are changes at the level of the cell surface, concurrent with the observed phenotypic changes.

The dramatic and reversible changes in HEp-3 phenotype with in vivo and in vitro passage provided a system with which to analyze specific changes in protein expression linked to phenotype changes. We were most interested in proteins whose abundance increased in HEp-3 cells with time in culture in order to identify potential negative regulatory proteins. Analyses of HEp-3 cellular proteins using single dimension SDS–PAGE yielded few if any detectable proteins whose expression correlated inversely with phenotype (Fig. 4). More than 75 distinct pro-

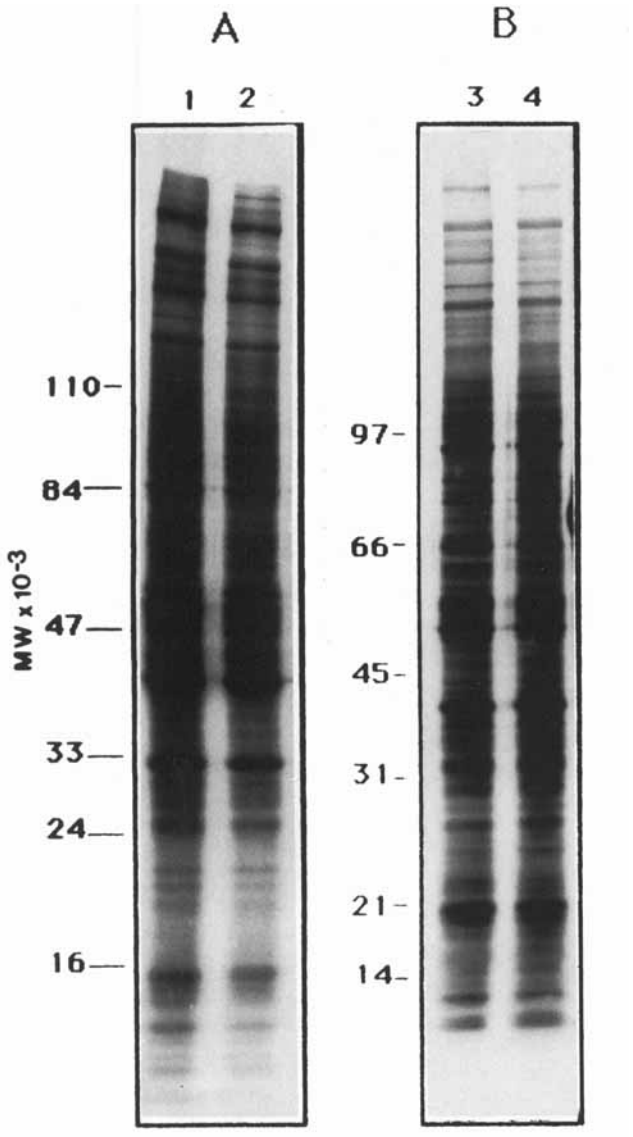

Fig. 4. Comparison between total cell lysates of metastatic and nonmetastatic HEp-3 cells by single dimension SDS-PAGE. Total cell lysates were prepared from metastatic (13 days in culture, **lanes 1 and 3**) and nonmetastatic (88 days in culture, **lanes 2 and 4**) HEp-3 cells. **A:** Autoradiograph of lysates generated from ^{35}S-methionine-cysteine labeled cells. Each lane was loaded with 250,000 cpm. **B:** Silver stain of nonradiolabeled companion cultures. The lysates were electrophoresed on a 6–15% gradient SDS–PAGE. The positions of molecular weight marker proteins are shown on the left.

tein bands were detectable by high-resolution SDS–PAGE (autoradiography and silver staining), and none showed more than a twofold increase during HEp-3 progression to a nonmetastatic phenotype.

To increase both the resolution and number of visible proteins on a gel, we employed large-format (22 cm × 22 cm) 2D-PAGE and computer-assisted (Quest Program) analyses. Representative autoradiographs of metastatic and nonmetastatic total cell lysates are shown in Figure 5. Autoradiographs from the 2D gels, subjected to computer analyses, distinguished approximately 1,000–4,000 protein spots compared to less than the 100 protein bands visible on a single dimension autoradiograph. Figure 5 illustrates two representative proteins (arrows) whose level increases upon in vitro progression to the tumorigenic nonmetastatic state.

The computer analyses enabled the determination of changes in specific protein ratios among the phenotypes of HEp-3 cells. Proteins spots that manifested greater than a two fold increase in abundance in the nonmetastatic cells over the metastatic cells were detected both visually and by Quest computer analysis. These protein spots were then analyzed in total cell lysates of the phenotypically reverted cells. Figure 6 illustrates these criteria in highly magnified areas of the large format 2D gel. A single example, protein spot #2121 (22 kD, 5.6 pI), increases in abundance in samples prepared from HEp-3 cells in culture 26 days (Fig. 6A) to 72 days (Fig. 6B) and 131 days (Fig. 6C). The level of this protein is then concomitantly reduced in HEp-3 cells that have reverted back to the metastatic phenotype (HEp-3 Rev. Fig. 6D) by two in vivo passages (see Fig. 2).

By 72 days in culture, when metastatic potential had been fully lost, there was a corresponding increase in abundance of the above-mentioned specific proteins in whole cell lysates. Therefore, it was of interest to investigate the level of these proteins in specific subcellular fractions and in a time frame more precisely coordinated to the loss of the metastatic phenotype (14–40 days in culture; see Fig. 1). HEp-3 Rev cells were carried in culture continuously from a metastatic primary tumor. Samples of cells were taken at 7- to 10-day intervals, sub-

Fig. 5. Two-dimensional PAGE autoradiographs of total cell lysates generated from ^{35}S-methionine-cysteine labeled HEp-3 cells grown in culture for 10 days (**A**) or 72 days (**B**). Each IEF gel contained a pH range of 4–8 and was loaded with 500,000 dpm. Isoelectric points are shown along the bottom and molecular weights are shown along the left side. The arrows indicate two proteins, #2121 (22 kD, 5.6 pI) and #2274 (36 kD, 5.7 pI), which increase in abundance upon the loss of metastatic potential. The arrowheads are spots of reference proteins used for orientation and indicate equivalent loading between the two gels.

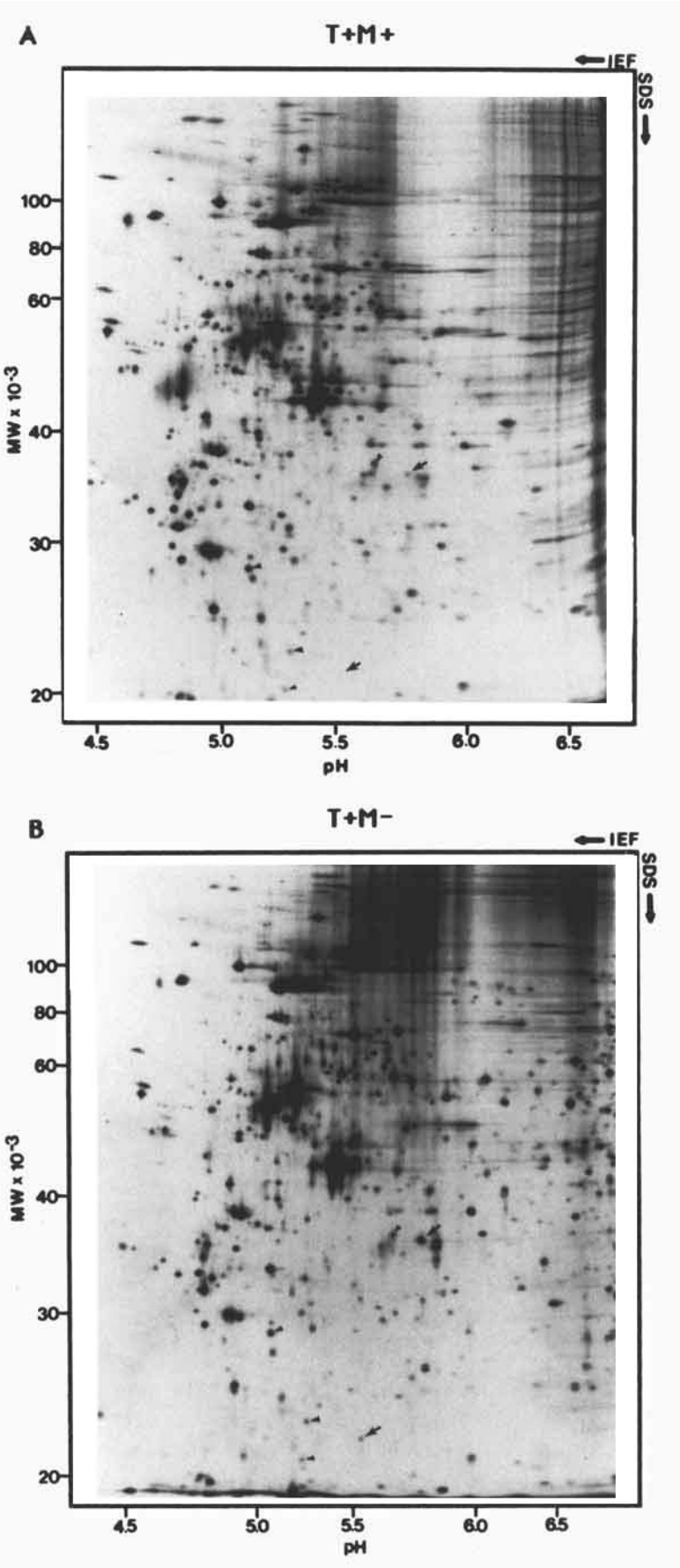
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Figure 5.

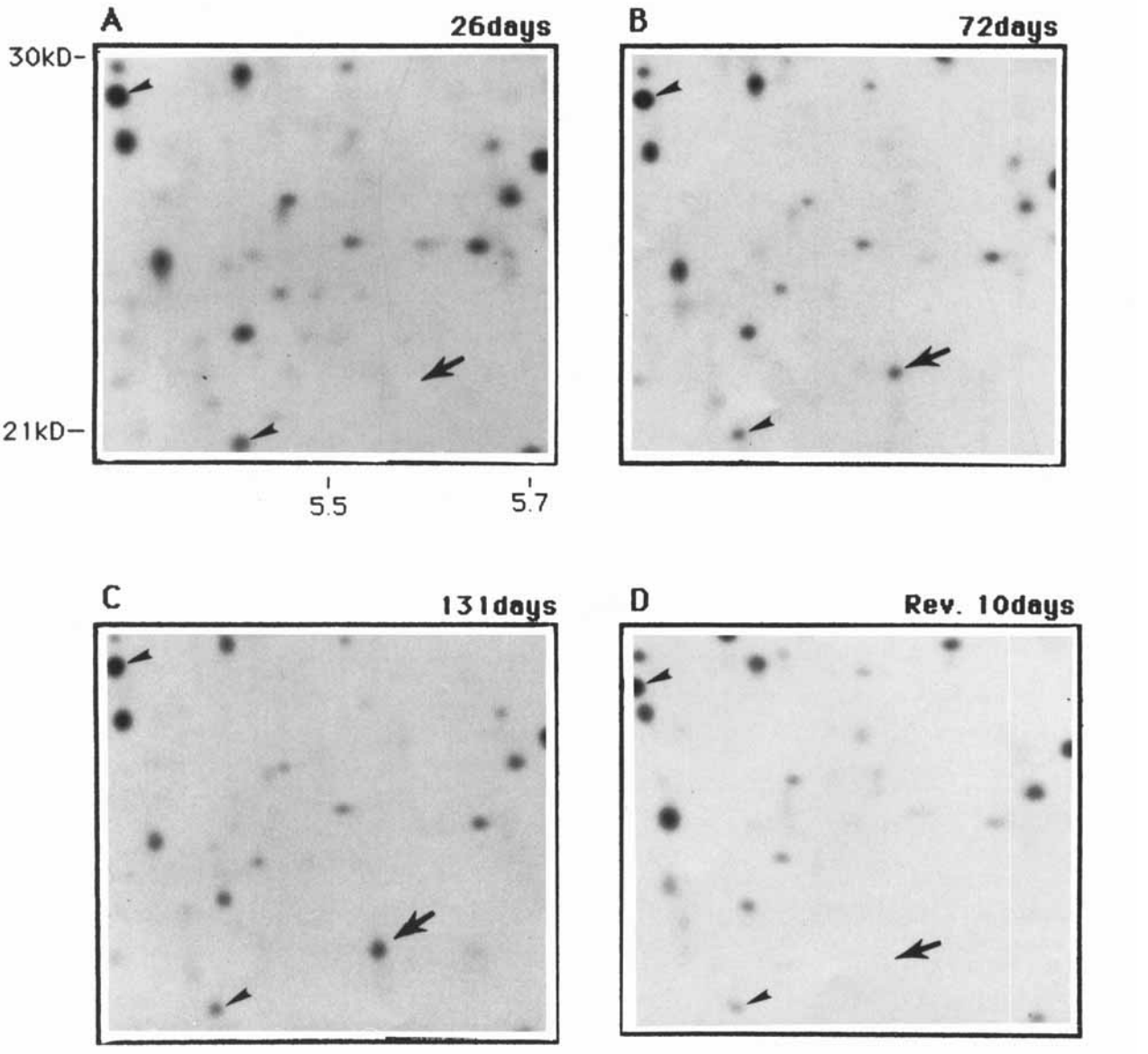

Fig. 6. Two-dimensional PAGE autoradiograph showing the relative abundance of protein #2121 in total HEp-3 cell lysates with increasing time in culture and upon reversion to the metastatic phenotype. Whole cell lysates labeled with ^{35}S-methionine–cysteine were generated from HEp-3 cells grown in culture: for 26 days, metastatic **(A)**; for 72 days, nonmetastatic **(B)**; and for 131 days, nonmetastatic–low tumorigenic **(C)**. Cells that had been in culture for 61 days (nonmetastatic) were grown in vivo to generate cells that had reverted to the full metastatic phenotype-HEp-3 Rev (see Fig. 2). The HEp-3 Rev cells were grown in culture for 10 days prior to cell lysis, metastatic **(D)**. Each panel represents an enlarged view taken of the region of interest from a full autoradiograph. Molecular-weight markers are along the left side and isoelectric points are along the bottom of A. The arrow indicates the position of protein #2121 (22 kD, pI 5.6) at each time point. The arrowheads point to protein spots used for orientation and that indicate equal loading between all four gels.

jected to subcellular fractionation and analyzed by 2D gel electrophoresis to ascertain more precisely at what stage of metastatic decline the specific proteins were seen to increase, and to localize these proteins to a particular subcellular fraction. To monitor the fractionation three specific subcellular markers were assayed in isolated membrane, cytoplasmic, and nuclear fractions. Plasminogen activator (PA) has been shown previously to be primarily associated with cellular membranes [Christman et al., 1975; Quigley, 1976]. The level of activity of this enzyme was assayed in all three fractions, and approximately 60% of the PA activity was found associated with the total membrane fraction (Fig. 7, solid black bars). Lactate dehydrogenase

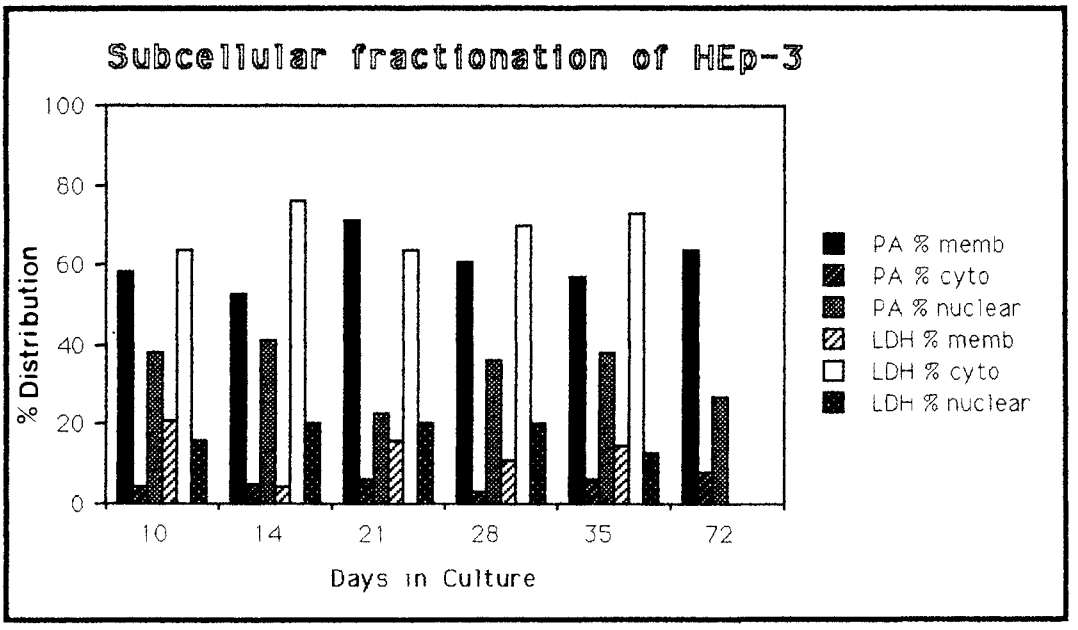

Fig. 7. Determination of membrane PA and cytoplasmic LDH activities to monitor the efficiency and reproducibility of HEp-3 subcellular fractionations. HEp-3 Rev cells were grown in culture and fractionated every 7–10 days for the first 35 days and again at 72 days in culture. At each time point, all subcellular fractions were analyzed for the level of human urokinase type PA and LDH activities. Each bar represents the level of activity as a percentage of the total enzymatic activity measured in three fractions; membrane, cytoplasmic, and nuclear.

(LDH) is a known cytoplasmic enzyme [Holbrook et al., 1975]. The cytoplasmic fractions isolated at each weekly interval were shown to contain 65–75% of the LDH activity (Fig. 7, white bars). It was also documented (Fig. 7) that the membrane fractions contained less than 20% of the total LDH activity, and the cytoplasmic fractions contained less than 10% of the total PA activity, indicating a relatively clean separation of these two subcellular fractions. Cells were also grown in the presence of ^{3}H-thymidine prior to fractionation to label DNA and serve as a nuclear marker. Greater than 95% of the TCA precipitable ^{3}H-thymidine counts were associated with the low-speed fraction, consistent with the predominance of intact nuclei in this fraction as visually observed (data not shown). The presence of 20–35% of the PA activity and 15–20% of the LDH activity in the low-speed nuclear fraction (Fig. 7) indicates that large membrane fragments and some unbroken whole cells (visually observed) were present in the nuclear fraction. The separate subcellular fractionations carried out at each 7- to 10-day interval showed consistent subcellular marker distribution (Fig. 7). These fractions were then examined by 2D-PAGE. Figure 8 shows the level of a particular protein (#2121) in each of the three subcellular fractions isolated from nonmetastatic HEp-3 cells (72-day culture). Protein #2121 appears to be primarily membrane associated, as it is highly enriched in the membrane fraction, absent from the cytoplasmic fraction and at low levels in the low speed nuclear fraction (Fig. 8). The level of protein #2121 was monitored in the membrane fractions isolated from HEp-3 cells grown continuously in culture during which time they lose their metastatic ability (Fig. 9). The most dramatic increase in abundance of protein #2121 was between 21 and 28 days of cell culture, as shown by the 2D gel analysis of the respective membrane fractions. This specific time period reflects the time frame during which the most dramatic decline in metastatic potential also takes place (see Fig. 1A).

A comparison relating the abundance of three other proteins between metastatic and nonmetastatic cells is shown in Figure 10 in the subcellular fraction with which they are associated. Membrane-associated protein #1274 (36 kD, pI 5.7) exhibits a pronounced increase in the nonmetastatic HEp-3 cells while a low abundance cytoplasmic protein #668 (65 kD, pI 6.2) also

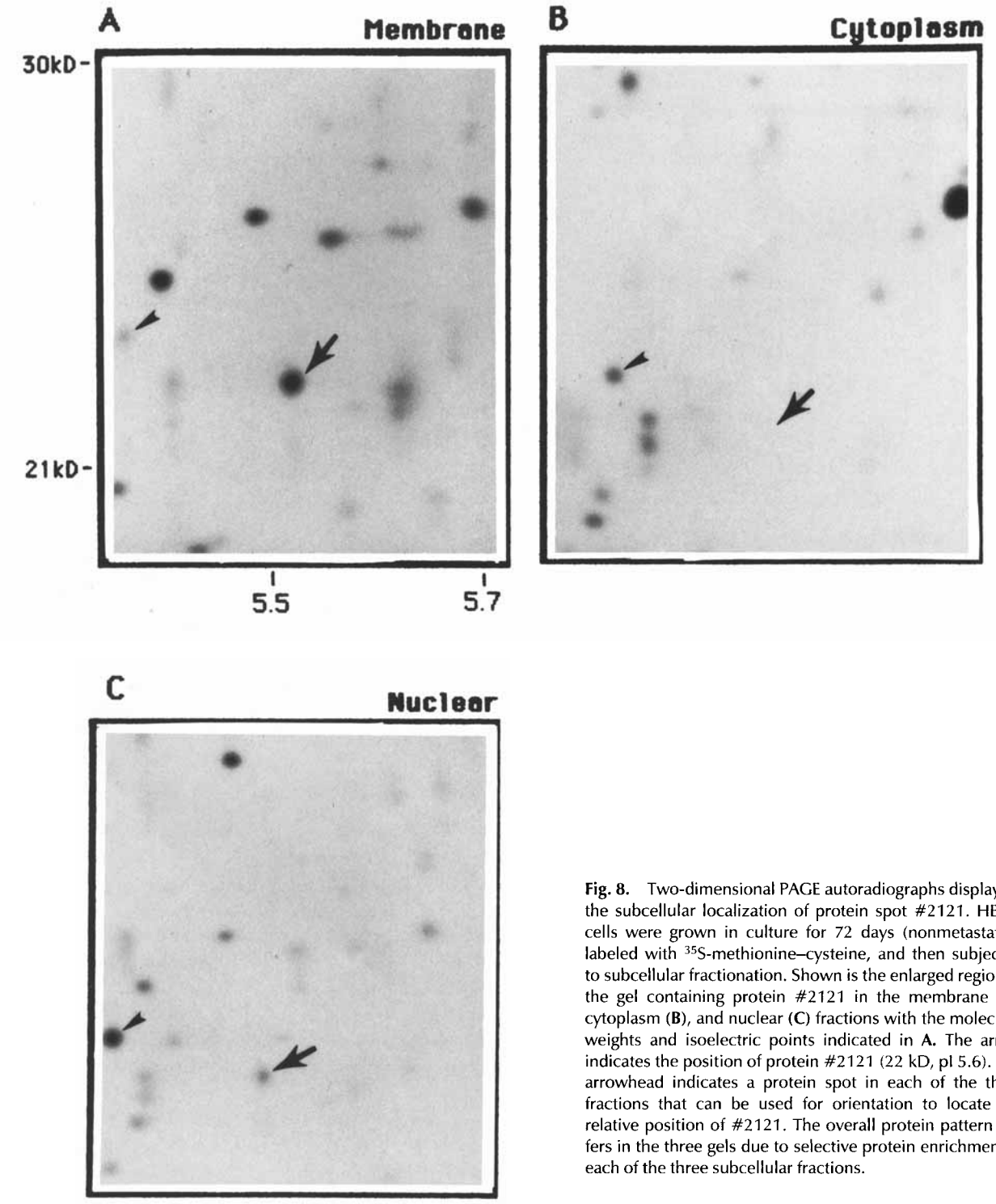

Fig. 8. Two-dimensional PAGE autoradiographs displaying the subcellular localization of protein spot #2121. HEp-3 cells were grown in culture for 72 days (nonmetastatic), labeled with ^{35}S-methionine–cysteine, and then subjected to subcellular fractionation. Shown is the enlarged region of the gel containing protein #2121 in the membrane (**A**), cytoplasm (**B**), and nuclear (**C**) fractions with the molecular weights and isoelectric points indicated in **A**. The arrow indicates the position of protein #2121 (22 kD, pI 5.6). The arrowhead indicates a protein spot in each of the three fractions that can be used for orientation to locate the relative position of #2121. The overall protein pattern differs in the three gels due to selective protein enrichment in each of the three subcellular fractions.

becomes manifested in the nonmetastatic cells from a virtual absence in the metastatic cells. A fourth protein, #1182, (32 kD, pI 5.8), found highly enriched in the nuclear fraction, exhibits a nearly 10-fold increase in the nonmetastatic HEp-3 cells. The combination of subcellular fractionation and 2D-PAGE thus allows for the detection of a limited number of proteins whose increased expression directly correlates with the loss of the metastatic phenotype.

A summary of the properties of four distinct proteins that have thus far been detected and

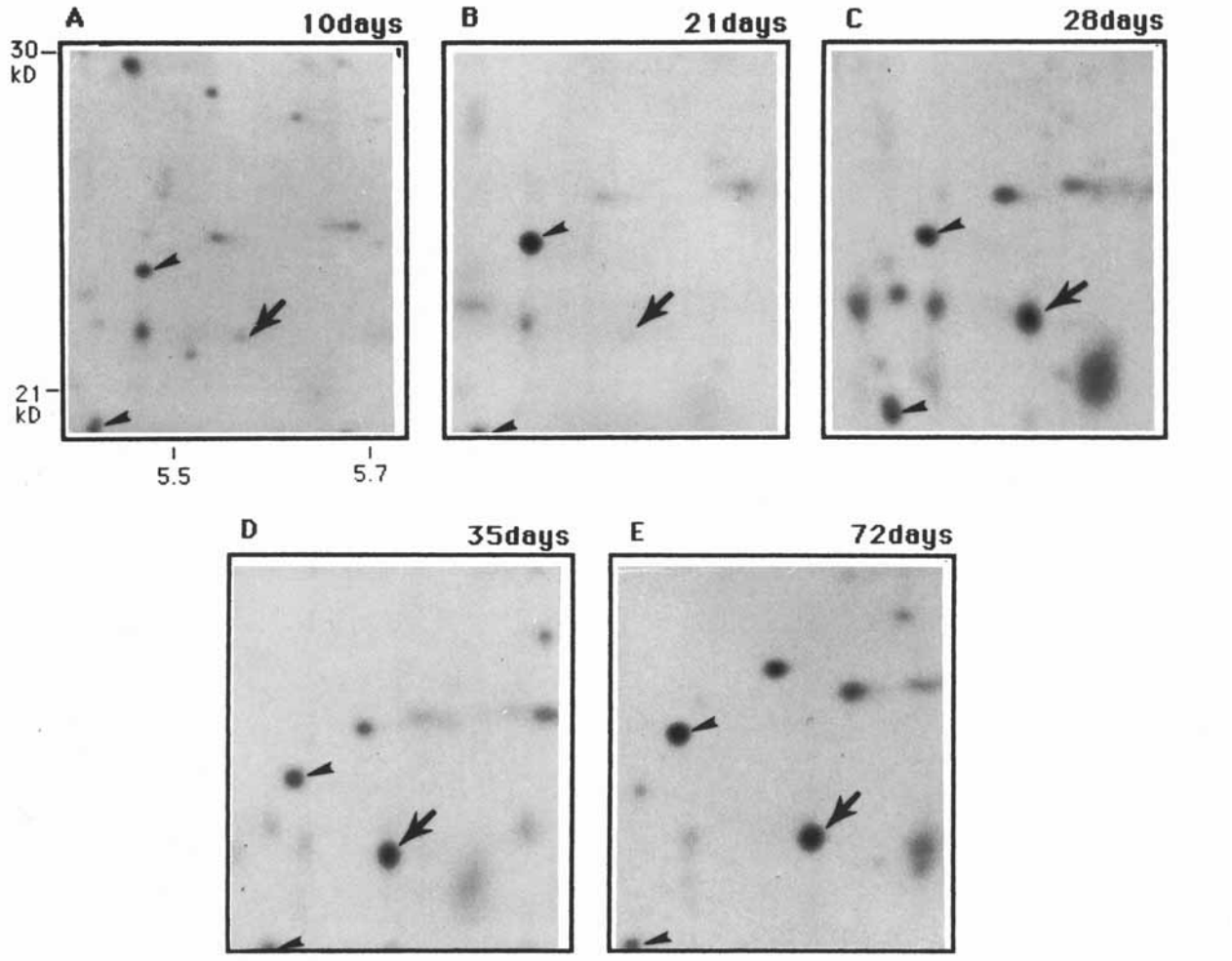

Fig. 9. Two-dimensional PAGE autoradiographs of HEp-3 membranes demonstrating the increase in abundance of protein #2121 with increasing time in culture of HEp-3 cells. HEp-3 cells were grown in culture continuously and at at 10(**A**), 21(**B**), 28(**C**), 35(**D**), and 72(**E**) days, the cultures were labeled with ^{35}S-methionine–cysteine and subjected to subcellular fractionation. The membrane fraction isolated at each time point was analyzed by two-dimensional PAGE. An enlarged view of the area of the gel containing protein #2121 (arrow) is shown with the molecular weights and isoelectric points indicated in **A.** The arrowhead indicates reference proteins used for orientation.

analyzed is shown in Table I. Three of the proteins fractionate into a distinct subcellular compartment, while one protein (#2121) is found in the membrane and nuclear fraction. All four proteins have an acidic isoelectric point and range in apparent molecular weight from 22 kD to 65 kD. All four proteins exhibit a greater than fivefold increase upon loss of the metastatic phenotype and one protein (#1274) shows a greater than 20-fold increase coincident with the change in phenotype. Their close correlation to the nonmetastatic phenotype is confirmed by their subsequent diminished abundance upon in vivo passage of HEp-3 when the reversion to the highly metastatic phenotype occurs.

DISCUSSION

The study of spontaneous metastasis requires a system that mandates that the cells perform the full range of events leading to metastatic lesions. These steps include the formation of a primary tumor, invasion from the primary site into surrounding tissue, intravasation and survival in the circulation, and finally arrest and growth at a secondary site [Hart and Saini, 1992; Liotta et al., 1991]. The HEp-3 system of metastasis in the chick embryo meets these requirements and has the advantage of being relatively rapid and quantitative. Although HEp-3 may be rather unique in its aggressive behavior

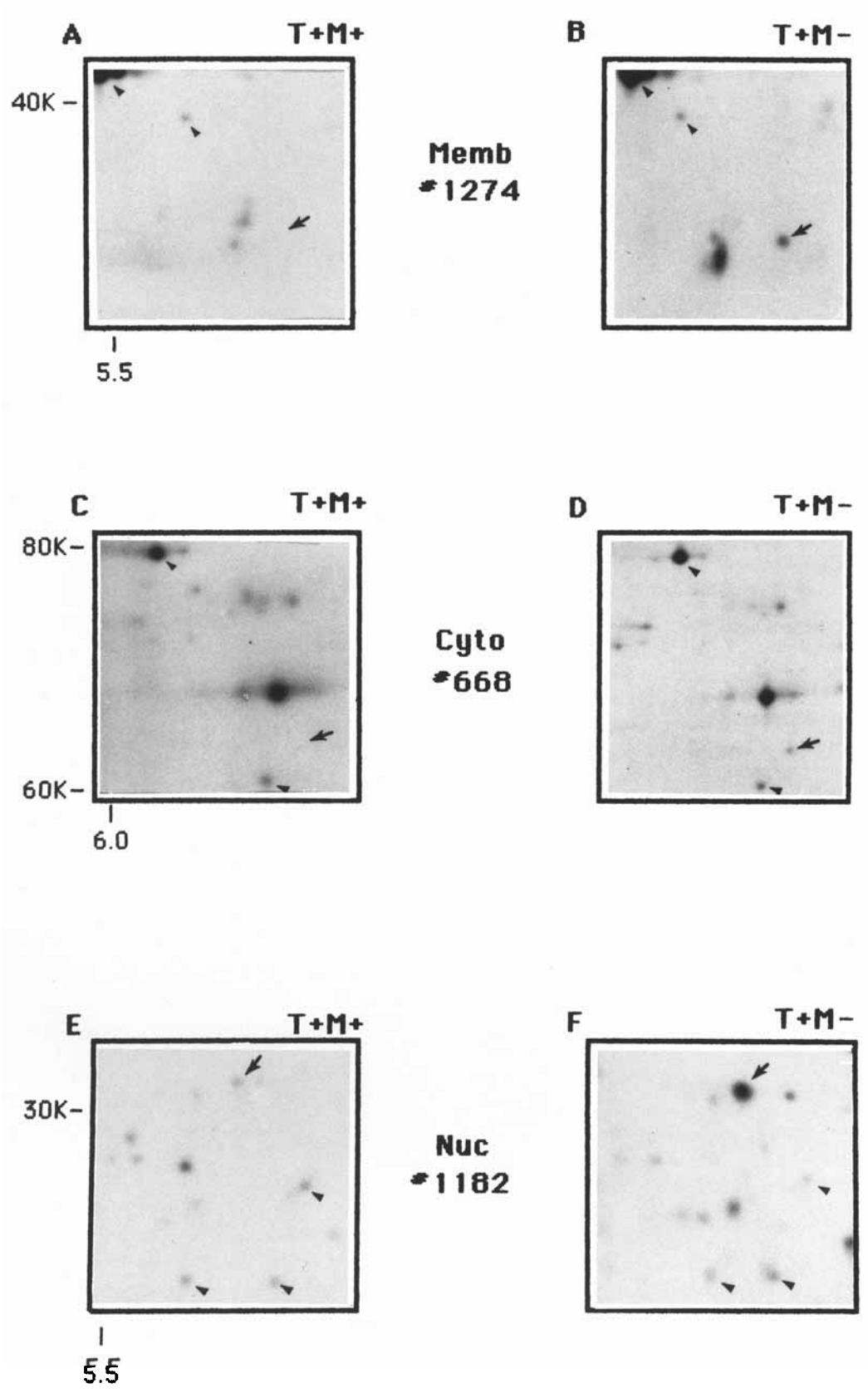
A
T+M+
40K –
5.5
Memb
#1274
B
T+M–
C
T+M+
80K–
60K–
6.0
Cyto
#668
D
T+M–
E
T+M+
30K–
5.5
Nuc
#1182
F
T+M–

Figure 10.

TABLE I. Summary of the Characteristics of Proteins Correlating With the Loss of the Metastatic Phenotype[a]

Protein spot number	Subcellular fraction	Molecular weight (kD)	Isoelectric point	Fold increase in protein abundance upon T^+M^+ to T^+M^-	Expression upon reversion of T^+M^- to T^+M^+
2121	Memb/Nuc	22	5.6	7	↓
1274	Membrane	36	5.7	23	↓
668	Cytoplasm	65	6.2	9	↓
1182	Nuclear	32	5.8	8	↓

[a]The subcellular fraction to which each protein has been localized is given as well as the molecular weights and isoelectric points. The increase in abundance of each protein from the metastatic to nonmetastatic phenotypes is given as a fold increase of parts per million (ppm) of each protein spot in nonmetastatic cell lysates divided by the parts per million value in metastatic cell lysates as determined by computer analysis of the radioactive intensity of the 2D gels (see Materials and Methods). All four proteins showed the indicated increase in abundance when T^+M^+ HEp-3 cells were carried in culture for 28 days. All four proteins also showed a diminished abundance (↓) when the cells reverted to the metastatic phenotype by two in vivo passages.

in the chick embryo, this behavior is also documented in the nude mouse [Ossowski et al., 1987] and other mammalian systems [Dagg et al., 1954]; therefore, it is not merely a function of the avian CAM environment.

The unique characteristics of the HEp-3 cell phenotype, i.e., progressive loss of metastatic ability in vitro with a rapid reversion to metastatic phenotype in vivo, allow for a precise correlation between the enhanced expression of specific cellular proteins within the time frame involved in the loss of the metastatic phenotype. Analysis of the unique ability of HEp-3 to revert from a metastatic to nonmetastatic phenotype is not meant to model a typical in vivo event, but it is an extremely useful phenomena with which to study differentially expressed molecules that may affect metastatic potential directly in an otherwise identical cell type. Since the loss of tumorigenicity occurs at a different rate than the loss of metastatic potential, it is possible to isolate distinct metastatic and nonmetastatic populations of cells, each being fully tumorigenic. This, in turn, permits the detection of specific proteins upregulated during the distinct progression from metastatic to nonmetastatic phenotype. These proteins may therefore be associated with metastatic suppression, distinct from tumorigenic suppression and may aid in the fundamental understanding of metastatic regulation. This specific analysis for molecules that concomitantly accompany the loss of metastasis, some of which may be negative regulators, will complement studies aimed at characterizing molecules, which may contribute to metastatic potential only by effecting the more broad phenotypes of transformation [Koi, et al., 1989; Lazaris-Karatzas et al., 1990] and tumorigenicity [Shuster et al., 1980; Trent et al., 1990].

Extensive analyses of proteins using high-resolution 2D-PAGE from HEp-3 cells during the progression from tumorigenic–metastatic to tumorigenic–nonmetastatic phenotype has enabled us to detect four distinct protein spots that are reproducibly and significantly upregulated (7- to 23-fold). The four proteins are clearly low abundance cellular proteins and were not detected in high-resolution single-dimension PAGE analysis (Fig. 4). Protein detection levels afforded by 2D-PAGE and the computer-assisted analyses of such gels allowed for quantitation of proteins that increase in abundance as HEp-3 cells progress to the nonmetastatic phenotype. These four proteins vary in subcellular compartmentalization, abundance, molecular weight, and isoelectric point. Because of their differences in subcellular localization, these four proteins may indicate multiple cellular mecha-

Fig. 10. Two-dimensional PAGE autoradiographs indicating the relative level of abundance of three proteins in metastatic (T^+M^+) and nonmetastatic (T^+M^-) HEp-3 cells. **A,B:** Arrows indicate the position of protein #1274 (36 kD, pI 5.7) in the membrane fraction. The arrowheads indicate a reference protein spot used for orientation and indicating equivalent loading. The protein constellation to the left of #1274 also seems to change in abundance with phenotype, but this finding was inconsistent and therefore not chosen for further study. **C,D:** Cytoplasmic fractions of HEp-3 cells reveal an increase in abundance of protein #668 (65kD, pI 6.2) in the nonmetastatic cells. **E,F:** Nuclear fractions of HEp-3 cells exhibit an increase in abundance of protein #1182 (32 kD, pI 5.8) in the nonmetastatic cells. A,C,E: From lysates of cells grown in culture or 10 days. D,F: From lysates of cells grown in culture for 35 days. B: From a lysate of cells grown in culture for 72 days. Each panel is an enlarged region of the total gel containing the proteins of interest. Molecular weights and isoelectric points are indicated in A, C, and E.

nisms involved in the control of the metastatic phenotype. Preparative isolation of the four proteins for microsequence analysis, cDNA cloning, and eventual identification is now ongoing.

A study of the actual protein levels in cells is in contrast to studies involving subtractive hybridization [Hendrick et al., 1984] and differential screening [Schalken et al., 1988], where enriched cDNAs representing differentially expressed mRNAs have been isolated. Although these techniques have been used to identify putative suppressor molecules [Dear et al., 1988, 1989; Lee et al., 1991], they are insensitive to any regulatory mechanisms that might take place post-transcriptionally. A major advantage of using 2D-PAGE to detect expressed gene products is that mechanisms which alter translation or post translational modifications can be detected, as well as transcriptional control mechanisms that give rise to altered protein levels.

An additional advantage of 2D-PAGE is that it can be used with little or no bias as to the type of molecules detected. Many survey studies linking gene expression with phenotype have been carried out to determine if highly specific gene products are involved in phenotype, examples of which are transfection of known oncogenes to alter malignant phenotype [Pohl et al., 1988; Pozzatti et al., 1987] or the use of antibodies to specific molecules to inhibit phenotype [Hendrick et al., 1984; Volmers and Birchmeier, 1983]. These studies are indeed informative but somewhat limited in that they focus on a specific gene or gene product to be analyzed for functional activity. In contrast, 2D-PAGE analysis as illustrated in this study allows an unbiased characterization of any differentially expressed proteins that may affect phenotype whether there is prior reason to suspect them. There has thus far been a very limited use of 2D-PAGE analysis to define the metastatic phenotype, and it should prove useful in identifying heretofore unknown proteins, or in characterizing new functions for known proteins. The HEp-3 malignant cell system with its inducible and fully reversible metastatic phenotype provides a unique system for initially detecting such proteins.

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