Journal of Cellular Biochemistry 63:174–184 (1996)

Estrogen Regulation of Nuclear Matrix-Intermediate Filament Proteins in Human Breast Cancer Cells

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Abstract The tissue matrix consists of linkages and interactions of the nuclear matrix, cytoskeleton, and extracellular matrix. This system is a dynamic structural component of the cell that organizes and processes structural and functional information to maintain and coordinate cell function and gene expression. We have studied estrogen regulation of nuclear matrix associated proteins, including the intimately connected cytoskeletal intermediate filaments, in T-47D5 human breast cancer cells. Three proteins (identified as cytokeratins 8, 18, and 19) present in the nuclear matrix-intermediate filament fraction (NM-IF) of cells grown in estrogen-replete conditions were dramatically reduced when the cells were grown in acute (1 week) estrogen-depleted conditions. Replacing estrogen in the medium of acute estrogen-depleted cells restored expression of these proteins. T-47D5 cells that are chronically depleted of estrogen (T5-PRF) are estrogen-nonresponsive in culture. These cells overexpressed these three proteins, compared to parent cells grown in the presence of estrogen. Treatment of the T5-PRF cells with estrogen did not lead to further up-regulation of these proteins. Treating T-47D5 cells in estrogen-replete conditions with the antiestrogens 4-hydroxytamoxifen and ICI 164 384 (100 nM, 3 days) resulted in a significant reduction in these proteins, while no effect was seen in long-term chronic estrogen-depleted T-47D5 cells. In conclusion, we have identified NM-IF proteins (*cytokeratins 8, 18,* and 19) in human breast cancer cells that are estrogen regulated and may play a role in estrogen action in human breast cancer cells.

Key words: cytokeratins, hormone independence, T-47D5, nuclear matrix, breast cancer

The tissue matrix system, consisting of dynamic linkages between the nuclear matrix (NM), the cytoskeleton, and the extracellular matrix (ECM) forms a structural and functional connection from the cell periphery to the DNA [Pienta and Coffey, 1992]. The cytoskeleton is composed, in part, of intermediate filaments (IFs). Cytokeratins are members of the intermediate filament family of structural proteins [Moll et al., 1982]. Direct connections (via intermediate filaments) between the cell periphery and NM have been demonstrated and this nuclear matrix-intermediate filament (NM-IF) system is altered by tumour promoters [Fey and Penman, 1981; Fey et al., 1984]. Direct evidence of a continuous network connecting the plasma membrane structure and cytoskeleton with the nucleoskeleton of eukaryotic cells is provided by data demonstrating that vimentin is anchored directly to the nuclear lamina via lamin B [Djabali et al., 1991; Georgatos and Blobel, 1987]. Evidence also suggests that the intermediate filaments, in particular the lamins, not only exist at the nuclear periphery but are also found as part of the internal nuclear matrix [Hozak et al., 1995; Martell et al., 1992].

Architectural alterations (defined by structural NM proteins and/or interactions with the cytoskeleton) within or associated with the nucleus may influence or control what genes or subsets of genes are actively transcribed. Nucleic acids can interact with IFs [Traub et al., 1983] and, disrupting the cytoskeleton with cytochalasin D (an actin microfilament inhibitor), induce specific gene expression [Zambetti et al., 1991]. Manipulation of the cytoskeleton can alter the pattern of gene expression [Blum and Wicha, 1988] and in mammary epithelial cells, the ECM has been shown to regulate tissue specific gene expression [Boudreau et al., 1995; Streuli et al., 1995]. Seely and Aggeler [1991] have demonstrated that modulating the cytoskeleton in cultured mouse mammary epithelial cells altered

Received February 8, 1996; accepted April 26, 1996.

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milk protein synthesis. Together these data demonstrate that not only is the cytoskeleton physically connected to, and may even be considered, an intimate part of the nucleus/NM structure, but that the nucleus can respond to signals from the structural organization of the cytoskeleton in order to modulate gene expression. Because of the intimate connection of the NM with the cytoskeleton, our study is concerned with examining the effects of hormone on NM proteins and IF proteins associated with the nuclei in human breast cancer cells.

The nuclear matrix plays an important role in many nuclear processes, including DNA organization [Getzenberg et al., 1991], replication [Cook, 1991], gene transcription and processing [Getzenberg and Coffey, 1991; Huang and Spector, 1991; Xing et al., 1993; Carter et al., 1993], and steroid hormone action [Kirsh et al., 1986; Alexander et al., 1987]. The expression of several NM proteins is cell [Fey and Penman, 1989], tissue [Getzenberg and Coffey, 1990], differentiation [Dworetsky et al., 1990], and hormonal state specific [Getzenberg and Coffey, 1990]. Differences in NM protein expression exist between normal tissues and their cancerous counterparts [Partin et al., 1993], and more specifically, differences have been shown between normal breast tissue and breast cancer [Khanuja et al., 1993]. Steroid hormone receptor complexes interact with the nucleus and subsequently modulate gene expression, but a comprehensive understanding of the mechanisms involved is lacking. Several steroid hormone receptors, including the estrogen receptor, have been shown to localize to the NM. Cell-free binding assays confirm that this localization is due to the presence of specific acceptor sites in the NM to which steroid–receptor complexes bind with high affinity and tissue specificity [Metzger and Korach, 1990]. Patterns of NM protein expression are hypothesized to be involved in changes in gene expression and it is believed that the specific proteins of the NM can influence gene expression [Stein et al., 1994; Bidwell et al., 1993]. The way in which the protein changes in the NM could influence gene expression is unclear, but transcriptionally active genes have been shown to be associated with the nuclear matrix, whereas inactive genes are not [Getzenberg et al., 1991; Gerdes et al., 1994].

Breast cancer is a hormonally responsive cancer and is dependent on estrogen for growth [Dickson and Lippman, 1991]. Estrogens promote the growth of human breast cancer and, as such, many therapies are aimed at blocking the growth promoting effects of estrogen (e.g., antiestrogens). The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of antiestrogen treatments [Clarke et al., 1990; Leonessa et al., 1991]. Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics.

Several breast cancer cell lines in culture also require estrogen for growth and long-term culture in estrogen-depleted conditions can result in these cells becoming independent of the requirement for estrogen for growth. Indeed, the development of estrogen-nonresponsive growth in human breast cancer is thought to be one of the initial steps in the progression to hormone independence [Clarke et al., 1994].

This study is concerned with examining the effects of estrogen on NM-IF expression in estrogen-responsive (ER+) and estrogen-nonresponsive (ER+) human breast cancer cells. We identified three NM-IF proteins—cytokeratin 8 (CK8), 18 (CK18), and 19 (CK19)—that are estrogen-regulated in T-47D5 human breast cancer cells. In T-47D5 cells that are estrogen independent in culture, these cytokeratins are overexpressed and are no longer sensitive to regulation by either estrogen or antiestrogens.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium (DMEM) and phenol red free DMEM powder were purchased from GIBCO/BRL (Burlington, Ontario). Fetal bovine serum (FBS) was purchased from UBI (Lake Placid, NY) and all other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). 4-Hydroxytamoxifen and ICI 164 384 were gifts from ICI (Macclesfield, Cheshire) and estradiol was purchased from Sigma. ^{3}H-Estradiol and ^{3}H-R5020 were purchased from NEN (Mississauga, Ontario).

Cells and Cell Culture

T-47D5 [Watts et al., 1992], T-47D, MDA MB 231, and HBL 100 human breast epithelial cells

were routinely grown in DMEM supplemented with 5% FBS, glucose, glutamine, and penicillin–streptomycin (5% CM) as previously described [Shiu, 1979]. T-47D5 chronically estrogen-depleted cells (T5-PRF) were routinely grown in phenol red-free DMEM supplemented with 5% 2× charcoal-stripped fetal calf serum (CS-FCS), glucose, glutamine, and penicillin–streptomycin (5% CS). T-47D5 cells acutely depleted of estrogen were grown in 5% CS medium for one passage. Cells were passaged at 70–80% confluency using Earle's EDTA solution. Cells for nuclear matrix isolation were harvested at ~80% confluency using a rubber policeman, pelleted by centrifugation and stored at −70°C until processing.

Development of T-47D5 Chronically Estrogen-Depleted Cell Line

T-47D5 parent cells were passaged into phenol red-free DMEM supplemented with 5% 2× charcoal-stripped CS-FCS, penicillin–streptomycin, glucose, and glutamine (5% CS). Cells were routinely passaged at 70–80% confluency using Earle's EDTA solution. Long-term chronically estrogen-depleted T-47D5 cells have been depleted of estrogen for at least 60 passages, while the short-term estrogen-depleted cells have been grown in estrogen-depleted conditions for at least 10 passages. Acutely estrogen-depleted T-47D5 cells are passaged once in 5% CS.

Growth Experiments

Growth experiments were routinely performed by setting up cells at 10^4 cells/35-mm dish [Murphy et al., 1990]. All growth experiments on chronically estrogen-depleted T-47D5 cells were performed in 5% CS. Estrogen growth experiments on T-47D5 cells were performed in 5% CS, while antiestrogen growth experiments were performed in 5% CM. Two days later, fresh medium was added, which contained the appropriate concentration of drug to be tested from 1,000× stock solutions in ethanol. After 5 days the cells were harvested in triplicate using trypsin/EDTA and counted using an electronic cell counter (Coulter Electronics, Burlington, Ontario). Results were expressed as proliferation rate (percentage control) using the equation

$$\text{Doubling time (DT)} = 2 \log n / \log (Tn/Ti)$$

where Ti is initial cell number, Tn is final cell number and n is the time (days) between T_i and T_n. Proliferation rate as a percentage of control was then calculated from the

$$\text{Proliferation rate} = \text{doubling time (control)} \times 100/\text{doubling time}$$

Isolation of NM-IF

Nuclei were extracted with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol) containing 1 mM PMSF and 0.5% (v/v) Triton X-100, and nuclear matrices were prepared essentially as previously described [Sun et al., 1994]. Briefly, nuclei (20 A_{260}/ml) were resuspended in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 1% (v/v) thiodiglycol, 0.5% (v/v) Triton X-100) and digested with DNase I (168 U/ml) for 20 min at room temperature. Ammonium sulfate was added dropwise from a 4 M stock, to a final concentration of 0.25 M, and the nuclear matrix was pelleted by centrifugation. The ammonium sulfate-extracted nuclear matrix was resuspended in digestion buffer and re-extracted by slowly adding NaCl to a final concentration of 2.0 M from a 4.0 M stock solution with mixing. This was left on ice for 30 min and then pelleted by centrifugation. The nuclear matrix was again re-extracted with 2 M NaCl and 1% (v/v) 2-mercaptoethanol for 30 min on ice and the insoluble nuclear matrix isolated by centrifugation. This fraction (NM-IF) contains nuclear matrix proteins and associated intermediate filaments [Fey et al., 1984]. Nuclear matrices were resuspended in 8 M urea and stored at −20°C. Protein levels were assayed using BioRad (Bradford) protein assay kit (Mississauga, Ontario).

Steroid Hormone Receptor Assay

Whole cell binding assays for estrogen receptors were performed as previously described [Murphy and Dotzlaw, 1989].

SDS–PAGE, Coomassie Staining, and Quantitation

Subcellular fractions were analyzed under reducing conditions by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) with 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method [Laemmli, 1970]. Gels were stained in 0.05% Coomassie Blue R-250. Quantitation of cytokeratin levels was performed on

Coomassie Blue stained gels using scanning densitometry and lamin bands (identified using immune detection along with purified lamins as standards, gifts from Dr. Y. Raymond) were used as loading controls.

2-D Gel Electrophoresis

First-dimension isoelectric focusing was performed according to the method of O'Farrell [O'Farrell, 1975] and the second-dimension (2-D) SDS–PAGE according to the method of Doucet [Doucet and Trifaro, 1988]. Gels were stained using the silver staining technique [Heukeshoven and Dernick, 1985].

Protein Purification

NM-IF samples were prepared as described and resuspended in 7 M urea, 20 mM Tris–HCl, pH 8. Samples were ran over 1 ml Poros PI (anion-exchange) column at a protein concentration of 8 mg/column. Proteins were eluted with a linear gradient of 0–0.5 M NaCl in 15 ml and 0.6 ml collected/fraction. Fractions were assayed by SDS–PAGE and samples containing protein bands of interest were pooled. 2-D gels were run on pooled samples (100 μg/gel), gels were coomassie stained and spots excised. Excised spots were sent to the WM Keck Foundation (New Haven, CT) for internal microsequencing.

Western Blotting and Immune Detection

NM-IF samples were run on 12% SDS–PAGE with 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method [Laemmli, 1970]. Gels were transferred to nitrocellulose as previously described [Delcuve and Davie, 1992] and transferred for 1½ hr at 120 V. Blots were blocked overnight at 4°C in 5% skim milk/Tris-buffered saline. Blots were incubated with 1° and 2° antibodies for 1 hr at room temperature in 2% skim milk/Tris-buffered saline containing 0.2% Tween-20. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

RESULTS

Development of Hormone-Nonresponsive T-47D5 Subline

T-47D5 human breast cancer cells are estrogen receptor positive (ER^+) and estrogen treatment in culture results in increased proliferation of these cells (Fig. 1A). An estrogen-nonresponsive cell line was developed from this parent line, by chronically depleting the cells of estrogen in long-term culture (~60 passages). These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line (Fig. 1A). Both the parent T-47D5 cells and the estrogen-nonresponsive (T5-PRF) cell line express the estrogen receptor, localized to the NM, as assayed by immune-detection (data not shown) and ligand-binding techniques (Table I). The T5-PRF cell line contained significant amounts of ER, although the absolute level of expression is reduced by ~50%, as compared to the parent line.

Antiestrogens antagonize the growth-stimulatory effects of estrogens and are often used in the treatment of human breast cancer [Jordan, 1988]. The antiestrogens ICI 164 384 and 4-monohydroxytamoxifen both result in the inhibition of T-47D5 cell growth (Fig. 1B,C). T-47D5 cells chronically grown in estrogen-depleted conditions still retain sensitivity to the growth inhibitory effects of 4-monohydroxytamoxifen and to a lesser extent to those of ICI 164 384 (Fig. 1B,C).

Total NM-IF Proteins in T-47D5 Cells

The T5-PRF cell line, while still retaining ER expression, is estrogen-nonresponsive, providing a model in which to examine the NM-IF profile between hormone-responsive and -nonresponsive human breast cancer cell lines. Total NM-IF protein expression was examined in T-47D5 human breast cancer cells. Subcellular fractions obtained during the NM-IF isolation procedure were analyzed by SDS–12% PAGE. Initially, the method for NM-IF preparation stopped at 0.25 M ammonium sulphate. However, the NM-IF fraction still contained significant amounts of histone contamination (Fig. 2, lane 4). This contamination was markedly decreased by sequential salt extractions of the NM-IF preparations as shown. The final protein composition of NM-IF isolated by sequential salt extractions of nuclei is shown in Figure 2 (lane 7). Arrows denote 41-, 45-, and 54-kD protein bands, which are enriched in the high-salt extracted NM-IF fraction.

Estrogen Regulation of NM-IF Proteins

NM-IF composition was compared between the parent T-47D5 cells and T5-PRF human breast cancer cell lines. The NM-IF of cells

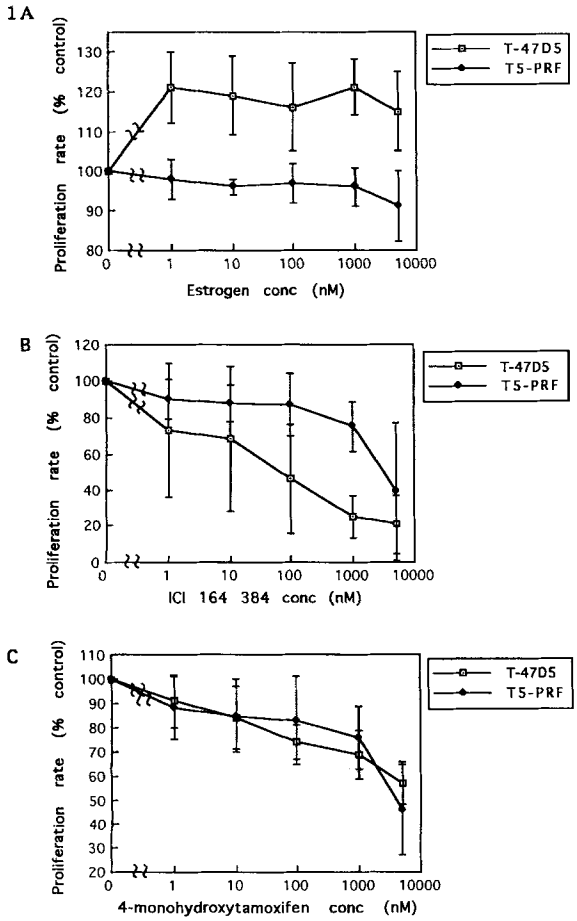

Fig. 1. Effect of estrogen and antiestrogen on proliferation of T-47D5 human breast cancer cells grown routinely in estrogen-replete (T-47D5) and estrogen-deplete (T5-PRF) conditions. The effects of increasing concentrations of estrogen (**A**) and antiestrogen (ICI 164 384 (**B**) and 4-monohydroxytamoxifen (**C**) on proliferation rate (percentage control). Approximately 10^4 cells were plated in duplicate on day −2. On day 0, three dishes were counted and cells were treated with complete medium containing estradiol or the appropriate antiestrogen to a final concentration of 0, 1, 10, 100, 1,000, and 5,000 nM. Five days later, cells were harvested and results expressed as proliferation rate as a percentage of control (see under Methods for formulae). Results represent the mean ±SEM, n = 3.

TABLE 1. Estrogen Receptor Levels in T-47D5 and T5-PRF Breast Cancer Cells.*

Cell line	ER sites/cell (±SEM)
T-47D5	186010 ± 37659
T5-PRF	71922 ± 29001

*Results are expressed as estrogen receptor sites/cell ±SEM, n = 5.

chronically depleted of estrogen (T5-PRF) contained elevated levels of the 41-, 45-, and 54-kD proteins compared to the parent, T-47D5, cell line grown in 5% CM (Fig. 3, lanes 1 and 3). This

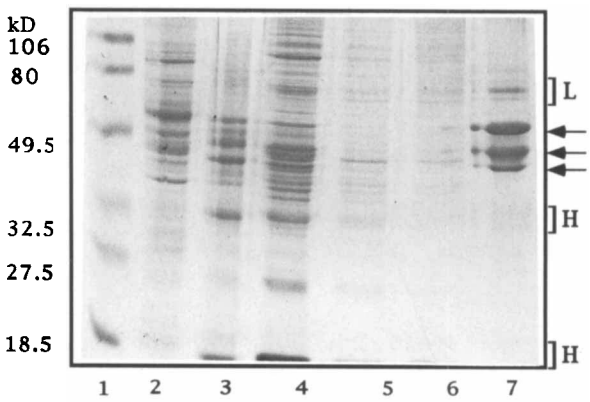

Fig. 2. NM-IF protein profile in T-47D5 human breast cancer cells. T-47D5 human breast cancer cells were grown in the presence of 10 nM estradiol for 72 h. NM-IF was isolated as described under Methods. *Lane 1,* MW standards (BioRad); *lane 2,* cytosol; *lane 3,* whole nuclei; *lane 4,* S1 = 0.25 M $(NH_4)_2SO_4$ NM-IF pellet; *lane 5,* S2 = 2 M NaCl solubilized fraction; *lane 6,* S3 = 2 M NaCl/1% 2-mercaptoethanol solubilized fraction; *lane 7,* NM-IF pellet. Subcellular fractions were run on 12% SDS–PAGE and gels were stained with Coomassie Blue to visualize protein bands. *Arrows,* 54-, 45-, and 41-kD protein bands. H, histones (identified based on relative mobility on SDS–PAGE against purified histones); L, lamins (identified using immune detection along with purified lamins as standards).

apparent overexpression appeared only in long-term chronic estrogen-depleted cells, since acute (one passage) estrogen withdrawal results in a marked reduction of these protein bands. T-47D5 cells passaged in estrogen-deplete conditions for ~10 passages, have levels comparable to those seen in the parent cell line (Fig. 3, lanes 5 and 7). The data suggested that the 41-, 45-, and 54-kD proteins may be estrogen regulated. This was confirmed by replacing estrogen in the medium of acutely estrogen-depleted cells, which resulted in increased expression of the 41-, 45-, and 54-kD proteins (Fig. 3, lanes 5 and 6). The expression of these three NM-IF proteins can also be further up-regulated by estrogen in T-47D5 cells and short-term chronic estrogen-depleted cells but exhibit little or no sensitivity to estrogen in the long-term chronic estrogen depleted T5-PRF cells (Fig. 3).

The effect of estrogen on these proteins was examined more closely. The results of a dose-response experiment, 72 h after estrogen treatment, are shown in Figure 4. Dose-response analyses show that as little as 0.1 nM estrogen resulted in increased levels of these proteins in the NM-IF of acute estrogen-depleted T-47D5 cells. Comparing the abundance of these proteins to the lamins (Fig. 4), it can be seen that the lamins are the most abundant NM-IF pro-

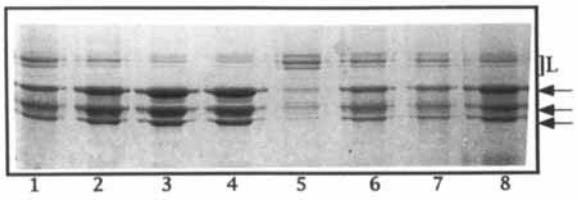

Fig. 3. NM-IF in estrogen-replete and estrogen-deplete conditions. NM-IF was obtained as described under Methods. *Lane 1,* T-47D5 NM-IF (ethanol vehicle); *lane 2,* T-47D5 NM-IF (10 nM estrogen (E2), 72 h); *lane 3,* T5-PRF NM-IF (ethanol vehicle); *lane 4,* T5-PRF NM-IF (10 nM E2, 72 h); *lane 5,* acute estrogen-deplete T-47D5 NM-IF (ethanol vehicle); *lane 6,* acute estrogen-depleted T-47D5 NM-IF (10 nM E2, 72 h); *lane 7,* short-term estrogen-depleted T-47D5 NM-IF (10 passages, ethanol vehicle); *lane 8,* short-term estrogen-depleted T-47D5 NM-IF (10 nM E2, 72 h). *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.

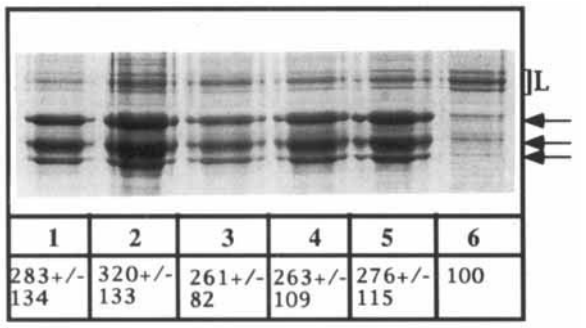

Fig. 4. Estrogen dose-response effects on NM-IF protein levels in acute estrogen-depleted T-47D5 cells. Cells were treated with estradiol at the appropriate concentration or vehicle control for 72 h. Dishes were harvested and NM-IF isolated as described under Methods. *Lane 1,* 1 μM E2; *lane 2,* 100 nM E2; *lane 3,* 10 nM E2; *lane 4,* 1 nM E2; *lane 5,* 0.1 nM E2; *lane 6,* ethanol vehicle. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins. Numbers below represent cytokeratin levels as a percentage of control ±SEM. Lamin bands were used as a loading control; n = 4.

teins in lane 6, but after estrogen treatment the 41-, 45-, and 54-kD protein bands are much more abundant.

Effect of Antiestrogen on NM-IF Proteins

The effects of the antiestrogens on the expression of the 41-, 45-, and 54-kD proteins were next studied. Treating T-47D5 cells, grown in the presence of estrogen, for 72 h with either 100 nM ICI 164 384 or 4-monohydroxytamoxifen (OT) resulted in a significant reduction in the NM-IF levels of these proteins (Fig. 5A). The amounts of these three proteins associated with the NM-IF fraction of long-term chronically estrogen-depleted T-47D5 cells (T5-PRF) did not appear to be affected by antiestrogen treatment (Fig. 5B). Thus, although antiestrogens inhibit the growth of these cells, the inhibitory effect on the abundance of the three NM-IF proteins seen in the parent T-47D5 cell line is no longer observed. The dose-response effects of antiestrogen were examined in T-47D5 cells grown in the presence of estrogen. Similar to what we observed with estrogen dose effects, the abundance of these proteins in the NM-IF fraction was sensitive to alteration by antiestrogen. With as little as 1 nM ICI 164 384 or 0.1 nM OT, significant reductions in the levels of these proteins in the NM-IF were seen (Fig. 6A,B).

Identification of 54-, 45-, and 41-kD Proteins

These proteins were subsequently identified as cytokeratin 8 (54 kD), cytokeratin 18 (45 kD), and cytokeratin 19 (41 kD). Identification was performed by chromatographically enriching for these proteins, isolating spots from 2-D gels, followed by microsequencing of the spots from T-47D human breast cancer cells (unpublished results). These protein bands were confirmed to be the same proteins identified in the NM-IF of T-47D5 human breast cancer cells after column purification of these proteins from T-47D5 cells followed by 2-D gel analysis of fractions (apparent molecular size and pI comparisons) from T-47D, T-47D5, and mixing experiments of the two sets of column fractions from each cell line (Fig. 7). 2-D gel analysis performed on samples with and without estrogen treatment confirmed that the spots identified as CK8, CK18, and CK19 increased in abundance with estrogen treatment as seen in SDS–PAGE (data not shown).

NM-IF Proteins in ER$^-$ Versus ER$^+$ Cells

To find out if a correlation exists between ER expression and expression of NM-IF proteins, total NM-IF composition was compared between ER$^+$ and ER$^-$ human breast cell lines. Figure 8 clearly shows a marked difference in total NM-IF composition between ER$^+$ and ER$^-$ cell lines. In particular, a notable decrease or absence of the cytokeratin 8, 18, and 19 bands was observed in some ER$^-$ breast cell lines.

DISCUSSION

We have identified three NM-IF proteins (CK8, CK18, and CK19) in T-47D5 human breast cancer cells that are regulated by estrogen. The

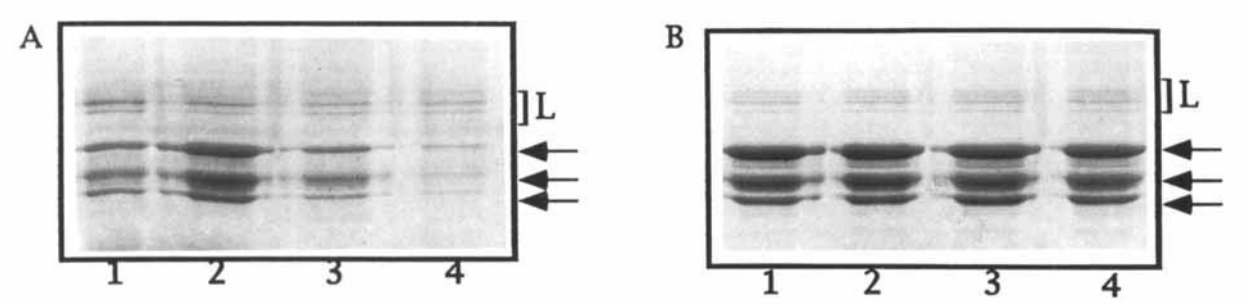

Fig. 5. Effects of antiestrogen on NM-IF proteins in T-47D5 human breast cancer cells. Cells were treated with drug at the appropriate concentration or vehicle control for 72 h before NM-IF isolation. **A:** T-47D5 NM-IF. *Lane 1,* vehicle control; *lane 2,* 10 nM E2; *lane 3,* 100 nM ICI 164 384; *lane 4,* 100 nM 4-monohydroxytamoxifen. **B:** T5-PRF NM-IF. *Lane 1,* vehicle control; *lane 2,* 10 nM E2; *lane 3,* 100 nM ICI 164 384; *lane 4,* 100 nM 4-monohydroxytamoxifen. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.

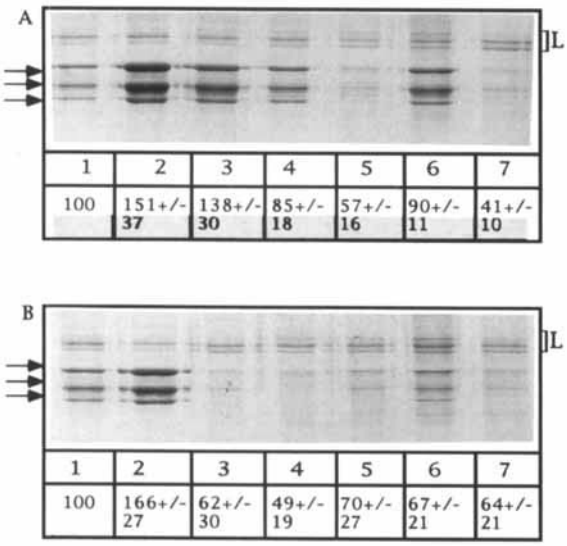

Fig. 6. Antiestrogen dose-response effects on NM-IF expression in T-47D5 human breast cancer cells. Cells were treated with antiestrogen at the appropriate concentration or vehicle alone for 72 h before isolation of the NM-IF. **A:** ICI 164 384 dose-response. *Lane 1,* ethanol vehicle; *lane 2,* 10 nM E2; *lane 3,* 0.1 nM ICI; *lane 4,* 1 nM ICI; *lane 5,* 10 nM ICI; *lane 6,* 100 nM ICI; *lane 7,* 1 μM ICI. **B:** 4-Monohydroxytamoxifen dose response. *Lane 1,* ethanol vehicle; *lane 2,* 10 nM E2; *lane 3,* 0.1 nM OT; *lane 4,* 1 nM OT; *lane 5,* 10 nM OT; *lane 6,* 100 nM OT; *lane 7,* 1 μM OT. *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins. Numbers below represent cytokeratin levels as a percentage of control ±SEM. Lamin bands were used as a loading control; n = 3.

abundance of these proteins in the NM-IF fraction is dramatically reduced upon acute withdrawal of estrogen from the cell culture medium, and re-addition of estrogen results in increased levels of these proteins in the NM-IF. The reduced levels of these three NM-IF proteins in the absence of hormone reflect the requirement of estrogen for upregulated expression of these NM-IF proteins (i.e., they are estrogen responsive proteins, or that the association of these proteins with the NM increases upon estrogen treatment). Antiestrogens downregulate the levels of these three NM-IF proteins consistent with an estrogen antagonistic activity of these compounds, suggesting the effect is mediated via the ER.

Chronic estrogen-depleted T-47D5 cells have overcome the requirement of estrogen for growth in vitro, while remaining ER^+. Despite the depletion of estrogen in the cell culture medium the NM-IF fraction from these cells has elevated levels of all three proteins. In the parent T-47D5 cell line addition of estrogen to the cell culture medium results in increased levels of the three NM-IF proteins. In the T5-PRF cells, the addition of estrogen at an equivalent dose does not increase the levels of the three NM-IF proteins. How these cells could have elevated levels of these estrogen regulated proteins in the absence of estrogen remains to be determined, but perhaps the cells are able to activate the ER through pathways other than classical estrogen/ER interactions. Recent research has demonstrated that the ER can be activated in a ligand-independent fashion [Aronica and Katzenellenbogen, 1993; Ignar-Trowbridge et al., 1993] and perhaps the ability to activate the ER in the absence of estrogen would confer a growth advantage to the cells and aid in the development of a hormone-independent phenotype. Our data suggest that as the length of time in estrogen-deplete conditions is increased, there is a concommitant increase in the levels of the 41-, 45-, and 54-kD proteins associated with the NM-IF. Although antiestrogens inhibit the growth of T5-PRF cells, they do not decrease the levels of CK8, CK18, and CK19 in the NM-IF as they do in the parent cell line, suggesting that both estrogen/antiestro-

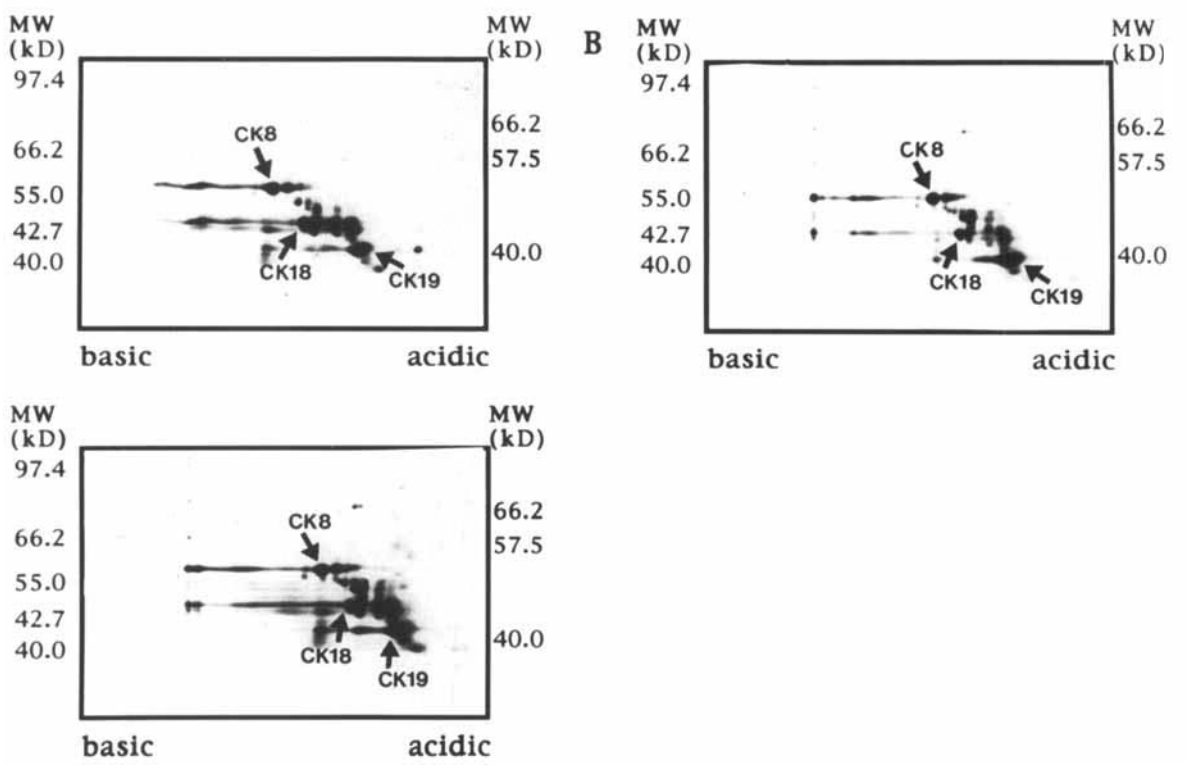

Fig. 7. 2-D gel electrophoresis of T-47D5 and T-47D column fractions. **A:** T-47D5 column fraction. **B:** T-47D column fraction. **C:** T-47D/T-47D5 mix; 15 μg protein/gel.

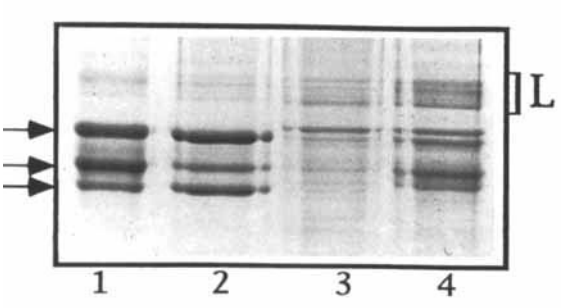

Fig. 8. ER^+ versus ER^- NM-IF protein composition. NM-IF composition was compared between ER^- and ER^+ human breast cancer cell lines. Cells were grown in 5% CM and NM-IF isolated as described in methods. *Lane 1,* T-47D5 (ER^+); *lane 2,* T-47D (ER^+); *lane 3,* MDAMB231 (ER^-); *lane 4,* HBL100 (ER^-). *Arrows,* 54-, 45-, and 41-kD protein bands; 5 μg protein/lane. L, lamins.

gen sensitivity, in terms of regulating the NM-IF levels of these proteins, has been lost in this cell line.

We have purified the p54, p45, and p41 NM-IF proteins and they have been identified by internal microsequencing as cytokeratin 8, 18 and 19, respectively [unpublished results]. That cytokeratins are estrogen regulated in T-47D5 cells is consistent with previous studies. In MCF-7 cells in culture estrogen and antiestrogen treatment both resulted in an apparent increase in cytokeratins [Sapino et al., 1986]. Studies in rat vaginal epithelium have also demonstrated estrogen-induced increases in cytokeratin expression, although antiestrogens did not have a significant effect in these studies [Kronenberg and Clark, 1985]. Androgen, but not estrogen, represses cytokeratin 8 and cytokeratin 18 mRNA expression in the rat prostate, and antiandrogens were shown to reverse this effect [Hsieh et al., 1992].

The ER^- cell lines MDA MB 231 and HBL 100 are estrogen-unresponsive cell lines and have very low levels of cytokeratin 8, 18, and 19 relative to the lamins in their NM-IF. This is consistent with other reports demonstrating in a subset of ER^- cell lines (including MDA-MB-231) an inverse relationship between cytokeratin and vimentin expression [Sommers et al., 1989, 1992]. Significant expression of cytokeratins may therefore be associated with an ER^+ phenotype.

The relationship between tumour growth and cytokeratin expression has been examined, with some studies suggesting a correlation between specific cytokeratin expression and tumour progression [Kannan et al., 1994a,b]. Changes in

the levels and subset of cytokeratin expression have been found with increasing tumour grade [Ferrero et al., 1990; Green et al., 1990]. Several studies have demonstrated enhanced expression of CK19 in tumour tissue, as compared to normal [Cooper et al., 1993; Broers et al., 1988]. Interestingly, in *ras*-transformed prostate cells three NMPs of 41, 46, and 55 kD were found to exhibit significant differences in their expression, as compared with their immortalized controls [Prasad et al., 1993]. These studies suggest that cytokeratins might be a biomarker for tumour stage and perhaps changes in cytokeratin expression are associated with altered tumour phenotype.

Evidence suggests that estrogen-independence in breast cancer cells is associated with changes in expression of a set of estrogen-regulated genes [Brunner et al., 1993; van Agthoven et al., 1993]. van Agthoven and coworkers have shown that in an estrogen-independent ZR-75-1 human breast cancer cell line changes in cell morphology occur along with increases in total cellular levels of cytokeratins 8, 18, and 19 [van Agthoven et al., 1992, 1994]. Although loss of the ER can accompany and/or explain hormone independence, loss of ER does not always occur in the hormone-independent phenotype. Katzenellenbogen and colleagues have demonstrated levels of ER comparable to parent MCF-7 cell lines or greater ER expression in hormone-independent MCF-7 cells obtained through prolonged growth in estrogen-deplete conditions [Katzenellenbogen et al., 1987; Read et al., 1989]. Clarke et al. [1989] isolated a series of hormone-independent MCF-7 sublines that still maintain ER expression at levels comparable to, or greater than, the parent cell line. In our model, continued expression of the ER allows us to examine expression of estrogen responsive genes, under conditions comparable in both the parent and hormone-nonresponsive cell lines.

Our knowledge of the function of the NM-IF proteins is still very limited. Our findings that cytokeratins associated with the nuclear matrix are regulated by estrogen in human breast cancer cells suggests that these structural proteins may be important to estrogen action. Preliminary data suggest that similar patterns of cytokeratin expression can be seen in the NM-IF preparations from some human breast tumour biopsy samples suggesting that these patterns of cytokeratin expression could be relevant to tumour phenotype in vivo.

In conclusion, we have demonstrated that estrogen can regulate the levels of NM-associated intermediate filament proteins. The abundance of these proteins in the NM-IF is increased in T-47D5 human breast cancer cells that are no longer responsive to estrogen in culture. It is hypothesized that such an alteration may result in nuclear architectural changes that could reflect alterations in gene expression associated with a hormone-independent phenotype.

ACKNOWLEDGMENTS

The authors thank Dr. G. Greene (Ben May Institute, Chicago, IL) for providing the hER antibody H226, Dr. Y. Raymond (Institut Du Cancer De Montreal, Montreal, PQ) for providing the lamin antibodies (119D5-F1 and 131C3) and purified lamin proteins, ICI (Macclesfield, UK) for providing monohydroxytamoxifen and ICI 164 384, and Norm Huzel for technical assistance in developing the T5-PRF cell line. This research was supported in part by the Medical Research Council of Canada (PG-12809), the Canadian Breast Cancer Research Initiative, and the U.S. Army Breast Cancer Research Program, with equipment grants from the H.E. Sellers Foundation and Canadian Women's Breast Cancer Foundation. A.S.C. holds an MHRC studentship and a Women's Health Research Foundation of Canada scholarship. L.C.M. and J.R.D. are MRC (Canada) Scientists.

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