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QUANTITATIVE CHANGES IN CYTOSKELETAL AND NUCLEAR ACTINS DURING CELLULAR TRANSFORMATION

Jian Yu RAO[1,7], Rebecca B. BONNER[1], Robert E. HURST[1,4,5], Yuan Yuan LIANG[1], Catherine A. REZNIKOFF[6] and George P. HEMSTREET III[1,2,3,5]*

[1]*Department of Urology, University of Oklahoma Health Sciences Center, Oklahoma City, OK*
[2]*Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK*
[3]*Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK*
[4]*Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK*
[5]*Department of Occupational and Environmental Health, University of Oklahoma Health Sciences Center, Oklahoma City, OK*
[6]*Department of Human Oncology, University of Wisconsin, Madison, WI*

Actin, a highly conserved protein comprising cell stress fibers and other cellular structures, is found in both the cytoplasm and nucleus of cells and responds to both epigenetic signals and altered gene expression occurring during tumorigenesis. We have previously shown that changes in the cytoplasmic F- and G-actin ratios reflect bladder cancer risk. To determine whether nuclear actin is also altered and how nuclear and cytoplasmic actin alterations are interrelated in transformation, an *in vitro* model of carcinogen-induced transformation consisting of 2 human uroepithelial cell lines immortalized by infection with SV-40 was studied. One line, HUC-PC, is tumorigenic in nude mice after incubation with the carcinogen 4-ABP, the other, HUC-BC, is not. Cytoplasmic and nuclear F- and G-actin were determined by QFIA on individual cells using fluorochrome-labeled phallicidin and DNase, I, respectively. Before exposure to 4-ABP, the PC cells had lower cytoplasmic F-actin content, higher cytoplasmic G-actin content, but similar levels of nuclear G- and F-actin in comparison to the BC cells. After incubation with 4-ABP, F-actin decreased and G-actin increased in both cytoplasm and nuclei of PC cells and cytoplasmic F-actin fibers were lost, but only cytoplasmic actin was altered in the BC cells. Northern blot analysis showed the expression of the β-actin gene was only approximately 20% lower in 4-ABP–treated PC cells than in untreated controls, indicating the cellular change in actin was attributed to a shift between F- and G-actin proteins rather than to net actin synthesis. *Int. J. Cancer,* 70:423–429, 1997.
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Carcinogenesis is a multi-stage process resulting in the eventual emergence of a malignant phenotype that often continues to progress to an eventually fatal metastatic phenotype. Although the genetic elements of carcinogenesis, the oncogenes and tumor suppressor genes, have been considered the central theme and are relatively well understood (Bishop, 1987; Weinberg, 1991), it is clear that cancer is a more complex process also involving epigenetic interactions among cells and their environment. Rubin (1992) described reversible transformation, including stable genetic heterogeneity of the cell population, simply by manipulating cell culture growth conditions. Pienta *et al.* (1989) have proposed that DNA organization in nuclei is an important epigenetic factor in cancer development, whereas others have observed that cell-cell interactions such as gap junctions and adherence are also involved (Trosko *et al.,* 1990). Other experiments studying the relationship between basement membrane and type IV procollagen mRNA expression support a direct interaction among extracellular matrix/cytoskeleton/nuclear matrix (Schlussel *et al.,* 1991). Other studies show a connection between cytoskeletal actin, the nucleus, and the cell periphery that is altered after transformation (Pienta and Coffey, 1992). Mutation of the β-actin gene has been shown to alter the metastatic phenotype (Leavitt, 1994). A number of genes are also known to regulate the multimolecular focal complexes associated with actin stress fibers and cell cycle progression through G_1 and DNA synthesis (Nobes and Hall, 1995; Olson *et al.,* 1995). One candidate for being the crucial integrative factor is the actin-containing nuclear matrix.

Qualitative alterations of cytoplasmic actin, the major cellular protein involved in cell-cell interaction, morphology, motility, differentiation and adhesion, have been widely investigated in cancer cells in response to interactions between cells and their environment (Rao and Cohen, 1991; Pollard and Cooper, 1986; Wessells *et al.,* 1971; Shyy *et al.,* 1989; Koyasu *et al.,* 1988; Lombardi *et al.,* 1990). We have previously extended these descriptive findings by identifying a quantitative relationship between cytoplasmic F- and G-actin, the polymeric filamentous and monomeric forms respectively, reflecting transformation and differentiation in bladder cells (Rao *et al.,* 1990; 1991; 1993). These changes can be used as markers to detect field changes occurring during cancer development and, hence, as markers to express individual cancer risk (Rao *et al.,* 1991; 1993).

Nuclear actin has been identified as an important nuclear protein involved in DNA cross-linking (Miller *et al.,* 1991), transcription control (Scheer *et al.,* 1984; Ankenbauer *et al.,* 1989), and chromosome morphology (Lachapelle and Aldrich, 1988; Kolber *et al.,* 1990). In neuronal cells, F-actin was found in association with nucleoli and most was in the G-actin form (Milankov and De Boni, 1993; Amankwah and De Boni, 1994). Actin redistributions during frog oocyte development suggest F-actin is associated with changes in the distribution of nuclear components (Parfenov *et al.,* 1995). Since most of these nuclear functions are altered in cancer cells, and because profound changes in nuclear structure and function are seen in transformed cells, it appears plausible that a quantitative alteration of nuclear actin might also occur. Understanding how alterations of actin in the cytoplasm and the nucleus in transformation occur may provide a unified mechanism that will enhance our understanding of the relationship between genetic and epigenetic mechanisms of carcinogenesis.

The objective of the current study was to investigate quantitative changes in both nuclear and cytoplasmic G- and F-actin using an established *in vitro* cellular transformation model (Loretz and Reznikoff, 1988) in relation to morphologic rearrangements of actin filaments. The molecular structure and characteristics of cytoplasmic and nuclear actin are similar but not identical (Bremer *et al.,* 1981; Milankov and De Boni, 1993); nevertheless, they can

*Correspondence to: Department of Urology, University of Oklahoma Health Sciences Center, 920 Stanton L. Young Boulevard, Oklahoma City, OK 73190, USA. Fax: 405-271-3118.

[7]Present address: Department of Pathology and Laboratory Medicine, University of California Center for Health Sciences, and Jonsson Comprehensive Cancer Center, Los Angeles, CA, 90024-1732, USA.

Abbreviations: QFIA, quantitative fluorescence image analysis; PF, paraformaldehyde; TCC, transitional cell carcinoma; 4-ABP, 4-aminobiphenyl; AGL, average grey level of fluorescence.

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still be quantified by similar molecular probes (Lachapelle and Aldrich, 1988; Milankov and De Boni, 1993). Human uroepithelial cell lines, HUC-BC and HUC-PC cells, immortalized by infection with SV-40 virus, were used as the *in-vitro* transformation model. The BC and PC lines have similar morphologic and biochemical features; however, the PC line can be induced to become tumorigenic by treatment with 4-ABP (Bookland *et al.,* 1992*b*) or transfection with mutant *ras* oncogene (Pratt *et al.,* 1992). The BC line, which was derived from the PC line (Bookland *et al.,* 1992*b*), does not become tumorigenic under these conditions and therefore serves as a control to identify effects in the HUC-PC cells that are specific for transformation. F- and G-actin contents were quantified by fluorescence image analysis (Rao *et al.,* 1993). Parallel biochemical actin analyses performed on the cell lysates both to demonstrate that fluorescent probe binding methods are accurate and to calibrate the QFIA methods in absolute actin content. The results of actin alterations observed by these 2 methods were further examined to determine the extent to which changes in actin content are controlled at the gene level using Northern analysis of expression of the β-actin gene. Our results confirmed the hypothesis that nuclear actin is altered in response to 4-ABP exposure. Because only the PC cells—the cell line with constitutively decreased cytoplasmic actin—can be transformed by exposure to 4-ABP and because only the 4-ABP–treated PC cells showed quantitative alterations of nuclear actin, we hypothesize that a pathway from extracellular matrix/cytoplasmic actin/nuclear actin is important in maintaining the homeostasis of a cell while alterations of the pathway are related to specific events in cellular transformation.

MATERIAL AND METHODS

Material

Texas red-conjugated DNAse I and Bodipy-phallacidin were obtained from Molecular Probes, Eugene, OR. Hoechst 33258 was obtained from Polysciences, Warrington, PA, and used to prepare a 2.0 mM stock solution in deionized, distilled water, pH 7.4. Paraformaldehyde, DMSO, and 4-ABP were obtained from Sigma (St. Louis, MO). QFIA fixative consisted of buffered 50% ethanol (Rao *et al.,* 1993).

Cell culture

The detailed procedures for cell culture were described previously (Rao *et al.,* 1990). In brief, promyelocytic leukemia cells (HL-60, ATCC 240), used as a reference for G- and F-actin quantification, were obtained from the ATCC (Rockville, MD); HL-60 cells were seeded at 1 to 2×10^5/ml in 25 cm^2 plastic culture flasks containing supplemented with 20% heat-inactivated fetal bovine serum and maintained in exponential growth by subculturing every 4 to 6 days. HUC-PC and HUC-BC cells were seeded at 1×10^6 viable cells grown in 100-mm plastic dishes (Corning, Corning, NY) in Ham's F-12 medium (GIBCO, Grand Island, NY) supplemented fetal bovine serum with 1% FBS (Sigma). Cell cultures were maintained in humidified incubators at 37°C in 5% CO_2 and 95% air.

Exposure to 4-ABP and cell collection

4-ABP was dissolved in DMSO to a stock concentration of 40 mM. BC or PC cells were seeded at 1×10^6 viable cells exposed to medium either containing DMSO (0.5% v/v) alone as a control or with 200 μM 4-ABP. After incubation for 24 hr, the medium was replaced with regular growth medium without DMSO or 4-ABP. Cells were passaged to a new dish every week for 4 weeks. At the 5th week, cells were collected by scraping, counted using a hemocytometer, and their viability was determined by Trypan blue exclusion.

Quantitative fluorescence analysis of cytoplasmic and nuclear actin

Cultured cells were collected and washed with PBS using micro-centrifugation, fixed with 1% formaldehyde for 15 min, and followed with either QFIA fixative for 2 hr (optional) or acetone for 3 to 5 min. Cells were washed again, pelleted, and incubated with a cocktail consisting of Texas red-conjugated DNase I (1:40 v/v) and Bodipy-phallacidin (1:25 dilution) for 30 min. The DNA was labeled with Hoechst 33258 (10 μM) to provide a nuclear signal to identify cells.

To avoid contamination of nuclear actin by cytosolic actin, cell nuclei were isolated, permeabilized, and labeled with the appropriate fluorescent probes for nuclear G- and F-actin. This assay enables simultaneous quantitation and imaging of nuclear G- and F-actin in the isolated nuclei. Single cell suspensions were prepared and the nuclei were isolated using an isotonic colloid solution, based on a modified protocol of Boyle and Baluda (1987) that contains 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM NaH_2PO_4, 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% Triton X-100, and 15% glycerin. To preserve the nuclear actin, this isotonic solution (rather than the commonly used hypotonic buffer) was used to isolate the nuclei. The nuclei were collected after centrifugation at 1000 g for 10 min at 4°C and washed 3 times with PBS. Nuclear F- and G-actin were labeled with the DNase I/phallacidin cocktail for 30 min, and then 10 μM Hoechst 33258 in 25% v/v ethanol was added for 2 min for labeling DNA. The labeled nuclei were then transferred to polylysine-coated Code-On slides and labeled for quantitative fluorescence measurements.

The detailed procedure for image analysis and marker quantification can be found elsewhere and was used with minor modifications (Rao *et al.,* 1993). Samples were fixed and triple labeled, as described above. Fluorescent intensities of G- and F-actin were quantified simultaneously using the IBAS image analysis system (Roche Image Analysis Systems, Elon College, NC) configured with a Zeiss Axiotron (New York, NY) fluorescent microscope. This instrument is equipped with a motorized, computer-controlled, 8-slide position stage, excitation/emission filters (Omega Optical quantitative image analysis quality, Brattleboro, VT), and autofocus. The software developed by us for this application is fully automated so that all cells are randomly selected by the computer, thereby removing any operator bias. Cells were identified by the presence of a nucleus that fluoresced with Hoechst-labeled DNA (excitation at 360 ± 50 nm, 400 nm DCLP, and 480 ± 50 nm emission filter) thresholded to detect nuclei that provided identification of all cells regardless of actin expression. Images of the same field at computer-controlled excitations of 485 ± 22 for Bodipy-labeled F-actin (505 DRLP, 530 ± 30 nm emission filter) and 560 ± 40 nm for Texas red-labeled G-actin (595 nm DRLP, 630 ± 23 nm emission filter) were captured using a SIT camera (Hamamatsu, NJ) and marked with the nuclear image. Measurements were made of the remaining objects including grey mean (average grey value of all pixels within the nucleus) and area calibrated in micrometers.

Conversion of fluorescence intensity into absolute actin content

The mean actin content per cell determined by fluorescence is directly comparable to the mean content per cell determined by biochemical analysis (see below), provided labeling is quantitative, *i.e.,* the fluorescence intensities of cells in a population are directly proportional to their content of the target biomolecule (Jones *et al.,* 1990; Hemstreet, III *et al.,* 1992). The proportionality constant is determined by simultaneously measuring the mean fluorescence intensity of some cell line chosen as an arbitrary standard and the biochemical content of biomolecule in an aliquot of the same cells as was used in the fluorescence measurement. In this case, HL-60 cells were chosen as the standard. The quantitative fluorescence method allows for the use of small numbers of cells and for investigations of cellular heterogeneity and the content of individual cells. The AGL is the average grey level of the pixels comprising an image and is proportional to the average concentration of the target molecule within a cell. Let $A_{HL\text{-}60}$ represent the mean content of actin (G or F as appropriate) in units of $\mu g/10^6$ cells in the HL-60 line as determined by biochemical assay, A_s represents the mean content of actin of the sample in the same units, and AGL_s and $AGL_{HL\text{-}60}$ represent the average grey levels of 500 cells

of the sample and HL-60 lines, respectively, then A_s is given by:

$$A_s = \frac{AGL_s}{AGL_{HL\text{-}60}} \times A_{HL\text{-}60}$$

A preliminary study was performed to measure the nuclear and cytoplasmic actin content of patient-derived samples to determine if the observed alterations in nuclear actin were observed naturally or were seen only in cultured cells. The nuclear actin was measured by creating an image mask using the nuclear signal identified by the blue Hoechst 33258 staining and then applying the mask for each cell to measuring the corresponding nuclear actin content. Normal uroepithelial cells were obtained from 4 normal urine specimens. Transitional carcinoma cells were obtained by swirling papillary tumors removed during transurethral resection in normal saline and then fixing the preparation as described above for cultured cells.

DNase I biochemical assay for actin content

To assure that the fluorescence methods measured F- and G-actin accurately, aliquots of cells were also independently analyzed by biochemical analysis as described by Blikstad *et al.* (1978). For clarity, the details of what is measured are described. A counted aliquot of cells was lysed, centrifuged, and the supernatant was divided into 2 aliquots. One aliquot was used to measure soluble G-actin directly from its inhibition of DNase I. In the second aliquot, F-actin was depolymerized into its constituent G-actin monomers and the DNase assay was repeated to quantify the total soluble actin. F-actin does not bind to DNase I and is measured indirectly from the difference between soluble G-actin and total soluble actin. The pellet, which consists of ''core actin'' (mainly nuclear actin), was depolymerized, and the G-actin content was measured by the DNase I assay. To measure separately the nuclear actin content, the above assay was also performed on isolated nuclei.

Northern blot analysis of β-actin gene expression

Northern blot analyses were used to compare the mRNA level of the β-actin gene in cells that were treated with 4-ABP and those that were not. Oligonucleotide probes for β-actin gene were obtained from Oncogene Science (Uniondale, NY). Total cellular RNA was extracted by the guanidinium thiocyanate-cesium chloride method, quantified by absorbance at 260 nm, and 10-μg samples were electrophoresed on a 1.2% agarose-formaldeyde gel. After staining with ethidium bromide, gels were visualized under ultraviolet irradiation to determine the positions of the 28S and 18S rRNA bands and to assess the integrity of the RNA before transfer to nitrocellulose. Filters were hybridized with probes radiolabeled with $\gamma^{32}P$ to high specific activity (1×10^9 cpm/ng) by nick translation of each tested gene fragment. Hybridizations were performed for 20 hr at 68°C in 4 × SSPE containing 0.2% SDS, 0.1% sodium pyrophosphate, and 5 μg/ml heparin. After washing for 1 hr each in 1 × SSPE + 0.1% SDS and 0.5 × SSPE + 0.1% SDS at 68°C, the filters were exposed at −70°C for 24 to 72 hr to films with intensifying screen. The mRNA level was estimated based on the band intensity assessed by densitometer reading.

Statistical analysis

Four independent experiments were performed for each experimental condition. The mean and standard deviation were calculated for each condition, and the Student *t*-test was used to test the difference between 2 conditions.

RESULTS

Determination of cytosolic G- and F-actin using quantitative fluorescence

Although HUC-PC and HUC-BC had different transformation potentials, they are reported to share similar morphologic and growth patterns as shown by phase-contrast microscopy (Bookland *et al.*, 1992*b*). However, when the cells were labeled with fluorescence probes for F- and G-actin (Bodipy-phallacidin and Texas red-DNase I, respectively), slightly different patterns were observed in the 2 cell lines. The cytoplasmic F-actin in HUC-BC cells (Fig. 1 *top left*) labeled more intensely with more prominent microspikes, whereas the HUC-PC cells presented more stress fiber structure (Fig. 1 *bottom left*). A G-actin pool was evident around the nucleus of both HUC-PC and HUC-BC cells, as shown by the yellow hue, whereas F-actin displayed the usual stress fiber morphology. After treatment with 4-ABP, both cell types became more rounded, smaller in size, and showed disruption of F-actin fibers and decreased F-actin labeling (compare Fig. 1 *top left* with 1 *top right* and Fig. 1 *bottom left* with Fig. 1 *bottom right*). The increased G-actin is evident from the increase of yellow in the cytoplasm and purple in the nucleus.

Quantitatively, as shown in Table I, the control HUC-BC cells had a nearly 2-fold higher F-actin and two-thirds lower G-actin content than HUC-PC cells ($p < 0.01$ and $p < 0.05$, respectively, by *t*-test). After 4-ABP exposure, cytoplasmic F-actin content decreased 2-fold in HUC-PC cells, and G-actin content increased compared with the PC controls (both at $p < 0.01$ by *t*-test in comparison with control). For HUC-BC cells, a similar alteration of cytoplasmic actin was also observed, although to a lesser extent ($p < 0.1$ for G-actin and $p < 0.05$ for F-actin by *t*-test). These data show that BC and PC cells, which have different transformation potentials, have different constitutive cytosolic G- and F-actin contents, and exposure of the cells to 4-ABP caused a further alteration of cytosolic actin in both types of cells.

Determination of nuclear G- and F-actin using quantitative fluorescence

Before 4-ABP treatment, the cytoplasmic actin of the transformable HUC-PC cells differed from the untransformable HUC-BC cells both quantitatively and qualitatively, although the nuclear G- and F-actin content of these 2 cell types were similar (Table I). After cells were exposed to 4-ABP, no change was seen in either the nuclear F- or G-actin content in the untransformable BC cells (Table I). In contrast, a 2-fold decrease of nuclear F-actin content ($p < 0.05$ by *t*-test) and a corresponding increase in nuclear G-actin ($p < 0.01$ by *t*-test) were observed in the transformable HUC-PC cells 5 weeks after incubation with 200 μM 4-ABP. The major effect noted was the quantitative difference between PC and BC cells seen subsequent to 4-ABP exposure. This difference was not manifested in any distinctive morphologic changes detectable by eye under fluorescence.

Determination of total and nuclear actin using DNaseI inhibition assay on HUC-PC cells

Table I also lists comparative DNase I biochemical assay data on HUC-PC cells. These assays were used as an additional validity check on the QFIA methods. These measurements agreed closely with those determined by QFIA and confirmed both the response of the PC cells to 4-ABP treatment and that the QFIA method yields accurate measurements of actin content.

Determination of β-actin gene expression using Northern blot analysis

To determine whether the alteration of actin in response to 4-ABP treatment in the PC cells was mainly due to effects on actin polymerization rather than changes in actin gene expression, Northern blot analysis was used to quantify expression of the β-actin gene in the cell lines. As shown in Figure 1, no significant changes in actin mRNA were detected. Densitometry of 2 independent Northern blots indicated no more than a 20% decrease in actin-specific mRNA level for the 4-ABP–exposed HUC-PC compared with the unexposed cells.

Preliminary study of nuclear actin content in bladder cancer urine specimens

The results of these studies, summarized in Table II, show similar alterations in nuclear and cytoplasmic G- and F-actins in patient samples as are seen in the cultured cell model. The differences are all highly statistically significantly different

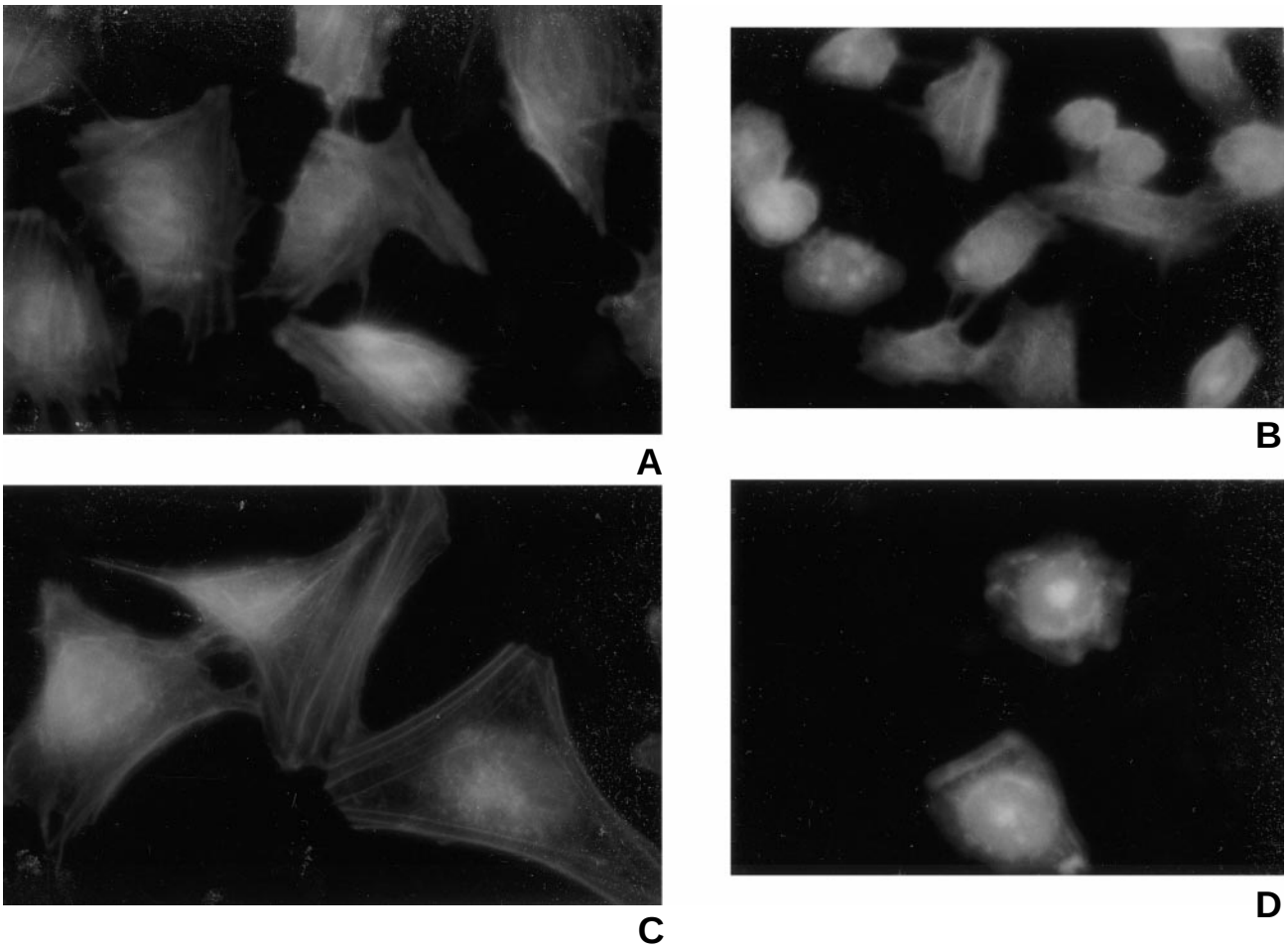

FIGURE 1 – Comparison of the effect of 4-ABP exposure on morphology and cytoplasmic actin patterns in HUC-PC and BC cells. This figure shows HUC-BC cells grown on slides (*top left*) without 4-ABP and (*top right*) with 200 μM 4-ABP as well as HUC-PC cells grown on slides (*bottom left*) without 4-ABP and (*bottom right*) with 200 μM 4-ABP. Photographs were taken with triple exposure at excitation wavelengths of 360 ± 50 for DNA (blue), 485 ± 22 nm for F-actin (green), and 560 ± 40 nm for G-actin (red) with automatic exposure control. The effects of 4-ABP on cytoplasmic actin of HUC-BC and PC cells were manifested as a prominent loss of F-actin structural features. No distinct morphology associated with nuclear F-actin was seen. G-actin did not show distinct morphologic pattern, being distributed fairly uniformly, and is therefore not shown separately. The increase in G-actin is shown by the increased yellow (red plus green) hue in the cytoplasm and purple (red plus blue) in the nucleus.

($p < 0.01$) and indicate that the nuclear actin changes are not simply a function of cell culture, but occur *in vivo* and in the *in vitro* model system.

DISCUSSION

The profound alterations in the anatomic distribution of actin stress fibers of transformed cells suggest a role for actin organization in cellular transformation. We have previously shown that quantitative changes in cytoplasmic F-actin content of HL-60 cells reflect differentiation (Rao *et al.*, 1990), and when measured in bladder cells, reflect risk for bladder cancer occurring in cells of the epithelial field (Rao *et al.*, 1991; 1993). Similar field changes occurring during lung carcinogenesis have also been reported (Carter *et al.*, 1994). In the current study, this approach was extended by measuring both G- and F-actin in both the cytoplasm and the nucleus to determine whether nuclear changes track or are independent of cytoplasmic changes and whether G- and F-actin concentrations are reciprocally related. Our major finding, that the transformable HUC-PC cell line shows a change in nuclear actin content in response to 4-ABP treatment while the untransformable HUC-BC line shows no change in nuclear actin, suggests that changes in nuclear actin content reflect specific events closely associated with transformation and not simply altered differentiation.

The model used in our studies consists of 2 lines of SV-40–immortalized bladder cells, one of which can be converted to a tumorigenic line by treatment with 4-ABP and the other of which is refractory to transformation. Although it might be argued that SV-40 might not be ideal for immortalization because it institutes a certain degree of genetic instability and that the large T-antigen has well-known effects on both p53 and Rb, we believe the use of this model is fully justified. Neither cell line is tumorigenic in nude mice (Bookland *et al.*, 1992*a* and *b*), thereby showing that SV-40 itself is not tumorigenic in these cells; moreover, one of the lines cannot be transformed by 4-ABP treatment, presumably because of inactivation of the enzymes that metabolize 4-ABP to its ultimate carcinogen (Bookland *et al.*, 1992*a*). The untransformable line serves as a control for effects that are not directly related to transformation, such as the changes in cytoplasmic actin.

A close correspondence was seen between total actin content of HUC-PC cells measured by quantitative fluorescence and the DNase I inhibition assay (200 *vs.* 208 μg/10^6 cells without 4-ABP

TABLE I – EFFECTS OF 4-ABP EXPOSURE ON CYTOPLASMIC AND NUCLEAR G- AND F-ACTIN CONTENTS (μg/10^6 CELLS)[1]

Cell	Treatment	G-actin	F-actin	Core actin	Total
QFIA-cytoplasmic actin					
HUC-BC	Control	40 ± 6	150 ± 7		190 ± 13
	4-ABP	64 ± 8	120 ± 5[2]		184 ± 13
HUC-PC	Control	61 ± 7	88 ± 9		149 ± 16
	4-ABP	92 ± 10[3]	44 ± 8[3]		136 ± 18
	$A_{HL\text{-}60}$	40	30		70
QFIA-nuclear actin					
HUC-BC	Control	21 ± 1	38 ± 7		59 ± 8
	4-ABP	20 ± 1	34 ± 4		54 ± 5
HUC-PC	Control	19 ± 2	32 ± 4		51 ± 6
	4-ABP	28 ± 2[3]	13 ± 2[2]		41 ± 4
	$A_{HL\text{-}60}$	12	45		57
QFIA-total cellular actin					
HUC-BC	Control	61 ± 7	188 ± 14		249 ± 21
	4-ABP	84 ± 9	154 ± 9		238 ± 18
HUC-PC	Control	80 ± 9	120 ± 13		200 ± 22
	4-ABP	120 ± 12[3]	57 ± 10[3]		177 ± 22
DNaseI-total cellular actin					
HUC-PC	Control	75 ± 3	82 ± 15	51 ± 19	208 ± 37
	4-ABP	107 ± 9[2]	38 ± 14[2]	44 ± 15	189 ± 38
DNaseI-nuclear actin					
HUC-PC	Control	16 ± 2	30 ± 6		46 ± 8
	4-ABP	42 ± 8	11 ± 2		53 ± 10

[1]Measured by 4 independent quantitative fluorescence and 3 independent experiments of DNase I assay (mean ± SD) for HUC-BC (untransformable) and HUC-PC (transformable) cells before and after 4-ABP treatment. Also shown are the actin values for the HL-60 cells that were used to standardized the quantitative fluorescence measurements in terms of absolute actin content.–[2]$p < 0.05$ in comparison with control.–[3]$p < 0.01$ in comparison with control.

TABLE II – NUCLEAR AND CYTOPLASMIC G- AND F-ACTINS IN BLADDER TUMOR PATIENT SAMPLES[1]

Parameter	Tumor	Normal control	*P*
Nuclear G-actin	5.53 ± 0.68	3.17 ± 0.45	0.0010
Nuclear F-actin	38.9 ± 2.2	48.1 ± 5.3	0.0092
Cytoplasmic G-actin	20.1 ± 2.2	12.9 ± 3.1	0.0044
Cytoplasmic F-actin	63.2 ± 3.7	76.8 ± 5.8	0.0038

[1]An image mask derived from the fluorescence of the Hoechst 33258 dye was used to measure separately the nuclear and cytoplasmic G- and F-actins in tumor cells derived from 4 transitional cell carcinomas and from urine cells from 4 asymptomatic controls. Values listed are the mean actin content for the 4 samples together with the standard deviation. Statistical significance was tested with the *t*-test.

treatment and 167 *vs.* 189 μg/10^6 cells after treatment), with similar close correspondence seen between nuclear F- and G-actin measurements. Several precautions were taken to assure the accuracy of nuclear actin quantification. One potential problem is the diffusibility of actin between cellular cytoplasm and nuclei (Paine, 1984). In this study, cells were permeabilized with a fixative containing 25% ethanol before nuclear isolation. The highly consistent results of nuclear G- and F-actin levels measured by quantitative fluorescence and the DNase I inhibition assay indicate that the cross-contamination problem was minimal and that the fluorescent label methods were as accurate as conventional biochemical analysis. Therefore, the much more convenient quantitative fluorescence assay can be used to measure nuclear actin accurately. This method has the additional advantage of being able to assay single cells, therefore making studies with heterogeneous cell populations feasible (Rao *et al.*, 1993).

Our major finding is that while the cytoplasmic actin content was altered and the ratio between G- and F-actin was shifted by 4-ABP treatment for both evaluated cell lines, only the nuclear actin content of the transformable HUC-PC line was altered by the carcinogen. That the nuclear actin content of the refractory HUC-BC cells was not altered, either in amount or distribution between F- and G-actins, while the actin content of the transformable HUC-PC cells was altered suggests that nuclear actin changes reflect events that occur specifically with transformation. This conclusion was supported by the finding that similar differences in nuclear actin were observed between transitional cell tumor cells and normal urinary transitional epithelial cells. Although both cell lines have similar morphologic and growth patterns, as reported previously (Bookland *et al.*, 1992*b*), we found here that the transformable PC cells constitutively express a lower cytoplasmic F- to G-actin ratio than BC cells, thereby suggesting that the PC line may be more altered, *i.e.*, more premalignant, than the BC line.

Another important finding was that the shift in total G- and F-actin content (cytoplasmic and nuclear actin) in the HUC cells induced by 4-ABP apparently is not managed by regulating the expression of the β-actin gene itself. This suggests that the changes seen reflect changes in an equilibrium between F- and G-actin and that regulation of this equilibrium may be a major homeostatic mechanism that reflects the effects of epigenetic factors (such as tumor promoters), stress and growth factors, or genetic factors (such as deletion of tumor suppressor genes or oncogene activation). Ridley and Hall (1992) demonstrated that actin assembly in the cytoplasm was regulated by the GTP-binding protein-related *ras* oncogene superfamily, with *rho* being more specific for stress fiber formation and *rac* being more related to membrane ruffling (Ridley *et al.*, 1992). However, the mechanisms for regulation of actin polymerization are not known.

Increasing evidence indicates a direct role for actin in transformation. First, a mutated β-actin gene contributed to a stable conversion of the transitions of normal human fibroblasts toward the stable tumorigenic state (Leavitt *et al.*, 1987; Leavitt, 1994). Second, a mutation in the actin-binding domain of the bcr-abl oncoprotein reduced the transformation ability of this oncoprotein, strongly indicating a direct involvement of actin in transformation

(McWhirter and Wang, 1993). In addition, Pienta and Coffey (1992) demonstrated a physical connection by actin between the nucleus and cell periphery that is altered in transformation. Finally, our preliminary results from 2 independent, large-scale, prospective molecular epidemiologic studies of workers who were occupationally exposed to bladder carcinogens showed that G-actin levels in urinary cells correlated with exposure, whereas more specific tumor markers did not. These data strongly indicated that the alteration of actin is a direct and early event in bladder cancer carcinogenesis. The current study suggests that the roles of actin changes in transformation are different in the nucleus and cytoplasm. The actin alterations in the cytoplasm reflect differentiation and are manifested as both a redistribution or disappearance of actin fibers and decreasing F-actin levels coupled with increasing G-actin content (Rao *et al.,* 1990). The current study provides evidence that nuclear actin is also altered in transformation. This alteration may represent a more specific event than the cytoplasmic actin change because it was observed only in the transformable HUC-PC line, whereas the cytoplasmic actin change was observed in both cell lines in response to 4-ABP treatment. Studies on human bladder cancer confirm that these events also occur in natural carcinogenesis.

The 2-stage model of carcinogenesis consists of initiation, which represents genetic mutation, and promotion, which consists of epigenetic alterations of the cell to a less-differentiated state. A speculative model is proposed linking actin changes to both epigenetic and genetic pathways, as shown in Figure 2 and explained in the Figure caption. Although loss of tumor suppressor genes is certainly a primary event in carcinogenesis (Couture and Hansen, 1991) and its tissue culture analog, transformation, the process seems to be more complex than the simple accumulation of genetic errors. Rubin (1992) has demonstrated reversible transformation in cell culture in the absence of stable genetic mutation but apparently involving changes in the cell population as a whole, thereby also implicating epigenetic effects in producing the transformed phenotype (Rubin, 1994). Moreover, many genotypes apparently yield similar phenotypes, probably because cellular signaling pathways are linked and not independent of each other (Roberts and Sporn, 1992). Exactly how genetic and epigenetic pathways toward transformation are linked is not known, but one possibility is that an altered nuclear matrix may support a higher rate of replicative errors. Genetic instability seems to be the main driving force in progression to clinically detectable tumors because it generates mutations for multiple rounds of selection and competition that characterize progression (Temin, 1988; Purnell *et al.,* 1987).

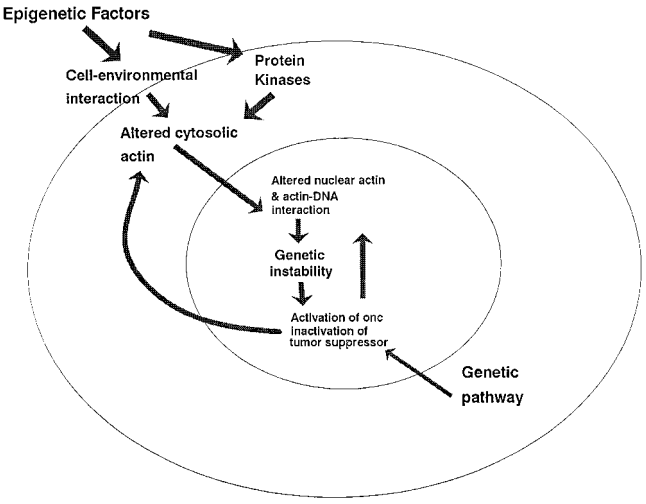

FIGURE 3 – Diagram of the hypothesis describing how actin is involved in malignant transformation. Interacting genetic and epigenetic pathways are involved in transformation. The genetic pathway involves loss of tumor suppressor genes and activation of oncogenes. This pathway is apparently driven by genetic instability, which generates additional mutations that are tested by selection and lead to further progression of initiated cells. Alteration of nuclear actin appears to be a marker for this genetic instability. The epigenetic pathway, which seems to function through dedifferentiation and/or growth stimulation, enhances the ability of cells to express mutations and thereby also contributes to the process of transformation.

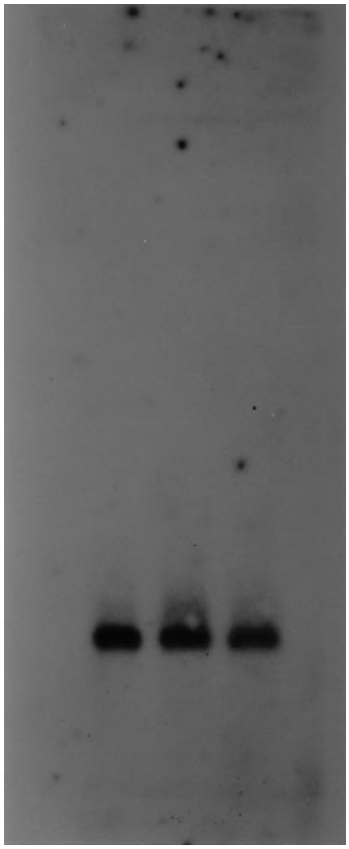

FIGURE 2 – Northern blot analysis of β-actin expression. Lane 1: control; lane 2: 24-hr exposure to 4-ABP; lane 3: 48-hr exposure to 4-ABP. For each lane, approximately 10 µg of total RNA were used.

The preliminary study with cells derived from urine samples (Table II) indicates that alterations in nuclear G-actin with transformation are a general property of cancer cells and not just associated with cell culture. Further study will not only facilitate understanding of oncogenesis, but should open new approaches for cancer management and control. Previous studies have indicated that cytoplasmic actin measurements are an intermediate endpoint marker for chemoprevention reflecting altered differentiation (Hemstreet, III *et al.,* 1992; 1996). Our current results suggest that nuclear actin measurements may provide a more specific marker for transformation by being less affected by differentiation. By combining nuclear and cytoplasmic actin measurements, a powerful marker profile may result that can be used to assess individual risk for cancer and to guide intervention and management.

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