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Advances in Brief

Hormone-dependent Regulation of BRCA1 in Human Breast Cancer Cells

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Abstract

BRCA1 mRNA and protein levels are regulated by the steroid hormones estrogen and progesterone in human breast cancer cells. BRCA1 mRNA and protein levels were significantly decreased in estrogen-depleted MCF-7 and BT20T cells and increased again after stimulation with β-estradiol. The increase in BRCA1 expression upon stimulation with estrogen was not coordinated with the early induction of the estrogen-dependent *pS2* gene but closely paralleled the delayed increase in the S-phase dependent marker cyclin A. T47-D cells deprived of steroid hormones and subsequently stimulated with progesterone also showed a delayed increase in BRCA1 mRNA expression. However, no change in BRCA1 protein was detected in these cells. When considered together, the data suggest that steroid hormones may affect BRCA1 expression indirectly by altering the proliferative status of the cells rather than acting directly on DNA sequences in the *BRCA1* gene itself.

Introduction

Breast cancer is the most common malignancy among women in North America, with the number of newly diagnosed cases increasing at a rate of about 1% a year (1). As one approach toward understanding the genetic basis of this disease, Miki *et al.* (2) have recently reported the cDNA sequence of a gene on chromosome 17q that that confers susceptibility to early onset familial breast and ovarian cancers. Analysis of the *BRCA1* gene in familial and sporadic breast tumors has suggested that mutation is not a common event in sporadic cases of breast cancer; however, a recent report has indicated that somatic mutations in the *BRCA1* gene may play a role in a subset of sporadic ovarian cancers (3). In addition to genetic factors, hormones of pituitary and ovarian origin, including estrogens, progestins, and prolactin, all play a role in the control of normal and breast tumor cell proliferation (reviewed in Refs. 4 and 5). The response to polypeptide growth factor and steroid hormones is abrogated as normal mammary epithelial cells progress to gain a malignant phenotype (6). Since estrogens and progestins can influence both the growth and differentiation of normal and breast cancer cells, we examined whether the breast cancer susceptibility gene *BRCA1* was regulated in response to these hormonal signals.

Materials and Methods

Cells and Cell Culture. MCF-7 cells were obtained from Dr. R. Buick (University of Toronto, Toronto, Ontario, Canada). The BT20T cells were a gift from Dr. K. Keyomarsi (New York State Department of Health, Albany, NY), and the T47-D cells were purchased from American Type Culture Collection. All cells were cultured in IMEM[3] (Biofluids, Inc.) supplemented with 10 mM HEPES, 2 mM glutamine, 0.1 mM nonessential amino acids, 10% fetal bovine serum, and 2 μg/ml insulin. The cells were routinely screened for *Mycoplasma* contamination and maintained at 37°C in an atmosphere of 6.0% CO_2. To study the effects of hormones on BRCA1 expression, the respective cells were plated at approximately 2×10^6 cells/150-mm tissue culture dish into phenol red-free IMEM (GIBCO) supplemented with 5% charcoal-stripped calf serum and incubated for 5 or 6 days. At this time the cells were refed with phenol red-containing IMEM supplemented with 5% charcoal-stripped calf serum and 10 nM β-estradiol or 10 nM progesterone (Sigma).

RNA Isolation and Northern Blot Analysis. Total RNA was prepared and analyzed as described previously (7). RNA (20 μg) was electrophoresed on denaturing formaldehyde gels, transferred to Magna NT membranes, and cross-linked with UV. A combination of two probes was used for Northern blot analyses. An 1179-bp fragment corresponding to cDNA sequences from 1025 to 2222 of BRCA1 was amplified by PCR and subcloned into the *Eco*RI and *Sma*I sites of pGem7. A 624-bp PCR product corresponding to cDNA sequences from 4388 to 5011 was generously provided by Dr. Roger Wiseman (NIEHS). The authenticity of the subcloned probes was verified by DNA sequence analysis using standard techniques (Sequenase; U. S. Biochemicals). The 36B4 probe was an 800-bp *Pst*I fragment (8), and the cyclin A probe contained the entire cDNA (generously provided by Dr. Tony Hunter). All probes were labeled with [α-^{32}P]dCTP to a specific activity of approximately 1×10^9 cpm/μg DNA using a random primed labeling kit (Boehringer Mannheim). The amount of radioactivity hybridized with specific bands on the RNA filters was quantitated using a Molecular Dynamics Phosphoimager.

Western Blot Analyses. Western blot analysis was performed as described previously (7). Protein lysate (100 μg/lane) was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with Tris-buffered saline containing 8% dried milk and 0.1% Tween 20, filters were probed using antibodies to BRCA1 (2 μg/ml immunoaffinity-purified C-20; Santa Cruz Biotechnology Inc.), cyclin A (4 μg/ml cyclin A antibody; Pharmingen), and actin (2 μg/ml actin antibody-1; Oncogene Science). Specific complexes were detected using a chemiluminescent technique according to the manufacturer's recommendations (New England Nuclear). The X-ray films were captured with a Fotodyne digital image system and quantitated using the NIH Image Analysis software program.

Results

BRCA1 mRNA expression was examined in estrogen receptor-positive MCF-7 cells that were depleted of estrogens for 6 days and subsequently refed with phenol red-containing medium supplemented with charcoal-stripped calf serum and 10 nM β-estradiol for the times indicated (Fig. 1*A*). We have shown previously that steroid depletion results in a significant decrease in the growth of these MCF-7 cells (9). Northern blot analysis was performed using a combination of two probes that hybridize to distinct regions of the BRCA1 cDNA. Both of these probes recognize the major 8.1-kb transcript (designated *M*) while only the 624-bp 3′ probe hybridizes to the lower abundance 4.6-kb BRCA1 mRNA (designated *m*).[4] BRCA1 mRNA levels were low in estrogen-deprived MCF-7 cells (time 0) and remained depressed for the first 8 h after feeding the cells with fresh medium containing β-estradiol. BRCA1 mRNA levels increased gradually 12 and 24 h after treatment. The kinetics of BRCA1 mRNA accumulation was strikingly similar to that observed for the S-phase-dependent marker cyclin A (Fig. 1*A*, *second panel*).

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[3] The abbreviation used is: IMEM, improved MEM.

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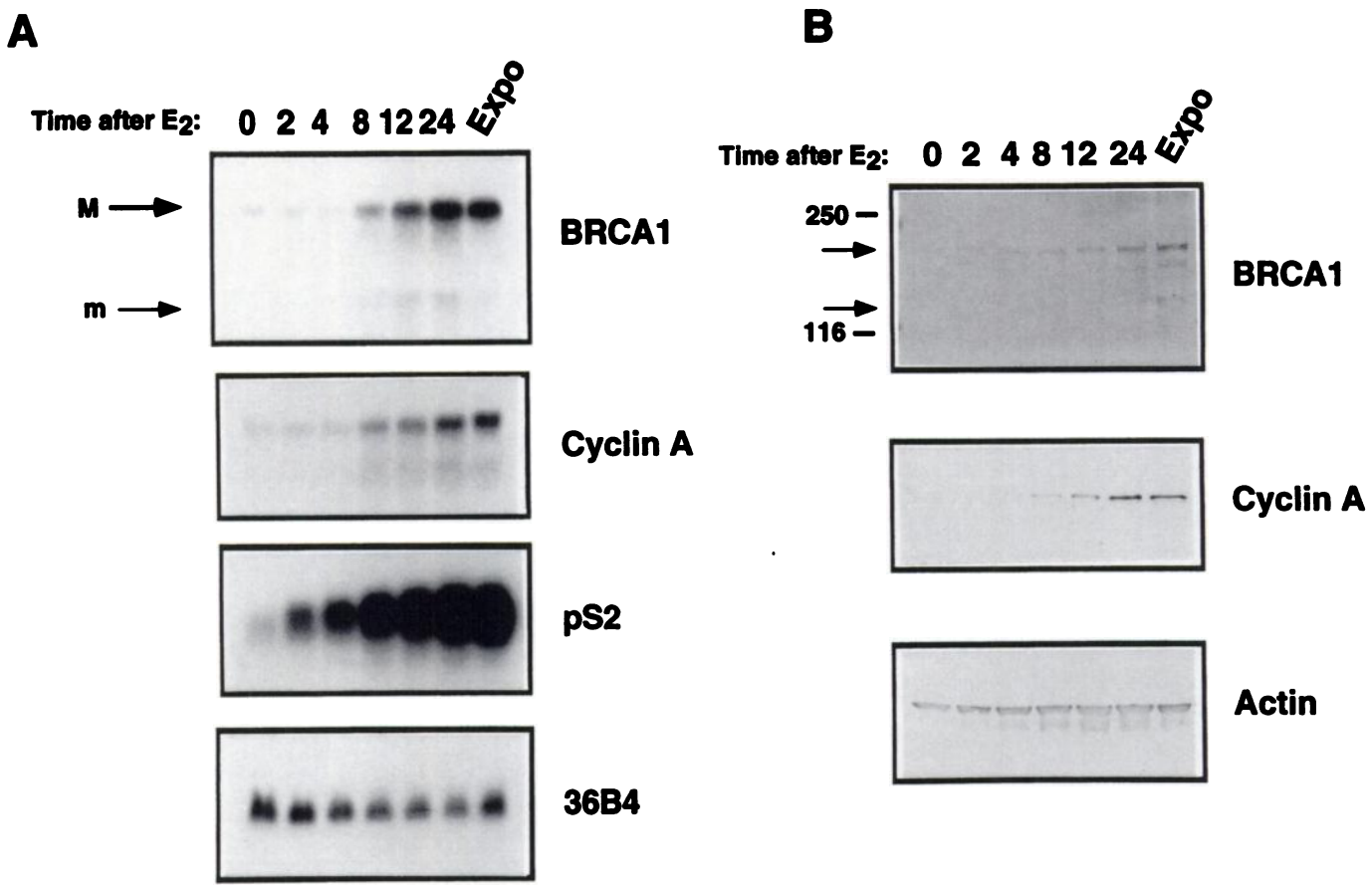

Fig. 1. *A*, BRCA1 mRNA expression in estrogen receptor-positive MCF-7 cells. MCF-7 cells were deprived of estrogens as described in "Materials and Methods." At time 0 the cells were stimulated by addition of IMEM supplemented with 5% charcoal-stripped calf serum and 10 nM β-estradiol. RNA obtained from cells at the times designated was electrophoresed on a denaturing formaldehyde gel, transferred to Magna NT membranes, and probed sequentially for the expression of BRCA1 (*first panel*), cyclin A (*second panel*), pS2 (*third panel*), and the constitutively expressed mRNA 36B4 (*fourth panel*). *M*, major 8-kb BRCA1 transcript; *m*, minor 4.6-kb transcript. *B*, Western blot analysis of proteins from estrogen-deprived and -stimulated MCF-7 cells. Total protein extracts were prepared from estrogen-depleted (time 0) cells and cells that were stimulated with β-estradiol for the times designated above each lane. After separation on SDS-polyacrylamide gels, the proteins were transferred to nitrocellulose and probed for BRCA1 (*upper panel*), cyclin A (*middle panel*), and actin (*lower panel*). *Arrows*, two BRCA1 proteins.

To compare the kinetics of BRCA1 mRNA accumulation with that of a gene containing a known estrogen-response element within its promoter, the same filters were stripped and reprobed for *pS2* gene expression. pS2 mRNA levels were increased earlier (approximately 2–4 h after estrogen treatment) and rose to much higher levels than the proliferation-associated BRCA1 and cyclin A mRNAs. As a control for RNA loading and transfer, these and all subsequent filters were reprobed with the estrogen-independent marker 36B4 (10).

Using a peptide antibody that recognizes an epitope at the carboxyl terminus of the BRCA1 protein, we have demonstrated the presence of two BRCA1 proteins having approximate M_rs 185,000 and 140,000, respectively, in most breast cancer cells.[4] Examination of BRCA1 protein levels in samples that were treated and harvested in parallel revealed a decrease in estrogen-depleted MCF-7 cells (Fig. 1*B*). Digitization and quantitation of the X-ray films revealed an approximate 4-fold increase in the amount of the M_r 185,000 protein after estrogen treatment. Twenty-four h after estrogen addition, the amount of the M_r 185,000 BRCA1 protein was similar to that present in exponentially growing cells. In contrast, the M_r 140,000 BRCA1 protein did not increase until 24 h after estrogen stimulation. Thus, the rise in BRCA1 protein levels was coordinated with the rise in cyclin A protein levels (Fig. 1*B*, *middle panel*). The results observed were specifically due to the effects of estrogen, as MCF-7 cells depleted of estrogen and refed with medium containing the antiestrogen tamoxifen showed no increase in either BRCA1 or cyclin A mRNA or protein levels (data not shown).

To determine whether the changes observed were specific for MCF-7 cells, the same experiments were performed using another estrogen-dependent cell line, BT20T. BRCA1 mRNA levels were significantly decreased in estrogen-depleted BT20T cells, but not to the same magnitude as observed in MCF-7 cells (Fig. 2*A*). Expression of both BRCA1 mRNAs increased approximately 8–12 h after restimulation with estrogen. The kinetics of BRCA1 mRNA accumulation was similar to that of the proliferation marker cyclin A. Both of these mRNAs were increased 12–24 h after estrogen stimulation. In contrast, the mRNA for the estrogen-inducible gene *pS2* was maximally increased 2 h after refeeding with estrogen (Fig. 2*A*, *third panel*).

BRCA1 protein levels were lower in estrogen-depleted BT20T cells when compared with an exponentially growing population (Fig. 2*B*). The amounts of the M_rs 140,000 and 185,000 BRCA1 proteins increased approximately 2.5-fold after estrogen stimulation for 24 h. Cyclin A protein levels were also decreased in the estrogen-depleted cells and did not increase significantly until 24 h after refeeding. When considered together, these data suggest that the increase in BRCA1 mRNA and protein levels after estrogen stimulation reflect a change in the proliferative status of the cells and may not be due to a direct effect of estrogen on DNA sequences in the *BRCA1* gene.

T47-D breast carcinoma cells are dependent on progesterone and insulin for their growth *in vitro* (11). We examined BRCA1 mRNA expression in T47-D cells that were depleted of steroids and subsequently restimulated with medium containing progesterone (Fig. 3*A*).

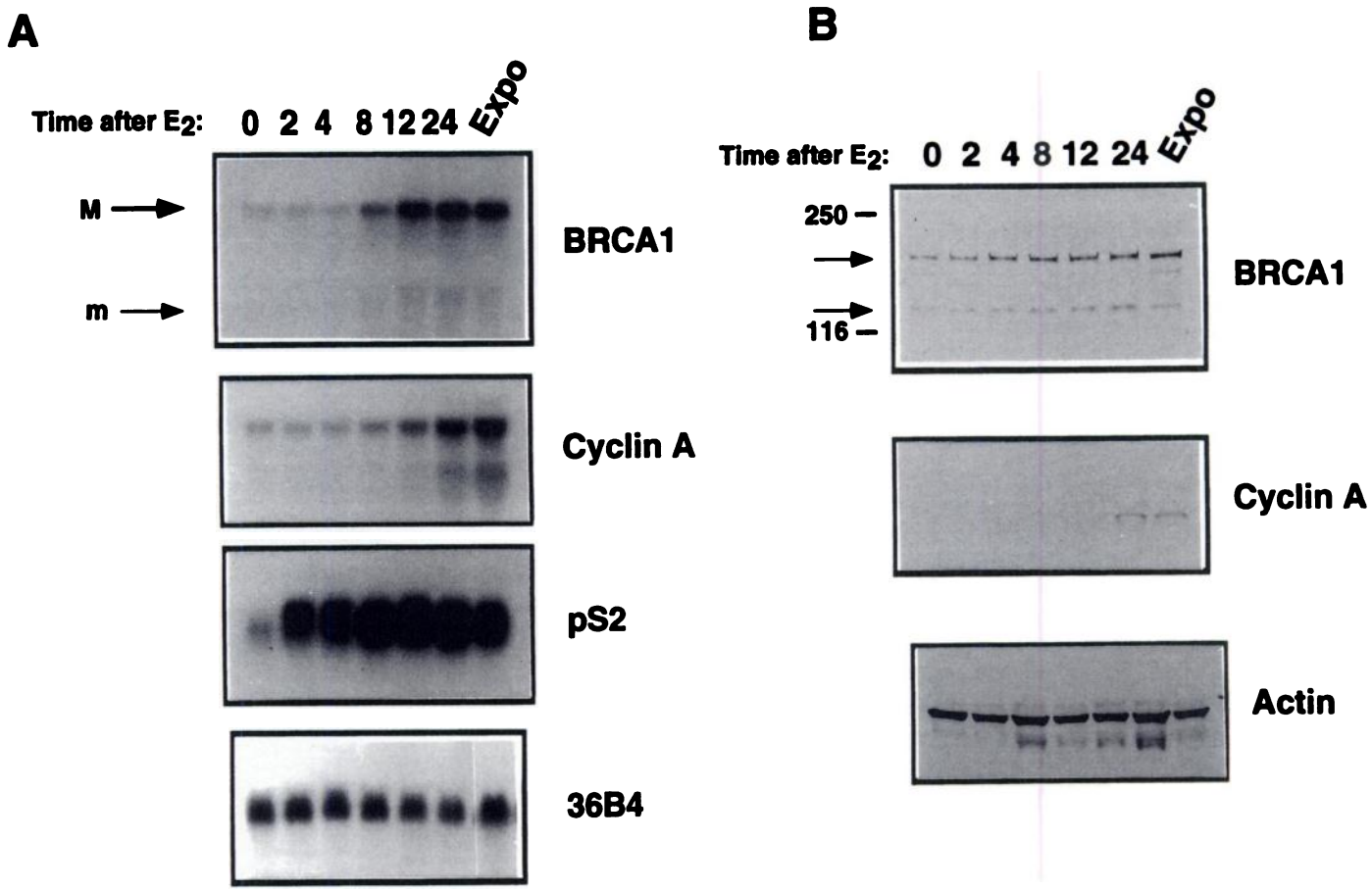

Fig. 2. *A*, BRCA1 mRNA expression in estrogen receptor-positive BT20T cells. BT20T cells were deprived of estrogens as described in "Materials and Methods." At time 0 the cells were stimulated by addition of IMEM supplemented with 5% charcoal-stripped calf serum and 10 nM β-estradiol. RNA obtained from cells at the times designated was analyzed for expression of BRCA1 (*first panel*), cyclin A (*second panel*), pS2 (*third panel*), and 36B4 mRNA (*fourth panel*) as described in the legend to Fig. 1*A*. *M*, major 8-kb BRCA1 transcript; *m*, minor 4.6-kb transcript. *B*, Western blot analysis of proteins from estrogen-deprived and -stimulated BT20T cells. Total protein extracts were prepared from estrogen-depleted cells (time 0) and cells that were stimulated with β-estradiol for the times designated above each lane. BRCA1 (*upper panel*), cyclin A (*middle panel*), and actin (*lower panel*) protein levels were determined as described in the legend to Fig. 1*B*. *Arrows*, two BRCA1 proteins.

The results show that BRCA1 mRNA was markedly decreased in steroid-depleted T47-D cells and increased significantly 8–12 h after progesterone stimulation. In these cells the increase in BRCA1 mRNA again correlated well with the respective increase in cyclin A mRNA levels (Fig. 3*A*, *second panel*). In contrast, the mRNA for a progesterone-regulated gene, fatty acid synthetase (12), was induced at earlier times (4 h after treatment), peaked by 12 h, and returned to basal levels 24 h after treatment.

Examination of BRCA1 protein levels in parallel cultures of T47-D cells revealed no change in the steroid-depleted *versus* progesterone-stimulated cells (Fig. 3*B*). However, analysis of cyclin A protein levels in the same extracts revealed a significant rise beginning 8–12 h after progesterone stimulation. Thus, in T47-D cells BRCA1 protein expression appears to be regulated independently of BRCA1 mRNA levels.

Discussion

In this study we show that BRCA1 mRNA levels are regulated in human breast cancer cells by the steroid hormones estrogen and progesterone. To determine whether this increase was correlated with a possible direct effect of estrogens or whether it was more closely associated with the proliferative effects of estrogens on breast cancer cells, the kinetics of BRCA1 mRNA accumulation was compared with that observed for pS2 and cyclin A mRNAs. *pS2* was originally isolated as a gene that was specifically induced by estrogens in MCF-7 cells (13). Subsequent studies showed that the effects of estrogens were mediated by consensus estrogen-response elements within the promoter region of this gene (14). Cyclin A is one of the regulatory components of the cell division kinases (cyclin-dependent kinase 2). Because its level and activity is specifically increased just prior to S-phase entry (15, 16), we chose to use this gene as a marker for late G_1-S-phase cells.

Our results demonstrated that BRCA1 mRNA and protein levels were regulated in concert in estrogen-deprived and restimulated MCF-7 and BT20T cells. The kinetics of BRCA1 induction was delayed when compared to the early rise in estrogen-regulated pS2 mRNA levels. In contrast, we observed a good correlation between the rise in BRCA1 mRNA and protein with that of the S-phase-regulated gene *cyclin A*. Therefore, the effects of estrogens on BRCA1 expression may be due to overall changes in the proliferative status of the cells as opposed to a direct effect of estrogen on DNA sequences in the *BRCA1* gene itself.

The increased BRCA1 mRNA levels observed in T47-D cells may also reflect an indirect effect of progesterone on cell proliferation rather than a direct effect on DNA sequences in the *BRCA1* gene itself. In these cells the kinetics of BRCA1 mRNA accumulation closely paralleled that of cyclin A rather than the increase in progesterone-regulated fatty acid synthetase mRNA levels. In contrast to estrogen-depleted and -stimulated cells, the level of BRCA1 protein was not decreased in progesterone-depleted T-47D cells. These results

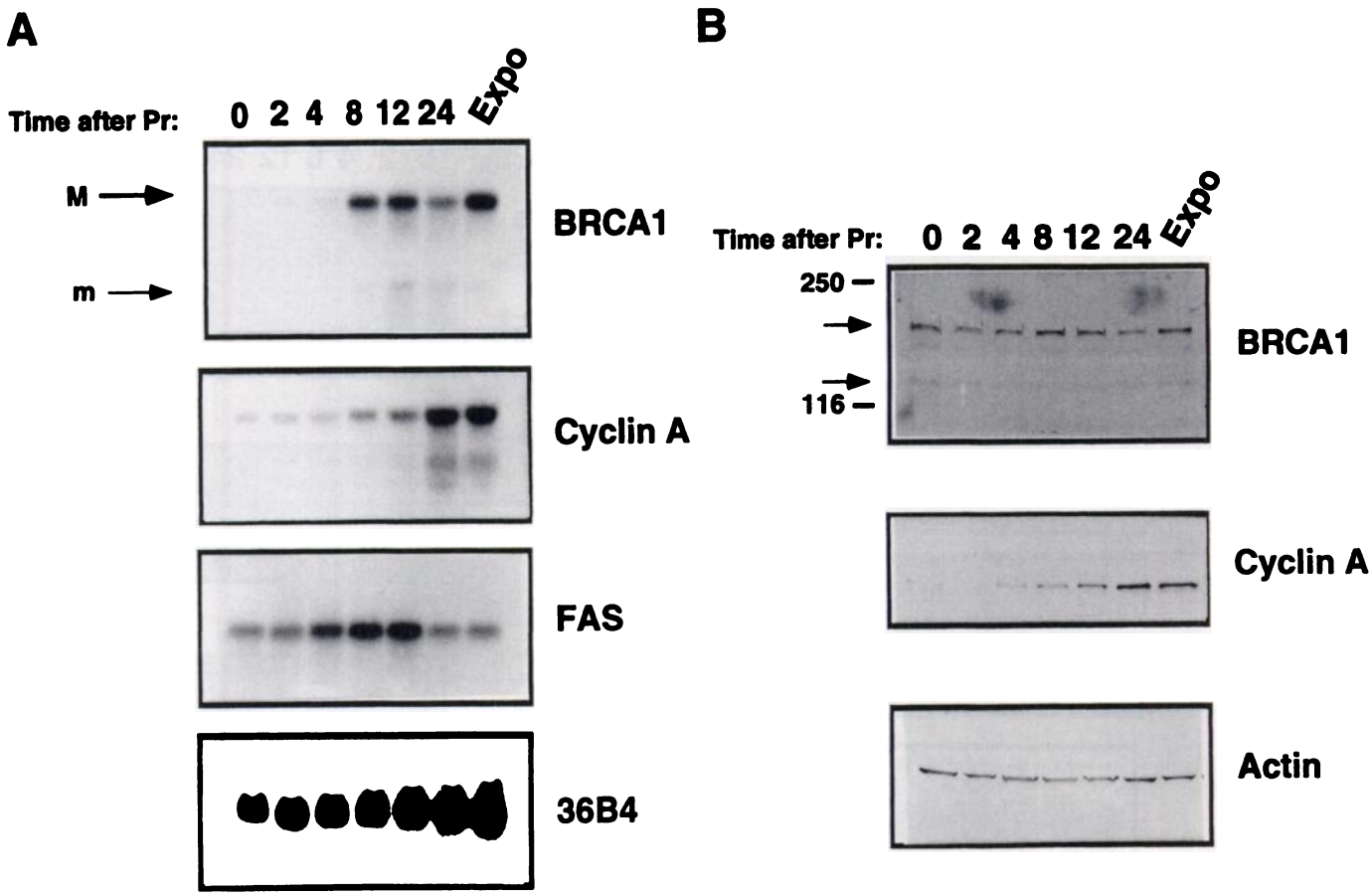

Fig. 3. *A*, BRCA1 mRNA expression in progesterone receptor-positive T47-D cells. T47-D cells were deprived of steroid hormones as described in "Materials and Methods." At time 0 the cells were stimulated by addition of IMEM supplemented with 5% charcoal-stripped calf serum and 10 nM progesterone. RNA obtained from cells at the times designated was analyzed for expression of BRCA1 (*first panel*), cyclin A (*second panel*), fatty acid synthetase (*FAS, third panel*), and 36B4 mRNA (*fourth panel*) as described in the legend to Fig. 1*A*. *B*, Western blot analysis of proteins from steroid hormone-deprived and -stimulated T47-D cells. Total protein extracts were prepared from hormone-depleted cells (time 0) and cells that were stimulated with progesterone for the times designated above each lane. BRCA1 (*upper panel*), cyclin A (*middle panel*), and actin (*lower panel*) protein levels were determined as described in the legend to Fig. 1*B*.

suggest an uncoupling of mRNA and protein levels under certain metabolic conditions.

A similar uncoupling of BRCA1 mRNA and protein levels was detected in synchronized populations of immortalized MCF10 and 184B5 cells.[4] In these two cell lines BRCA1 mRNA levels were tightly regulated during the cell cycle while BRCA1 protein levels remained constant. There are several possible explanations for discrepancies between mRNA and protein levels under different physiological conditions. BRCA1 proteins may have an increased half-life in T47-D cells. Alternatively, the low level of BRCA1 mRNA in the progesterone-depleted cells may be translationally regulated by other cellular proteins or antisense RNA transcripts. Precedents for both mechanisms of regulation exist for other genes (17). Interestingly, many developmentally regulated genes exhibit regulation at the level of mRNA translation (17).

The underlying mechanisms responsible for the changes in BRCA1 expression remain unknown. However, this report provides evidence to indicate that BRCA1 mRNA and protein levels can be regulated in response to steroid hormones. If the BRCA1 protein(s) functioned as a feedback regulator of growth in proliferating breast epithelial cells, women who are at increased risk of developing breast cancer due to nonsense or missense mutations in the *BRCA1* gene may have an altered cellular response to hormonal stimuli. Future studies aimed at determining the role of this gene in the growth and/or differentiation of human breast epithelial cells should provide insights into novel approaches for the prevention and treatment of these cancers.

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