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Changes in *BRCA2* Expression during Progression of the Cell Cycle

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It has been shown that genetic alterations in *BRCA1* and *BRCA2* can predispose an individual to develop breast cancer. We investigated the expression of both *BRCA2* and *BRCA1* during the progression of the cell cycle by northern blot analysis. In MCF-10F (normal breast epithelial cell line) and MCF-7 (breast cancer cell line) cells the expression of *BRCA2* RNA was low in G0 and early G1 phases then up-regulated at the G1/S phase junction. Expression of *BRCA2* was maintained at relatively high levels when cells progressed through S and G2/M phases. For MCF-7 cells, the level of *BRCA2* transcript decreased as cells were released from nocodazole-mediated metaphase arrest. This is consistent with the observation of low but detectable *BRCA2* RNA level in G1 phase of the cell cycle. For both cell lines, the patterns of RNA expression of *BRCA1* and *BRCA2* were similar during the proliferation phase of cell cycle. However, the transcripts from both genes were undetectable in quiescent cells. These results suggest important functions for both *BRCA2* and *BRCA1* in regulation of cell growth.

The *BRCA2* gene, like *BRCA1,* behaves as a dominantly inherited breast cancer susceptibility gene and contributes to a significant fraction of familial breast and ovarian cancers (1, 2, 3). Involvement of *BRCA2* in the development of familial breast cancer has been demonstrated by germline mutations of this gene which predispose to breast cancer and the accompanied loss of heterozygosity (LOH) at this locus in the breast tumors which subsequently developed in these patients (4, 5). However, *BRCA2,* like *BRCA1,* is not frequently mutated in sporadic breast and ovarian cancers, and only rare *BRCA2* mutations have been detected in established cell lines derived from other types of cancer (6, 7, 8, 9). The role played by *BRCA2* in tumor progression remains a challenge to our understanding of cancer biology. Therefore, strategies other than molecular genetic studies are required to further explore the biological functions of *BRCA2* (and *BRCA1*) and the mechanism of tumorigenesis in *BRCA2*-mutated cells.

It has been well established that deregulated cell proliferation can lead to tumor development. Some tumor suppressor genes, such as *p53* and *RB,* have been shown to play important roles in protecting cells from chaotic deregulation by functioning as regulators at check points of the cell cycle before committing to active cell proliferation (10, 11, 12). Recently, it has been reported that the *BRCA1* gene is expressed in a cell cycle-dependent manner. The RNA and protein levels are up-regulated at the G1/S junction and maintained at relatively high levels in S and M phases, suggesting that BRCA1 functions as a regulator of cell proliferation through the cell cycle (13, 14, 15). This hypothesis is further supported by the observation that the *BRCA1* protein is phosphorylated during cell proliferation and is able to bind to other cell cycle regulators including cdk2, cyclin A and cyclin D (13). In this report, we describe the cell cycle-dependent RNA expression of *BRCA2* and *BRCA1.* These results suggest that *BRCA2* (and *BRCA1*) may play roles in controlling the progression of latter stages of the cell cycle.

MATERIALS AND METHODS

Cell culture and synchronization. MCF-10F cells (a normal breast epithelial cell line) were maintained in DMEM/F12 containing 5 % horse serum, insulin 10 μg/ml, EGF 20 ng/ml, cholera toxin 100 ng/ml and hydrocortisone 0.5 μg/ml. MCF-7 cells (a breast cancer cell line) were maintained in DMEM/F12 medium containing 10 % fetal bovine serum. Synchronization in G0 phase was attained by serum starvation using DMEM/F12 medium containing 0.5 % horse serum (for MCF-10F cells) and 0.5 % fetal bovine serum (for MCF-7 cells). Cell cultures were enriched at the G1/S junction by double thymidine block using 2 mM thymidine for the first 16-hour block, release for 9 hours in standard media with serum, then followed by a second 16-hour block with 2 mM of thymidine. S phase synchronization was

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attained by releasing the serum-starved cells for 18 hours in standard media. M phase synchronization was achieved by nocodazole (0.15 μg/ml) arrest for up to 15 hours. For the starvation-releasing experiments, cells were grown in medium containing 0.5 % horse serum (for MCF-10A) or fetal bovine serum (for MCF-7) for 24 hours. Cell cultures were then washed with PBS and released by adding complete growth medium for different time intervals (0 hr, 6 hr, 12 hr, 18 hr, 24 hr and 30 hr). For the M phase release experiment, the nocodazole-arrested M phase cells were collected by shake-off and re-plated in complete medium without nocodazole for 0 hour, 2 hours, and 4 hours.

RNA analysis. Total RNA was isolated from cells using the Trizol reagent (Life Technologies, Gaithersburg, MD) according to the protocol recommended by the manufacturer. Total RNA (20 μg) isolated from cells synchronized at different stages of the cell cycle was size-fractionated on an 1.2 % agarose gel containing formaldehyde. was transferred to a nylon membrane and hybridized with the ^{32}P-labeled *BRCA2* cDNA probe cloned by RT-PCR. To detect the *BRCA1* RNA, the membrane was stripped and re-probed with a ^{32}P-labeled 1.6 kb *Bam*HI-*Kpn*I fragment of the *BRCA1* cDNA (a generous gift from Dr. Wen-Hwa Lee).

Cell cycle analysis. A duplicate culture for each synchronized sample was examined for DNA content by staining with propidium iodide followed by analysis with an EPICS PROFILE flow cytometer (Coulter) to confirm the cell cycle stage (16). The data was then processed with the MultiCycle computer program (Phoenix Flow System).

RESULTS

In order to determine whether *BRCA2* RNA expression is coordinately regulated with the progress of the cell cycle, total RNA was isolated from MCF-10F cells synchronized at different cell cycle stages and subjected to northern blot analysis using a full length *BRCA2* cDNA probe (unpublished data; Figure 1). Cells synchronized in G0 phase by serum starvation had no detectable *BRCA2* message, while cells synchronized at the G1/S boundary by double thymidine block expressed high levels of *BRCA2* RNA. High levels of *BRCA2* RNA were maintained in cells synchronized at S phase, either by releasing from serum starvation (78% S phase cells, Figure 1) or by treatment with aphidicholin (data not shown). M phase cells synchronized by nocodazole treatment and collected by shake-off also contained relatively high levels of *BRCA2* RNA (90% M phase cells, Figure 1). The pattern of *BRCA2* RNA expression in MCF-7 was similar to that observed in MCF-10F cells (data not shown). Undetectable in serum-starved cells, the *BRCA2* RNA expression in MCF-7 cells was high at the G1/S boundary after double thymidine block, and the intensity of the signal remained relatively high through S phase and M phase.

A different method of cell cycle synchronization was used to confirm these results and further explore the kinetics of *BRCA2* RNA accumulation during the cell cycle. MCF-10F cells were synchronized by serum starvation for 24 hours and then released by adding complete growth medium. The *BRCA2* RNA levels were determined at different time points after release (Figure 2A). A duplicate cell culture at each time point was

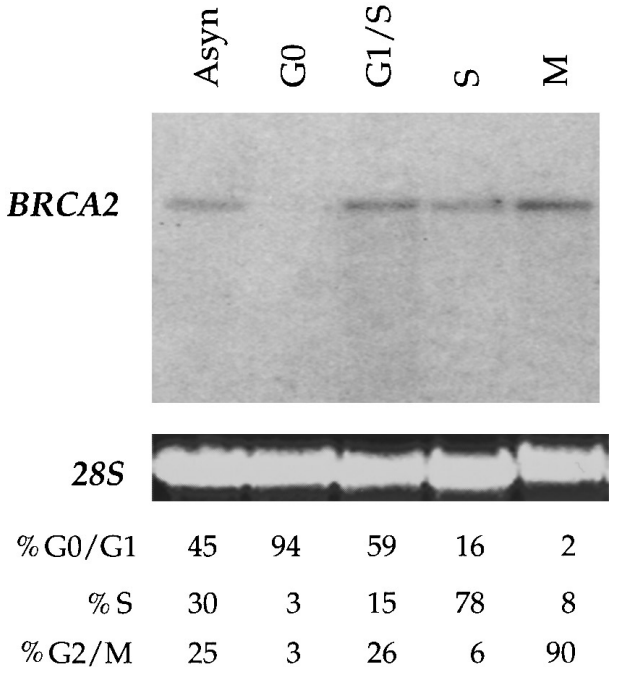

FIG. 1. Cell cycle-dependent expression of *BRCA2* in MCF-10F cells. 20 μg of total RNA isolated from MCF-10F cells synchronized at different stages of the cell cycle was size-fractionated on a 1.2% agarose gel containing formaldehyde. The RNA was transferred to a nylon membrane and hybridized with the ^{32}P-labeled *BRCA2* cDNA probe. A duplicate culture for each synchronized sample was examined for DNA content by staining with propidium iodide followed by cell cycle analysis with a flow cytometer. The percentage of total cells at each stage (G1, S and G2/M) is indicated under each lane. The 28S ribosomal RNA stained with ethidium bromide was used as an internal control for equal loading. Asyn—asynchronous cell culture.

subjected to fluorescence activated cell sorting (FACS) analysis to examine the progress of the cell cycle (Figure 2B). The *BRCA2* RNA level remained undetectable at 6 hours following release from serum starvation. The level increased by 12 hours as the proportion of S phase cells began to increase. The *BRCA2* RNA remained at the same level at the 18 hr time point, although the S phase population (78%) at 18 hr was more predominant than at 12 hr (21 %) or 24 hr (36 %). However, the *BRCA2* RNA level further increased by the 24 hr time point when the proportion of cells in G2/M phases increased (47 % G2/M cells).

The cell cycle-correlated pattern of *BRCA2* expression observed in this starvation-release time course experiment is not unique to the MCF-10F cell line. A similar pattern of *BRCA2* expression was observed for the breast cancer cell line MCF-7 (Figure 2C). Low but detectable levels of the *BRCA2* RNA appeared by 6 hours after release. Induction of the *BRCA2* RNA accumulation was detected before cells entered S phase (12 hr), the level remained elevated during S phase (24 hr), and further increased at the 30 hr time point where the proportion of G2/M cells (25%) was relatively high

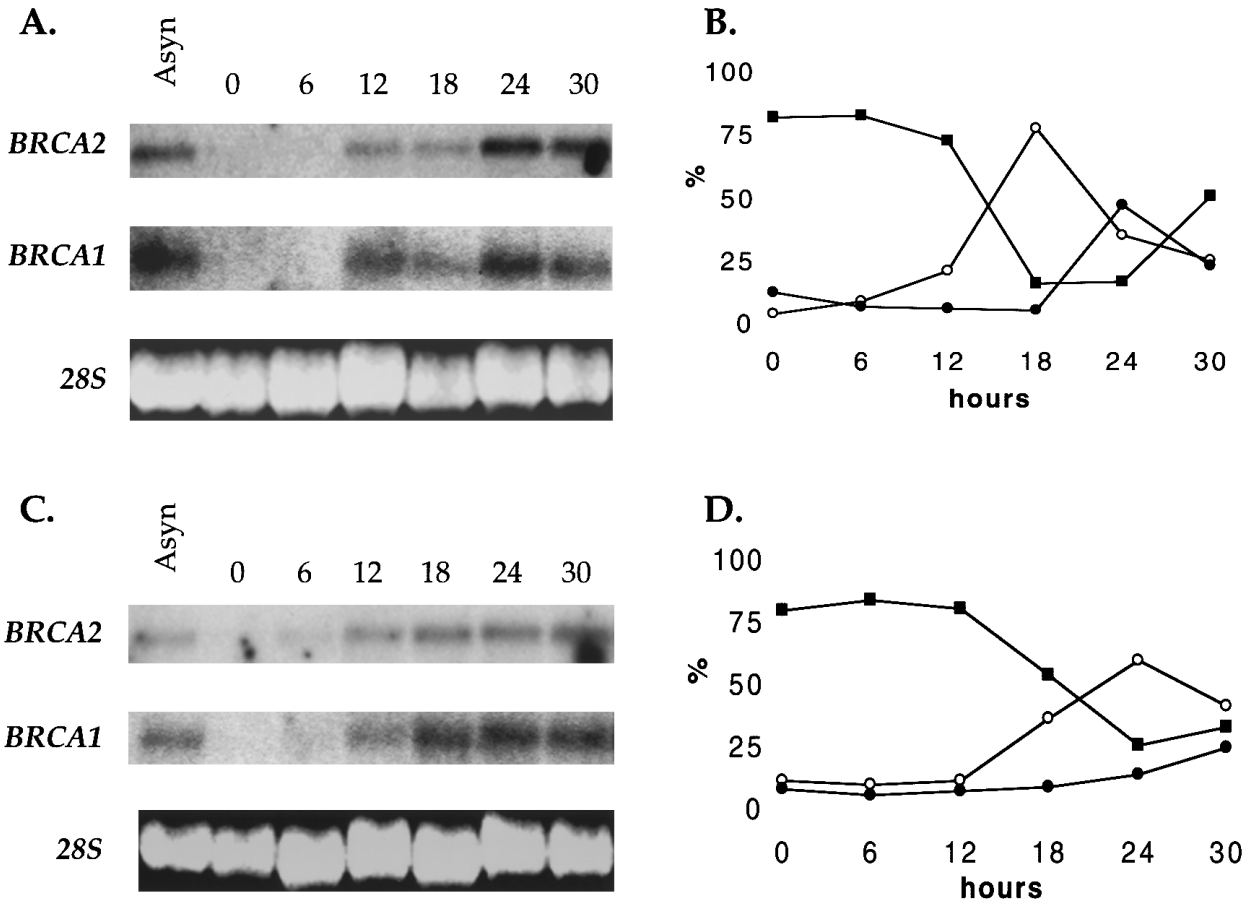

FIG. 2. *BRCA2* expression in cells released from serum starvation. **A.** The expression of *BRCA1* and *BRCA2* showed similar patterns of induction during cell proliferation in MCF-10F cells. MCF-10F cells were grown in medium containing 0.5 % horse serum for 24 hours. Cell cultures were then released by adding complete growth medium for different times (0 hr, 6 hr, 12 hr, 18 hr, 24 hr and 30 hr). Northern analysis was performed as described in Figure 1. Following analysis with the *BRCA2* probe, the same membrane was stripped and re-probed with a DNA fragment of the *BRCA1* cDNA. The ethidium bromide-stained 28S ribosomal RNA was used as an internal control. Asyn—asynchronous cells. **B.** A duplicate culture for each time point in the experiment described in "A" was examined with a flow cytometer, and the percentage of cells in G0/G1 (solid squares), S (open circles) and G2/M (solid circles) phases were plotted. **C.** The expression of *BRCA1* and *BRCA2* showed similar patterns of induction during the cell cycle of MCF-7 cells. MCF-7 cells were serum-starved and then released by adding normal growth medium for different times as described in "**A**". Northern analysis was performed as described in "**A**". **D.** A duplicate culture for each time point in the experiment described in "**C**" was examined using a flow cytometer. The percentage of cells in G0/G1 (solid squares), S (open circles) and G2/M (solid circles) phases were plotted.

compared with the previous time points (Figure 2D). To summarize, the level of *BRCA2* RNA is low in G0 and early G1 phases and begins to increase at the G1/S junction. These high levels were maintained through the remaining proliferation stages. The northern blots of the MCF-10F and MCF-7 time course experiments were stripped and then hybridized with a cDNA probe of *BRCA1*. In both cases, the cell cycle-dependent expression of *BRCA1* was parallel to the pattern observed for *BRCA2* expression, consistent with the earlier findings that *BRCA1* RNA expression is cell cycle-dependent and is elevated when cells enter S phase.

A different approach was used to better characterize the cell cycle-dependent manner of the *BRCA2* RNA expression in G2/M phases. MCF-7 cells arrested in metaphase by nocodazole were collected and plated in fresh medium without the drug. FACS analysis showed that most cells exited M phase within two hours of release (G1 71%), and this change was accompanied by a modest decrease in the level of *BRCA2* RNA. The *BRCA2* RNA band intensity decreased by 50 % within four hours after release from the metaphase arrest (Figure 3). These results demonstrate that, in contrast to cells in G0 phase which have undetectable levels of the *BRCA2* transcript, cells exiting M phase and re-entering G1 phase contained low but detectable levels of the *BRCA2* RNA. This experimental approach could not be successfully performed with MCF-10F cells due to poor cell viability after being released from nocodazole treatment.

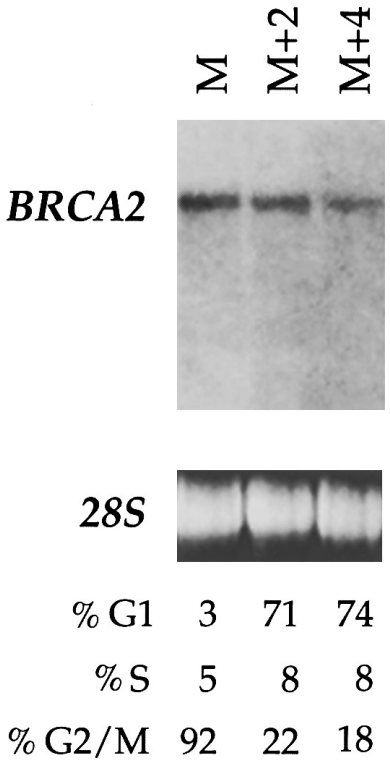

FIG. 3. Decreasing expression levels of *BRCA2* in cells leaving M phase. Nocodazole-arrested M phase cells were collected by shake-off and re-plated in complete medium without nocodazole for 0 hour (M), 2 hours (M+2), and 4 hours (M+4). Total RNA isolation and the northern blot analysis for *BRCA2* were carried out using a *BRCA2* cDNA probe. A duplicate culture for each time point was examined for DNA content to confirm the cell cycle stage. The percentage of total cells examined in each stage (G1, S and G2/M) is indicated as numbers under each lane. The 28S ribosomal RNA stained with ethidium bromide was used as an internal control for equal loading.

DISCUSSION

We have demonstrated that breast cancer and breast epithelial cells arrested in G0 or early G1 have low levels of *BRCA2* (and *BRCA1*) transcript while, in contrast, high levels of the *BRCA2* RNA were produced after releasing into late G1, S and M phases. These results together suggest that *BRCA2* (and perhaps *BRCA1*) is expressed in cells engaged in active proliferation and its expression is regulated in a cell cycle-dependent manner as suggested by Vaughn *et al.* (17). However, our current studies have further extended the observation to G2/M and post-mitotic phases by using two experimental methods (serum starvation and nocodazole arrest). MCF-7 and MCF-10F cell cultures enriched with G2/M phase cells contained high levels of *BRCA2* transcript relative to that of S phase. The fact that *BRCA2* is universally expressed in proliferating cells and the observation of the sustained *BRCA1* and *BRCA2* RNA levels during the G2/M phases would imply potential roles for these genes in controlling and/or monitoring the latter stages of the cell cycle. The potential importance of *BRCA2* in G2/M phases is further implicated by the fact that in G2/M phases, like in G1/S phases, reside a number of surveillance mechanisms to ensure the normal ordering and processing of mitotic cell proliferation. Examples include the "checkpoints" of DNA damage (18), spindle assembly (19), and spindle pole body duplication (20).

Whether the cell cycle-correlated RNA expression of *BRCA2* has any biological significance and whether *BRCA2* and *BRCA1* are functionally related given the parallel expression patterns await further investigation. Nevertheless, the findings reported here provide additional information which will be important in determining the biological functions of *BRCA1* and *BRCA2*.

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