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Alterations in Cellular Gene Expression Without Changes in Nuclear Matrix Protein Content

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Abstract Cell metabolism and function are modulated in part by cell and nuclear shape. Nuclear shape is controlled by the nuclear matrix, the RNA-protein skeleton of the nucleus, and its interactions with cytoskeletal systems such as intermediate filaments and actin microfilaments. The nuclear matrix plays an important role in cell function and gene expression because active genes are bound to the nuclear matrix whereas inactive genes are not. It is unknown, however, how genes move on and off the matrix, and whether these events require compositional protein changes, i.e., alterations in protein content of the nuclear matrix, or other, more subtle alterations and/or modifications. The purpose of this investigation was to begin to determine how nuclear matrix protein composition is related to gene expression. We demonstrate that gene expression can change without apparent changes in the protein composition of the nuclear matrix in MCF10A breast epithelial cells. © 1994 Wiley-Liss, Inc.

Key words: MCF10A, nuclear matrix, gene expression, ras, actin

It has been previously demonstrated that cell metabolism, i.e., DNA, RNA, and protein synthesis, is modulated in part by cell and nuclear shape [Benecke et al., 1978; Folkman and Moscona, 1978; Maness and Walsh, 1982; Getzenberg et al., 1991a; Vincent et al., 1993]. Nuclear shape is controlled by the tissue matrix, the dynamic skeletal network of the cell that consists of linkages between the nuclear matrix, the cytoskeleton, and the extracellular matrix [Bissell et al., 1981, 1982; Getzenberg et al., 1990; Isaacs et al., 1981; Wittelsberger et al., 1981; Ben-Ze'ev et al., 1980]. Each part of the tissue matrix system contributes to the control of nuclear shape and function. The nuclear matrix contributes to DNA organization, gene function, and overall nuclear organization and control [Berezney and Coffey, 1974; Fey and Penman, 1988; Stuurman et al., 1990; Getzenberg et al., 1991b; Pienta et al., 1991a]. The interaction of nuclear structure and function with cytoskeletal systems, especially intermediate filaments and actin microfilaments, has been reported by several investigators [Fey et al., 1984; Ben-Ze'ev, 1985; Zambetti et al., 1991; Pienta and Coffey, 1992; Crossin and Carney, 1981]. The contacts a cell makes with its external environment have been shown to profoundly effect nuclear structure and function [Raz and Ben-Ze'ev, 1987; Bissell et al., 1981; Fujita et al., 1986; Gospodarowitz et al., 1978; Murphy et al., 1992; Pienta et al., 1989, 1991b].

We have previously demonstrated, in Normal Rat Kidney (NRK) cells, that nuclear shape is altered dramatically by disruption of the actin microfilament network, either through treatment with cytochalasin D or by cell transformation with the Kirsten-ras oncogene [Pienta and Coffey, 1992]. Furthermore, the Stein research group has demonstrated that disruption of the actin cytoskeleton alters gene expression [Zambetti et al., 1991]. It has been demonstrated that active genes are bound to the nuclear matrix while inactive genes are not [Ciejek et al., 1982; Cockerill, 1990]. It is unknown, however, how genes move on and off of the matrix and whether these events require compositional protein changes in the nuclear matrix or other, more subtle alterations and/or modifications. The purpose of this investigation was to begin to determine how nuclear matrix protein composition is related to gene expression. We treated immortalized MCF10A breast epithelial cells and their

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H-ras transformed counterparts MCF10AneoT with cytochalasin D and monitored nuclear shape, nuclear matrix protein composition, as well as select gene expression [Soule et al., 1990].

MATERIALS AND METHODS

Materials

Cytochalasin D was obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin/EDTA was obtained from Gibco BRL.

Cell Lines

The MCF-10 cell lines mortal (MCF10M), immortal (MCF10A), were derived and maintained as previously reported [Khanuja et al., 1993]. The MCF10 transfected cell line MCF10AneoT was obtained from Dr. Linda Watkins (Michigan Cancer Foundation, Detroit, MI, 48201).

Immunofluorescence

Cells were plated in double well chamber slides (Nunc, Inc., Naperville, IL) at a concentration of 1×10^4 cells per well. Cells were incubated at 37°C for 24 h. Cells were then treated with 10 μg/ml cytochalasin-D drug for 30 min to 1 h. Cells were then washed twice with phosphate buffered saline (PBS) and fixed in 3.75% formaldehyde/PBS for 10 min at room temperature. Cells were then washed twice with PBS and extracted with −20°C acetone for 5 min. The slides were allowed to air dry after which the cells were stained with DAPI and counterstained with rhodamine-phalloidin for 20 min, each at room temperature. Slides were then washed twice with PBS, mounted with 1:1 PBS/glycerol, and viewed with a Zeiss fluorescent microscope equipped with 10×, 40×, and 100× objectives (Zeiss Inc., Thornwood, NY).

Morphometric Assay

Cells were plated in double well chamber slides (Nunc, Inc., Naperville, IL) at a concentration of 1×10^4 cells per well. Cells were incubated at 37°C for 24 h, and then treated with 10 μg/ml cytochalasin-D for 1 h. After incubation, chambers were transferred to a 37°C heated microscope stage (Zeiss TRZ 2700, Thornwood, NY) for analysis. Images of control and treated cells were captured utilizing the Zeiss confocal image analysis system. One hundred cells and nuclei for each treatment group and controls were captured and the X-Y-Z three-dimensional volume coordinates of the nuclear boundaries were stored using the Zeiss software. Nuclear volume in cubic microns and perimeter in microns were measured for each nucleus. Cross-sectional images of nuclei were collected along the Z-axis in one micron steps. Perimeter and area of the area of largest cross-section as judged by the microscope operator were stored and recorded. Volumes were reconstructed in three dimensions and calculated utilizing the Zeiss Imaging Software.

Statistics

Statistical analysis was performed using Statgraphics v5.1 (Statistical Graphics Corp.). Statistical significance was determined using the Student's *t*-test.

Analysis of Nuclear Matrix Proteins

Nuclear matrix preparation. Nuclear matrices were prepared as previously described [Khanuja et al., 1993]; 5×10^7 control and cytochalsin-treated cells growing in culture were detached utilizing trypsin. Trypsin was neutralized by treating media with 10% fetal calf serum, spun at 800× for 10 min to produce a pellet, and then resuspended on ice with 0.5% Triton X-100 to release the lipids and soluble proteins in a buffered solution containing 2 mM vanadyl ribonucleoside, an RNAase inhibitor. Salt extraction with 0.25 M ammonium sulfate with vanadyl ribonucleoside is added to release the soluble cytoskeletal elements. DNAase-1 and RNAase-A at 25°C are used to remove the soluble chromatin and RNA. The remaining fraction contains the intermediate filaments and nuclear matrix proteins. This fraction is disassembled with 8 M urea, and the insoluble components, which consist principally of carbohydrates and extracellular matrix components, are pelleted. The urea is then dialyzed out, and the intermediate filaments then reassemble and are separated out by centrifugation. The soluble nuclear matrix proteins are then ethanol precipitated. All solutions contain freshly made 1 mM phenylmethylsulfonylfluoride (PMSF) to inhibit serine proteases. The protein composition is determined by resuspending the proteins in 0.1 N sodium hydroxide and using the BCA protein assay (Pierce, Rockford, IL), with BSA as a standard.

Electrophoresis. Two-dimensional electrophoresis was performed by Kendrick Labs, Inc. (Madison, WI) according to the method of O'Farrell as follows: Isoelectric focusing was

carried out in glass tubes of inner diameter 2.0 mm using 2.0% pH 4–8 ampholines (BDH from Hoefer Scientific Instruments, San Francisco, CA) for 9,600 v-h. Forty nanograms of an IEF internal standard, tropomyosin protein, M.W. 33,000, and pI 5.2 was added to the samples. This standard is indicated with an arrow on the 2D gel pattern. After equilibration for 10 min in buffer "O" (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M tris, pH 6.8), the tube gel was sealed to the top of a stacking gel which is on top of a 10% acrylamide slab gels (0.175 mm thick) and SDS slab gel electrophoresis carried out for about 4 h at 12.5 mA/gel. The slab gels were fixed in a solution of 10% acetic acid/50% methanol overnight. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as M. Wt. standards to the agarose which sealed the tube gel to the slab gel: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). These standards appear as horizontal lines on the silver stained 10% acrylamide slab gel. The gels were then dried between sheets of cellophane paper with the acid edge to the left. Only protein spots clearly and reproducible observed in all gels of a sample type are counted as actually representative of the nuclear matrix components. To ensure accuracy of observations, gels were analyzed on a Molecular Dynamics 300A Computing Densitometer.

Northern Blot Analysis

RNA was isolated from subconfluent cell cultures by the guanidinium isothiocyanate method, as previously described [Ausubel et al., 1989]. Ten micrograms each total RNA per cell line and one microgram lambda DNA digested with HindIII (as an approximate molecular weight marker) were electrophoresed through a 0.8% agarose/7% formaldehyde gel, stained with ethidium bromide to visualize the 28S and 16S rRNAs and lambda DNA, and then transferred to a membrane (Biotrans, ICN) by capillary action in 20× SSC (20× SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.0). The RNA was fixed to the membrane by UV crosslinking and dried under vacuum. Probes comprised a 2.4 kb ClaI fragment of EGFR plasmid pE7 (American Type Culture Collection, ATCC), a 3.8 kb HindIII fragment of KRAS plasmid pJ819 (ATCC), an 8.4 kb HindIII/EcoRI fragment of CMYC plasmid pHSR.1 (ATCC), a 0.5 kb HindIII fragment of GAPDH plasmid pHcGAP (ATCC), and a 2.0 kb BamHI fragment of βActin plasmid pHF-BCA46 (ATCC). The probes (25 ng each) were labeled utilizing the random primer method and α^{32}PdCTP (>3,000 Ci/mmole; NEN). The blot was hybridized 12–16 h in 50% formamide, 5× SSC, 250 μg/ml denatured salmon testes DNA and 1× buffer (250 mM Tris-HCl, pH 7.5; 0.5% w/v sodium pyrophosphate; 5% SDS; 1% polyvinylpyrrolidone; 1% ficoll, 25 mM EDTA, and 5% BSA) with probe at 50–55°C (probe-dependent), washed twice in 1× SSC, 0.1% sodium pyrophosphate and 0.1% SDS, and then twice in 0.2× SSC, 0.1% sodium pyrophosphate and 0.1% SDS for 30 min each at 60°C, and autoradiographed for 1 or 3 days with screens at −70°C. Between hybridizations, probe was washed from the blot in a solution consisting of 70% formamide, 10 mM sodium pyrophosphate, and 1.0% SDS for 2–3 h at 55°C, rinsed in 5× SSC, and checked by autoradiography to ensure complete removal of probe.

RESULTS

Normal cells and their transformed counterparts often display very different cell and nuclear morphologies. The NRK and KNRK cells, for example, demonstrate both different cell shapes as well as altered nuclear morphologies [Pienta and Coffey, 1992]. These alterations in nuclear shape are accompanied by alterations in nuclear matrix composition [Getzenberg et al., 1991b,c; Pienta et al., 1991b]. We investigated the three-dimensional cell and nuclear shape of MCF10A cells and their H-ras transformed counterparts, MCF10ANeoT cells, to determine the effect of transformation on these cells. As can be seen in Table I, H-ras transformation did not alter nuclear size and/or volume significantly. Figure 1 demonstrates that the MCF10A cells as well as the MCF10AneoT cells exhibit similar morphology, although the cytoskeletal actin networks of the ras transformed cells appears to be more disorganized with less stress fibers. We investigated nuclear matrix protein composition of MCF10A cells and their transformed counterparts and could detect no significant differences between them by two-dimensional gel electrophoresis (Fig. 2), confirming our earlier published results [Khanuja et al., 1993].

Utilizing Northern blot analysis, we monitored the expression of the genes c-myc, EGFR, H-ras, beta actin, and GAPDH. Transfection of the H-ras oncogene appeared to cause an increase in myc and ras expression as well as

TABLE I. Nuclear Volume, Perimeter, and Area of Cytochalasin-D Treated and Control MCF10A and MCF10A-Ras Transfected Cells

Nuclear parameter	10A (untreated)	10A (treated)	10A-Ras (untreated)	10A-Ras (treated)
Volume[a]	1,777 ± 715	1,253 ± 575	1,715 ± 851	1,753 ± 756
Area[b]	170 ± 51	163 ± 4	174 ± 45	174 ± 47
Perimeter[c]	51 ± 7	49 ± 6	53 ± 7	52 ± 7

[a]Nuclear volume in cubic microns ± standard deviation.
[b]Nuclear area of largest nuclear cross-section in μm^2 ± standard deviation.
[c]Nuclear perimeter of largest nuclear cross-section in μm ± standard deviation.

smaller increases in EGFR and actin expression (lanes 1A and 2A, Fig. 3; Table II).

Cells were then treated with cytochalasin-D to disrupt their actin microfilament networks. As can be seen from Table I, microfilament disruption of the MCF10A and MCF10aneoT cells did not affect nuclear shape and/or volume in a significant manner. Nuclear matrix protein composition did not change by two-dimensional electrophoretic analysis (Fig. 2). Gene expression, however, did change. In the 10A cells, myc and ras expression increased and EGFR expression was lost. In the MCF10AneoT cells, the most significant change was the loss of EGFR expression.

DISCUSSION

The data presented here raises several provocative points about the control of nuclear shape, the nuclear matrix, and gene expression. We had previously implicated actin microfilaments as a key component in the control of nuclear shape and function, linking the nucleus to the cell periphery in nontransformed cells [Pienta and Coffey, 1992]. The data collected on the MCF10A cells would suggest that this may not be a universal phenomenon. The MCF10A cells exhibit clear actin stress fibers and yet nuclear shape does not change when these fibers are disrupted by cytochalasin treatment. This is opposite of what was previously demonstrated in the NRK cells. In the NRK system, transformation with Kirsten-ras (KNRK cells) resulted in disruption of the actin microfilament system and dramatic changes in nuclear shape. In the MCF10A system presented here, transformation with H-ras cell disrupted the actin microfilament system but did not alter nuclear shape (Table I, Fig. 1). It appears that the nucleus is connected to the cell periphery in different ways which may be dependent on cell type.

The nuclear matrix protein composition, as investigated by two-dimensional electrophoresis, was not altered, either by cell transformation with H-ras or by treatment with cytochalasin (Fig. 2). The protein composition of the nuclear matrix has been demonstrated to be altered by cell transformation in several systems [Fey and Penman, 1981; Getzenberg et al., 1991b; Khanuja et al., 1993]. We have previously demonstrated that the MCF10A cells exhibit a nuclear matrix protein composition pattern that is intermediate between normal breast epithelial cells and breast cancer cells [Khanuja et al., 1993]. Both normal breast tissue as well as tissue obtained from human breast adenocarcinoma contain unique nuclear matrix proteins. MCF10A nuclear matrices contain the proteins that are found in normal breast as well as cancerous breast tissue. These data suggest that nuclear matrix alterations in the acquisition of the cancer phenotype may, at times, be a relatively early event in the transformation pathway.

The stability of nuclear matrix protein composition in MCF10A system, despite alterations in gene expression, may reflect the stability of nuclear shape throughout this experiment. It is generally believed that the nuclear matrix changes with nuclear shape, but it is unclear if this requires protein composition changes or simply modifications in existing proteins within the nuclear matrix. This study does not answer this question and the issue certainly warrants further study.

It has been well recognized that active genes are bound to the nuclear matrix by matrix-associated regions (MARs) and inactive genes are not [Ward and Coffey, 1990]. It remains unclear, however, how gene expression relates to the protein composition of the nuclear matrix. This investigation gives two insights into this question. First, transfection with ras, as well as treatment with cytochalasin, altered the expression of both c-myc and H-ras in the MCF10A cells. These genes, however, were already "on." These data suggest that overexpres-

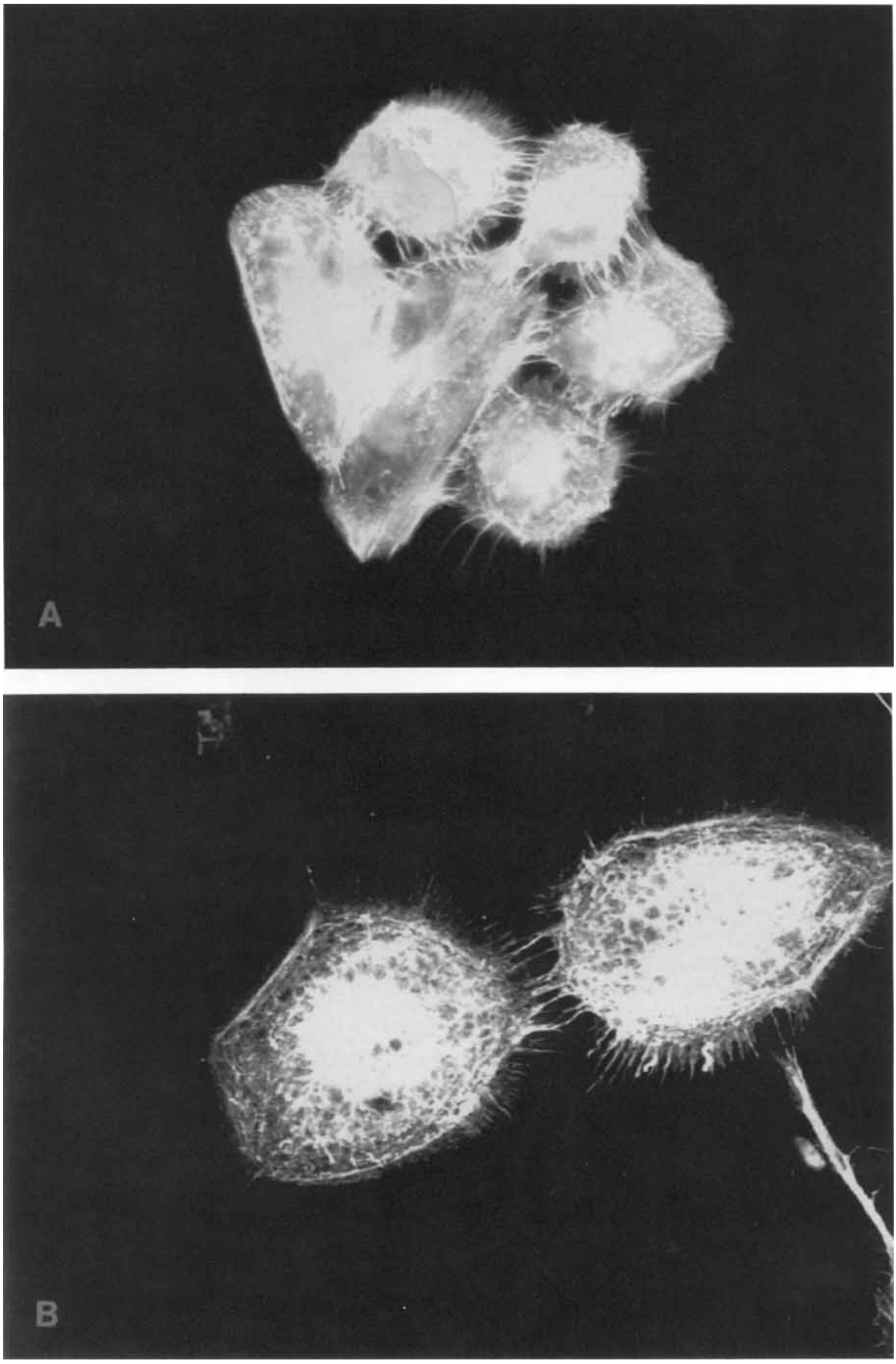

Figure 1

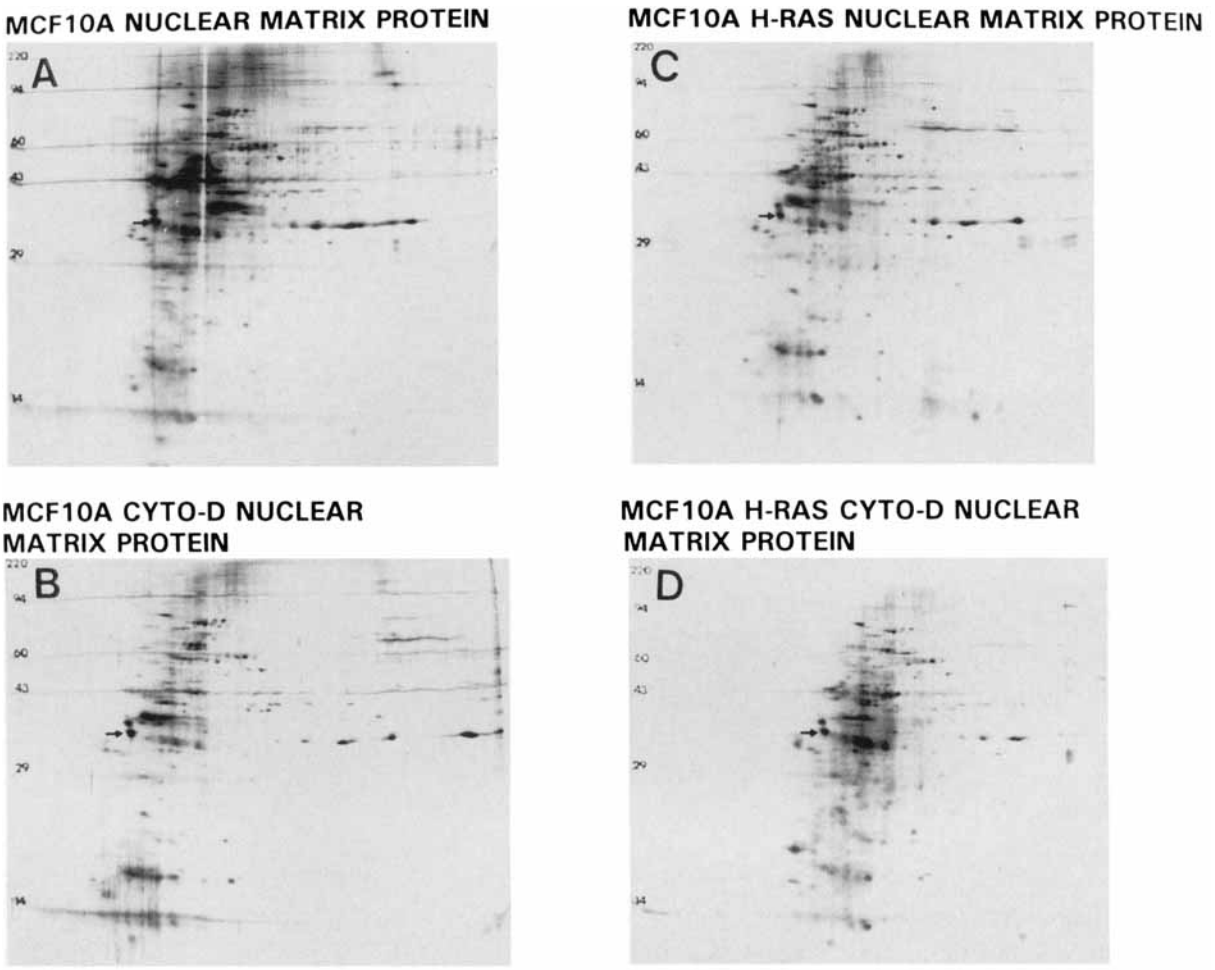

Fig. 2. Two-dimensional gel electrophoresis of nuclear matrix proteins. **A:** MCF10A cells, untreated. **B:** MCF10A cells treated with cytochalasin-D. **C:** MCF10AneoT cells (MCF10A cells transfected with H-Ras), untreated. **D:** MCF10AneoT cells (transfected with H-Ras), treated with cytochalasin-D.

sion of a gene does not require a protein compositional change in the nuclear matrix (as can be measured by two-dimensional protein gel electrophoresis). This is not unexpected since alterations in expression of a gene already "on" are thought to be the result of other cis- and trans-acting factors [Getzenberg et al., 1990; Pienta et al., 1993]. It is interesting to note, however, that EGFR expression was turned off (as opposed to down- or up-regulated) by cytochalasin treatment in both the MCF10A and MCF10AneoT cells (Fig. 2, Table II, Lane B1 and B2). Although we did not confirm that this gene had moved off the matrix by Southern analysis, this would suggest that genes can move on and off of the matrix without changing the protein composition of the nuclear matrix [Ward and Coffey,

Fig. 1. Morphology of immortalized MCF10A cells and their H-ras transformed counterparts MCF10AneoT stained with rhodamine phalloidin. **A:** MCF10A, ×63 objective, 1.4 zoom. **B:** MCF10AneoT, ×63 objective, 1.9 zoom.

TABLE II. mRNA Expression by Northern Analysis in Cytochalasin-D Treated and Control MCF10A and MCF10A-Ras Transfected Cells (MCF10AneoT)*

Onco-gene	10A (untreated)	10A (treated)	10A-Ras (untreated)	10A-Ras (treated)
myc	+	+++	+++	++
egf-r	+	−	++	−
ras	−	++	+++	++
b-actin	++	+	+++	++
rRNA	+	±	++	++
GAPDH	++	+	++	++

*See Figure 3 for actual Northerns.

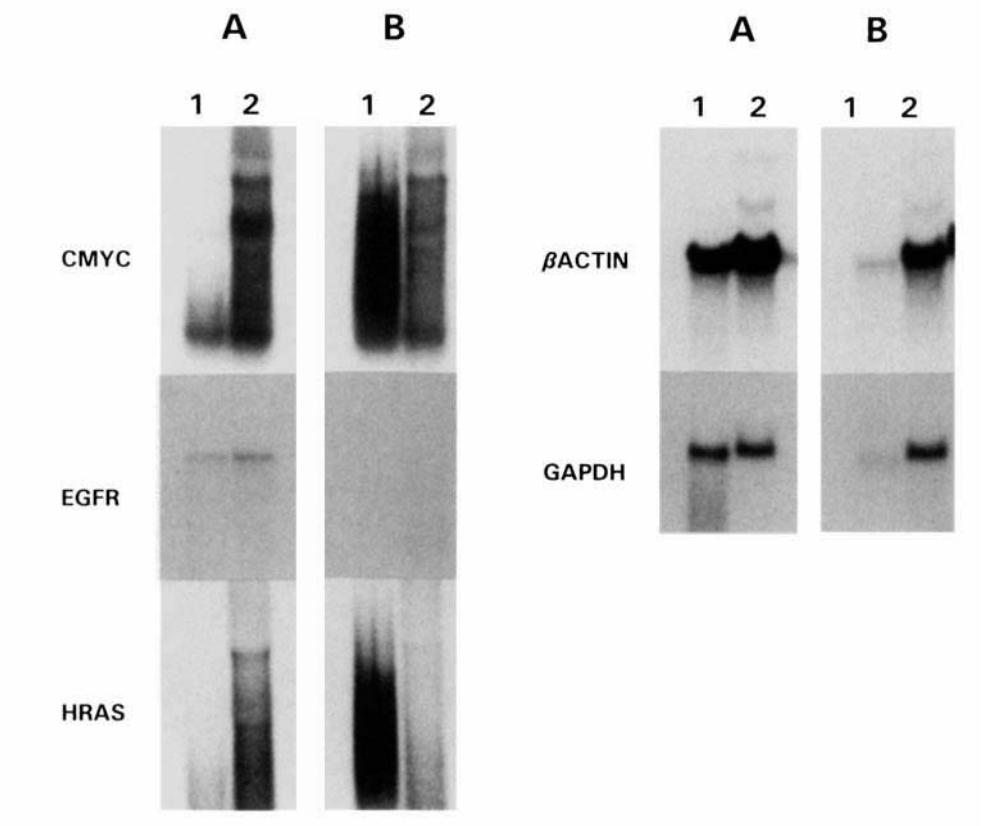

Fig. 3. Northern blots of MCF10A and MCF10AneoT cell lines. Lane 1, MCF10A. Lane 2, MCF10AneoT. Part A represents untreated cells. Part B represents cytochalasin treated cells.

1990]. The mechanism by which genes move on and off the matrix needs to be explored. An alternative explanation of these findings would be that cytochalasin treatment altered the stability of the association of the gene product mRNA with the cytoskeleton. The stability and importance of the association of mRNA with the cytoskeleton cannot be over emphasized and deserves further study.

Finally, this investigation further confirms the intimate relationship between ras and actin. It is worth noting that both transfection of the MCF10A cells with H-ras and treatment with cytochalasin-D of the MCF10A cells resulted in similar overexpression of H-ras and c-myc. It has been demonstrated that actin stress fibers appear to be regulated in part by ras through the GTP-binding protein rho [Ridley and Hall, 1992; Yang and Watson, 1993]. Furthermore, the actin-binding protein profilin appears to contribute to a signaling pathway that is regulated by ras [Goldschmidt-Clermont and Janmey, 1991]. The data presented adds to the growing body of evidence that cell signaling pathways and cell structure are intimately related. Further characterization of the relationship between ras and actin may lead to a better understanding of the events which contribute to cell regulation and transformation.

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