The human BRCA1 tumor suppressor interacts with transcriptional machinery, including RNA polymerase II (RNA pol II). We demonstrated that interaction with RNA pol II is a conserved feature of BRCA1 proteins from several species. We found that full-length BRCA1 proteins universally fail to activate transcription in classic GAL4-UAS one-hybrid assays and that the activity associated with the human BRCA1 C terminus was poorly conserved in closely related homologs of the gene. Fractionation studies demonstrated that BRCA1 proteins from all species tested interacted specifically with hyperphosphorylated pol II (IIIO), in preference to hypophosphorylated RNA pol II (IIA) expected at promoters. BRCA1-RNA pol II complexes showed evidence of a multiply phosphorylated heptad repeat domain in the catalytic subunit (p220) of RNA pol II, and the complex was highly functional in transcriptional run-off assays. Interestingly, endogenous BRCA1 associated with a large fraction of the processive RNA pol II activity present in undamaged cells, and the interaction was disrupted by DNA-damaging agents. Preferential interaction with processive RNA pol II in undamaged cells places BRCA1 in position to link late events in transcription with repair processes in eukaryotic cells.

Mutations in the BRCA1 tumor suppressor gene are associated with an increased risk of breast and ovarian cancer and an elevated incidence of certain other cancers (1–3). Genetic and biochemical data place BRCA1 as a downstream target of ATM/ATR in cellular responses to genotoxic stress, and the BRCA1 protein has been implicated in chromatin remodeling and homologous recombination functions in mitotic and meiotic cells (4–8). BRCA1 complexes contain E3 ubiquitin-ligase activity, which may be involved in regulation of various preinitiation steps, including, but not limited to, recruitment of RNA pol II to promoters, transcriptional activation (29, 30), transcriptional co-activation (31, 32), and transcriptional inhibition (23). A distinct, but not mutually exclusive, model would suggest that BRCA1 interacts with elements of the RNA pol II holoenzyme linked to postpromoter activities of the complex. Evidence for this model includes interactions of BRCA1 with the elongation factor NELF/B/COBRA1 (33) and the CstF-50 component of the polyadenylate complex (34, 35).

A distinct literature has evolved linking BRCA1 to roles in transcription. BRCA1 protein has been shown to associate with RNA polymerase II (RNA pol II) and various transcriptional regulators (13). Early on, several groups demonstrated that the C-terminal domain (CTD; amino acids 1380–1863) of human BRCA1 scored positively in transcriptional activator trap experiments using various forms of the so-called “one-hybrid” assay (14–18). While not elucidating a specific function, ectopic expression of full-length human BRCA1 was then shown to increase expression of stress-responsive genes including p21 (19), GADD45 (20), and p27 (21) and decreased expression of other genes, including c-Myc-regulated genes (22) and certain estrogen-regulated genes (23). However, it remains an open question whether endogenous BRCA1 directly mediates recruitment of RNA pol II to promoters of these genes in unmanipulated cells (24–26).

Previous experiments indicate that BRCA1 associates with RNA pol II through its acidic C terminus (27) and may do so by binding RNA helicase A (13, 28). Current models of BRCA1 function(s) in transcription are poorly defined but favor regulation of various preinitiation steps, including, but not limited to, recruitment of RNA pol II to promoters, transcriptional activation (29, 30), transcriptional co-activation (31, 32), and transcriptional inhibition (23). A distinct, but not mutually exclusive, model would suggest that BRCA1 interacts with elements of the RNA pol II holoenzyme linked to postpromoter activities of the complex. Evidence for this model includes interactions of BRCA1 with the elongation factor NELF/B/COBRA1 (33) and the CstF-50 component of the polyadenylate complex (34, 35).

Negative charge can lead to false positives in one-hybrid assays (36–38). Following our observation that the CTD of bovine BRCA1 had less charge and less one-hybrid activity than the same region of human BRCA1 (39), we decided to search for structural and functional features of the BRCA1-RNA pol II complex that are more broadly conserved in mammalian cells. In the present study, we compared full-length and truncated BRCA1 proteins from four species for their ability to activate transcription in one-hybrid transcription assays and then assessed their association with cellular RNA pol II complexes. Although BRCA1 proteins from all four species associated strongly with the hyperphosphorylated (IIIO) form of RNA pol II, none of the full-length BRCA1 proteins displayed the ability to correctly initiate pol II-dependent transcription. Importantly, we observed that BRCA1 co-purified with a large percentage of hyperphosphorylated (IIIO) RNA pol II found in cycling epithelial cells, but interacted minimally with hypophosphorylated (IIA) forms of the enzyme, which are more...
EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 and T47D (human mammary epithelial cell lines), Mv1Lu and C57MG (mouse mammary epithelial cell lines), bovine aortic endothelial cells and Mardin-Darby bovine kidney cells (bovine epithelial cells), 293T (human fibroblasts), and NIH-3T3 (mouse fibroblasts) were maintained in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Plasmid Constructs—The human BRCA1 CTDs, representing amino acids 1388–1863, previously shown to have the highest activity in GAL4 transactivation assays (16), was generated by PCR using full-length human BRCA1 cDNA as the template (a gift of E. Solomon, Guy’s Hospital, London, UK) and the primers 5’-CAAGGACTTCCGATTGAGCC-3’ and 5’-CTCATGCTTGTC-3’.

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BRCA1 (either Ab4 or C-20). To remove phosphate from precipitated proteins, immunoprecipitated proteins were treated with CIP (New England Biolabs) prior to elution with SDS-PAGE loading buffer. To test for protein-DNA interactions, some immunoprecipitations were performed in the presence of 50 μM EDTA. Eluted proteins were subjected to SDS-PAGE and immunoblotting as described above.

**DNA Damage**—Randomly cycling HBL100 cells were treated with 4 μM adriamycin, 2 μM camptothecin, or 1 μM hydroxyurea. Alternatively, cells were exposed to the indicated dose of ionizing radiation (0.5–10 grays) using a 137Cs source (Mark 1 irradiator; Shepherd and Associates, San Fernando, CA). Following treatments, cells were returned to a 5% CO2 incubator for 1 h, then lysed, and immune complexes were prepared as above.

**p220 Enzyme-linked Immunosorbent Assay**—To quantify the amount of p220 catalytic subunit present in various immunoprecipitates, immobilized substrate assays were developed. Cell lysates were immunoprecipitated with rabbit polyclonal antiserum to either p220 subunit of RNA pol II (N-20) or BRCA1 (C-20), and volumes were normalized to cell number. Equivalent volumes of normalized cell extract were then diluted in plating buffer (50 mM carbonate buffer, pH 9.5) and adsorbed to 96-well plates (ELA8 plates; Costar). Plates were washed, blocked (0.1% bovine serum albumin in phosphate-buffered saline), and incubated with mouse monoclonal antibodies to p220 catalytic subunit (8W916). Bound monoclonal antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse antibody (Pierce) followed by the addition of color-producing 3,3′,5,5′-tetramethylbenzidine substrate (Sigma). The reaction was stopped after 10 min with 10 mM (final) H2SO4, and the reaction product was read at 450 nm on a 96-well plate reader (Molecular Devices). Values were used from dilutions that were within the linear range of the assay as established from a standard curve created with immunopurified p220.

**Run-off (C-tailed) Transcription Assays**—The run-off transcription assay used was a modification of previously reported C-tail assay (43, 44). Oligonucleotides were purchased from MWG (Highpoint, NC). The C-tailed oligonucleotide was a 65-mer (5′-ATTGGTGAAGAGAGA-GAGATTTGAGCGGAGGACAGTACTGGTCGCCCCCCCCCCCCC-CGGCCGCGCCG-3′) that was annealed to a complementary 45-mer (5′-GACCCCCGAGGATCTGGTCGCCCCCCCCCCCCC-CGGCCGCGCCG-3′) that was annealed to a complementary 45-mer (5′-G-ACCCCGGAGGATCTGGTCGCCCCCCCCCCCCC-CGGCCGCGCCG-3′). The run-off template was created by annealing 50 pmol of each oligonucleotide in a 200-μl reaction mixture containing 20 μM Tris (pH 7.4), 1 mM EDTA, and 0.2 mM NaCl. Run-off transcription reactions contained 8.25 mM MgCl2, 250 μM of bovine serum albumin, 250 μM NTPs, 5 units of RNase inhibitor, 50 ng of poly(dI-dC), 0.05% Nonidet P-40, 1 pmol of annealed oligonucleotides, and 0.5 μCi of [3H]-TPTP. Following the addition of test immunoprecipitations (containing nuclear extracts from equivalent numbers of cells), reactions (20 μl) were incubated for 40 min at 30 °C and then stopped with 50 μl of PK buffer (300 mM sodium acetate, 0.2% SDS, 10 mM EDTA, 100 ng of tRNA, and 10 μg of protease K). Transcription reactions were then incubated at 55 °C for 1 h with phenol/chloroform, and ethanol precipitated. Single-stranded RNA transcripts were resolved under denaturing conditions on 12% polyacrylamide/urea gels, electrophoresed for 1.5 h at 300 V, dried, and visualized by autoradiography on x-ray film, as described (43, 44). Dried gels were also exposed to PhosphorImager plates, and the signals were quantified on a Storm™ PhosphorImager (Amersham Biosciences).

**RESULTS**

**The CTD of Mouse BRCA1 Lacks One-hybrid Transcriptional Activation Activity, and the Limited Activity Associated with CTDs from Human, Dog, and Cow BRCA1 Correlate in Part with Their Respective Charge**—To determine whether classic one-hybrid activation activity is a conserved feature of BRCA1, we created a panel of GAL4 fusion proteins with BRCA1-CTDs from humans, mice, cows, and dogs. This panel is informative, since mouse and human BRCA1 genes share only 60% amino acid identity (40), whereas cow and dog BRCA1 are ~80% identical to each other and to both mouse and human proteins (39). Initially, regions homologous to amino acids 1380–1863 of human BRCA1 were tested in the MCF7 one-hybrid assay (Figs. 1C and 2A), since this region had been shown to be the most active in previous reports (16).

When tested in human MCF-7 breast cancer cells, the human GAL4-BRCA1-CTD fusion activated reporter activity 20.8-fold over vector alone, similar to values previously reported for this domain (16), whereas the mouse BRCA1-CTD produced base-line responses (1.7-fold over vector alone) (Figs. 1C and 2B). Homologous regions of cow and dog BRCA1 showed intermediate activities. The level of activation achieved by the human BRCA1-CTD was 300-fold lower than that observed for the classic activation domain of VP16 (Fig. 1C). Interestingly, exposing the cells to rates of DNA damage (1–10 grays of ionizing radiation) known to increase phosphorylation of the BRCA1-CTD did not affect readings in these assays (data not shown).

The sequence of each BRCA1-CTD was analyzed for total charge and compared with reporter activation (Fig. 1C, inset). Total charge was assessed by subtracting the number of negatively charged amino acids (Asp and Glu) from positive amino acids (Lys and Arg). Inclusion of mildly basic histidines in the equation had no significant effect on the slope of the curve, since there are similar numbers of this amino acid in all BRCA1-CTDs analyzed. The human and dog BRCA1-CTDs had the largest net negative charge (~28.9 and ~24.0, respectively) and the highest level of activity in all cell types tested. In contrast, the cow and mouse BRCA1-CTDs had lower net negative charge (~14.6 and ~15.4, respectively) and scored poorly in this assay, suggesting some correlation between charge and one-hybrid activity.

One-hybrid transactivation activities for all GAL4-BRCA1-CTD fusions were tested in several additional human cell lines with similar results (MCF7 (Fig. 1C) and 293T and T47D (Fig. 1D)). Although the maximal -fold induction varied between human cell lines, the relative order of activity (human CTD = dog CTD >> cow CTD > mouse CTD) remained consistent. The highly negative human CTD (12.3–20.8-fold) and dog CTDs (14.4–21.7-fold) always gave higher activity in human cell lines than the less charged cow (2.3–5.3-fold) and mouse sequences (1.7–2.2-fold) (Fig. 2).

To test this notion further, we conducted a series of assays on various size fragments of BRCA1 and on disease-associated mutant BRCA1 alleles. As previously reported (16), amino acids 1380–1863 from the CTD of human BRCA1 displayed the highest degree of one-hybrid transcriptional activation activity when compared with shorter segments of the C terminus (Fig. 2A). The drop in activity of shorter fragments of the human BRCA1-CTD correlated with charge. Similar fragments of mouse BRCA1 showed consistently low (1.7–2.7-fold) induction (Fig. 2B). Dog sequences were consistently high in various human cells (Fig. 2D) but were not tested in canine cells. Cow sequences scored consistently low in all human cell lines (2.3–5.3-fold; Fig. 2C), and the cow BRCA1-CTD was inactive in bovine cells (1.7-fold; Fig. 1E).

The CTD from two disease-associated mutations of human BRCA1 (1853ter (46) and 5382insC (change of frame at amino acid 1755) failed to activate transcription (Fig. 2A). However, immunoblotting analysis of these proteins showed that they were less stable in mammalian cells when compared with proteins containing the native C-terminal ends (data not shown). These results support an alternative interpretation, that the activity of the human (and dog) BRCA1-CTD in one-hybrid assays reflects, to some extent, acidic content of the sequence and may not elucidate a direct biological function of the BRCA1 protein in transcriptional activation (36, 45).

**Full-length BRCA1 Proteins Lack One-hybrid Transcriptional Activation Activity**—Fusion of the GAL4 DNA binding domain to full-length BRCA1 constructs from humans, mice, and cows or to the naturally occurring mouse splice variant lacking exon 11 (∆11) registered similarly to disease-associated BRCA1 mutant BRCA1-CTDs in all cell lines in which they were tested (Figs. 2, A, B, and C). The full-length fusion proteins...
were stable in mammalian cells and were detected at the predicted molecular weight in immunoblotting experiments with an antibody to the GAL4 DBD (data not shown). It is important to emphasize that assayed values for full-length BRCA1s compare precisely for all species and that these in turn correlated with the values of mutant CTD domains assayed in this study (0.4 – 0.5 of control). Previous reports used similar human disease allele BRCA1-CTDs as standards of inactivity (14 – 16).

Therefore, full-length BRCA1 proteins do not act as direct transcriptional activators or inhibitors in this assay.

Since mouse and human BRCA1 proteins are interchangeable in a number of assays (11, 12, 48), we hypothesized that chimeric fusions of mouse and human BRCA1-CTD sequences should have a modest effect on activities that are important for gene function. Using conserved restriction sites within the mouse and human CTD, we constructed chimeric proteins in which mouse sequences (referred to as regions A, B, and C in Fig. 2E) were fused in-frame with human sequences. Constructs that contained any mouse BRCA1 sequence proved to be less active in the GAL4 one-hybrid transcriptional activation assay when compared with the native human CTD, even if this included substitution of the similarly charged region C (Fig. 2E). In T47D cells, the fusion protein using human region A and murine regions B and C activated the reporter assay by 4.1-fold, and the reverse fusion protein activated 12.9-fold. Replacing a longer region from human BRCA1 (amino acids 1380 – 1651; regions A and B) in the mouse BRCA1-CTD increased the one-hybrid transcriptional activation activity in 293T cells and MCF-7 cells but not to the level of the continuous human BRCA1-CTD. All fusions were stable and expressed at equivalent levels as determined by immunoblot experiments (data not shown).

A comparison of charge clusters in human versus mouse CTDs was conducted based on the swapped segments (Fig. 2E). Regions A and B contained the most significant accumulations of net negative charge within the human CTD. Region A had a net negative charge contribution of –12 in humans versus –8 in mice; region B had a net negative contribution of –13 in
humans versus ~3 in mice. Region C, which corresponds to the tandem BRCT repeats, had a net negative contribution of ~5 in humans versus ~4 in mice. This result would predict that fusing mouse regions A and B with the human region C would result in activity similar to the wild type mouse BRCA1-CTD, as is the case (Fig. 2E). The results of the swapping experiment showed that the addition of any charged region from the human to the less charged mouse sequence increased the activity when compared with the mouse BRCA1-CTD alone. Conversely, reduction of negative charge in the human by the addition of homologous mouse sequence decreased the activity in a stepwise manner. However, charge was not the only factor. The addition of human regions A and B with mouse region C gave values that were significantly lower (3.9–6.8-fold) than those present in the contiguous human CTD (12.3–20.8-fold) but higher than the mouse CTD (1.7–2.2-fold). Therefore, charge appears to be a strong contributing factor but is probably not the only factor in the one-hybrid response of various CTD domains.

Neither Increased Nuclear Localization nor Alteration of the Reporter Construct Reveal Measurable Transcriptional Activity in Murine BRCA1-CTD Fusions—In addition to charge and phosphorylation state, we considered several additional potential reasons for a lack of activity by mouse CTD-GAL4 fusions. First, we tested several different GAL4 reporters that have been used in various studies of one-hybrid activity. The human BRCA1-CTD had the highest level of one-hybrid activation when assayed against reporters with the c-fos promoter (pWHGG). The mouse BRCA1-CTD had consistently minimal activity with all reporter constructs (these data are provided as supplemental Fig. 1). Next, we added nuclear localization signals (NLS) to the GAL4 constructs to assess the effect of improved nuclear localization on reporter activation. The nuclear localization of both human and mouse BRCA1-CTDs was significantly improved by the addition of exogenous NLS sequences. NLS addition to the GAL4 vectors resulted in no increased activity in control GAL4 or mouse GAL4-BRCA1-CTD constructs but resulted in significant enhancement of the activity of the human CTD (these data are provided as supplemental Fig. 2).

These data confirm that mouse BRCA1 has minimal activity in one-hybrid transcriptional activation assays. Furthermore, the activity of the human BRCA1-CTD construct is limited, requiring defined sequence composition and specific chimeric reporter design for efficient detection. From previous work with this assay (14–17, 49, 50), it is clear that one-hybrid type assays can be useful in characterizing disruptive mutations in the CTD of human BRCA1. The present study provides direct evidence that this strategy would be significantly less informative about the role of amino acid differences in other species and may not measure a conserved or native function of the BRCA1 protein.

BRCA1 Interacts with Hyperphosphorylated RNA Pol II Complexes in Preference to the More Abundant Hypophosphorylated Pol II—In normal cells, promoter-bound RNA pol II complexes contain a hypophosphorylated p220 subunit (form IIa) that is sequentially phosphorylated during initiation to generate the hyperphosphorylated p220 (form I0), which predominates in elongation complexes (51–53). Since human
BRCA1 has been shown to associate with RNA pol II (13, 27, 28, 32), we considered it important to test for this interaction with mouse and bovine BRCA1. We set out to determine whether the biology and timing of this interaction was consistent with BRCA1 acting in association with promoter-bound RNA pol II, as would be expected in the promoter and prepro- moter models discussed above. We immunoprecipitated the endogenous BRCA1 from undamaged human epithelial cells (HBL100, H9262, or H9251) to RNA pol II (N20) or BRCA1 (BR). Immunoblotted material was then probed (WB) with α-pol II (N20) or α-phospho-pol II-specific antisera (S2 or S5). C, minimally phosphorylated BRCA1 was found in complex with hyperphosphorylated pp220. Nuclear lysates were mock-immunoprecipitated (lane 1) or immunoprecipitated with antisera to BRCA1 (lanes 2 and 3), p220 (N20; lanes 4 and 5), phosphoserine 5 p220 (S5; lanes 6 and 7), or phosphoserine 2 p220 (S2; lanes 8 and 9). Precipitated material was split into two equal fractions and treated with or without phosphatase (CIP). Immunoblotted material was then probed with BRCA1 antisera (Ab4). D, the interaction between RNA pol II and BRCA1 decreases after DNA damage. Cells were either untreated (c) or treated with adriamycin (adr), camptothecin (cmt), or hydroxyurea (HU) and lysed after 60 min. Proteins were immunoprecipitated with BRCA1-specific serum (IP) and immunoblotted (WB) with α-pol II (8WG16). E, the reduced mobility of BRCA1-associated p220 is due to phosphate. Nuclear fractions were immunoprecipitated with antisera to BRCA1 or RNA pol II (8WG16). The immunoprecipitated material was divided into two equal fractions and treated with or without phosphatase (CIP), as indicated. Immunoprecipitated proteins were probed (WB) with α-pol II (8WG16). F, the isolated BRCA1-RNA pol II complex is stable in the presence of DNA denaturants and contains FCP1, a marker of processive RNA pol II. Immune complexes were prepared with or without 50 μM EtBr and subsequently treated with phosphatase prior to immunoblotting with α-pol II (8WG16) or α-FCP1. For all panels, immunoprecipitated complexes were run on a 5% SDS-PAGE gels prior to immunoblotting with the indicated sera. Hyperphosphorylated (IIO) p220 is identified with solid arrowheads. Hypophosphorylated (IIA) p220 is indicated with open arrowheads.

Fig. 3. Minimally phosphorylated BRCA1 proteins interact preferentially with hyperphosphorylated RNA pol II. A, a schematic diagram of the catalytic subunit (p220) of RNA pol II showing epitopes identified by various antisera used in this study. N20 and 8WG16 are raised against minimally modified peptide fusions, whereas S2 and S5 were raised against phosphopeptides representing the heptad repeats of processive RNA pol II. B, nuclear lysates from human HBL100 epithelial cells were prepared in the presence of phosphatase inhibitors and then immediately immunoprecipitated (IP) with antisera (α) to RNA pol II (N20) or BRCA1 (BR). Immunoblotted material was then probed (WB) with α-pol II (N20) or α-phospho-pol II-specific antisera (S3 or S2). C, minimally phosphorylated BRCA1 was found in complex with hyperphosphorylated pp220. Nuclear lysates were mock-immunoprecipitated (lane 1) or immunoprecipitated with antisera to BRCA1 (lanes 2 and 3), p220 (N20; lanes 4 and 5), phosphoserine 5 p220 (S5; lanes 6 and 7), or phosphoserine 2 p220 (S2; lanes 8 and 9). Precipitated material was split into two equal fractions and treated with or without phosphatase (CIP). Immunoblotted material was then probed with BRCA1 antisera (Ab4). D, the interaction between RNA pol II and BRCA1 decreases after DNA damage. Cells were either untreated (c) or treated with adriamycin (adr), camptothecin (cmt), or hydroxyurea (HU) and lysed after 60 min. Proteins were immunoprecipitated with BRCA1-specific serum (IP) and immunoblotted (WB) with α-pol II (8WG16). E, the reduced mobility of BRCA1-associated p220 is due to phosphate. Nuclear fractions were immunoprecipitated with antisera to BRCA1 or RNA pol II (8WG16). The immunoprecipitated material was divided into two equal fractions and treated with or without phosphatase (CIP), as indicated. Immunoprecipitated proteins were probed (WB) with α-pol II (8WG16). F, the isolated BRCA1-RNA pol II complex is stable in the presence of DNA denaturants and contains FCP1, a marker of processive RNA pol II. Immune complexes were prepared with or without 50 μM EtBr and subsequently treated with phosphatase prior to immunoblotting with α-pol II (8WG16) or α-FCP1. For all panels, immunoprecipitated complexes were run on a 5% SDS-PAGE gels prior to immunoblotting with the indicated sera. Hyperphosphorylated (IIO) p220 is identified with solid arrowheads. Hypophosphorylated (IIA) p220 is indicated with open arrowheads.

When compared with p220 catalytic subunit precipitated directly from human epithelial cells (N-20 antiserum; Fig. 3B, lane 1), it was clear that BRCA1 interacted preferentially with the slower migrating IIO form of p220. Interaction of BRCA1 with hyperphosphorylated p220 was confirmed with antisera that recognize specific phosphorylation events within the heptad repeats of the p220 catalytic subunit. p220 co-purifying with BRCA1 showed evidence of phosphorylation at both the serine 5 (S5, Fig. 3B, lane 4) and serine 2 (S2, lane 6) positions of the heptad repeat, as well as showing a size shift consistent with maximal phosphorylation of the repeat domain (solid arrowhead, IIO).

When RNA pol II was directly precipitated from cells with anti-p220 antisera, it was evident that a majority of RNA pol II was present in a minimally phosphorylated state (open arrowhead, IIA; Fig. 3B, lane 1). By binding the hyperphosphorylated form of p220, BRCA1 shows considerable selectivity for the less abundant hyperphosphorylated form (IIO) found in these cells (compare Fig. 3B, lanes 1 and 2). By comparison of RNA pol II immunoreactivity co-migrating with the minimally phosphorylated form (IIA) before or after phosphatase treatment, we estimated that only 5–10% of total p220 was present in BRCA1 complexes in these cells (see also Fig. 5C). In contrast, a large percentage of hyperphosphorylated (IIO) p220 co-precipitated with BRCA1 (Fig. 3B).

To examine the specificity of BRCA1 interactions with RNA pol II, we also examined the BRCA1 that co-purified with different forms of p220 (Fig. 3C). Equivalent volumes of nuclear extract were immunoprecipitated with antisera to BRCA1, p220, or two distinct forms of phospho-p220. Following immunoprecipitation, each fraction was split equally, and half was treated with phosphatase (CIP). Subsequently, all fractions were immunoblotted with monoclonal antisera to BRCA1 (Ab4; Oncogene Sciences). Two interesting results were evident from this experiment. First, similar amounts of BRCA1 (arrow) were precipitated with BRCA1 and p220 antisera. Second, the mobility of the BRCA1 bound by RNA pol II was not affected by phosphatase treatment (CIP treatment). These results provide evidence that a
large fraction of BRCA1 associates with RNA pol II in undamaged epithelial cells and that the BRCA1 present is unphosphorylated or minimally phosphorylated.

Following exposure of randomly cycling epithelial cells to DNA-damaging agents known to cause phosphorylation of BRCA1, interaction with RNA pol II decreased (Fig. 3D). In this experiment, cells were treated with the topoisomerase II inhibitor adriamycin (adr), the topoisomerase I inhibitor camptothecin (cpt), or the ribonucleotide reductase inhibitor hydroxyurea (HU) for 1 h prior to cell lysis. It has been shown previously (54–56) that these agents induce double-stranded DNA breaks and ATM/ATR-dependent phosphorylation of BRCA1 (not shown). Following treatments, there was a clear reduction of in phospho-p220 associated with the BRCA1 immune complexes. The residual interaction that was detected in damaged cells still targeted the hyperphosphorylated (IIO) form of p220 and presumably represented residual unphosphorylated BRCA1 in these cells.

To further characterize the BRCA1-pol II complex, we examined its susceptibility to phosphatases and to the DNA denaturant EtBr. In Fig. 3E, immunoprecipitated material was divided into two equal fractions, and both fractions were incubated at 37 °C for 1 h in the presence or absence of phosphatase (Fig. 3E). The cellular fraction of p220 that associated with BRCA1 (IIO form) was reduced to the lower molecular weight (IIA form) following phosphatase treatment (Fig. 3E, lane 2). Although some degradation of p220 was apparent following 37 °C incubation (compare Fig. 3E, lane 1, with Fig. 3B, lane 2, or Fig. 3D, lane 1) it is clear that phosphatase treatment effectively returns the BRCA1-bound p220 (IIO) to a form that co-migrates with unphosphorylated p220 (IIA). In F, immunoprecipitations were conducted in the presence of 50 μM EtBr, a DNA denaturant that is useful in distinguishing DNA-dependent and DNA-independent interactions (57). Immunoprecipitates were subsequently treated with CIP and immunoblotted for p220. From these experiments, it is clear that EtBr did not affect the ability of BRCA1 to bind hyperphosphorylated RNA pol II (Fig. 3E, compare lanes 1 and 2).

In every preparation of BRCA1-RNA pol II complex, we observed a very minor IIA-like immunoreactive band (see the minor IIA-like band in Fig. 3B, lane 2). Although the precise nature of the band is unknown, it may represent doubly phosphorylated p220 (S5 + S2) that is not yet hyperphosphorylated (note reactivity of S2 and S5 antisera to similarly migrating bands in lanes 3 and 5). Alternatively, the IIA-like band could represent the actions of endogenous phosphatases on the isolated IIO complex. We have documented the presence of FCP1 in BRCA1-RNA pol II immunoprecipitates (Fig. 3F, lower panel) using antisera provided by Dr. Michael E. Dahmus (University of California, Davis). FCP1 is an active p220 phosphatase required for maximum processivity and recycling of RNA pol II (58). Interestingly, FCP1 is not efficiently inactivated by the phosphatase inhibitors used in our extracts (NaF and Na2VO4). More complete inactivation of FCP1 requires EDTA and EGTA (59), which are absent from our buffers due to the requirement for divalent cations in some BRCA1 structures (60, 61). Since this IIA-like band represents a minor percentage of the total p220 present in BRCA1 complexes, we have not been able to fully document its origin, although this remains an interest.

Unphosphorylated C-terminal Domains from Various Mammalian BRCA1 Orthologs Interact Preferentially with Phosphorylated RNA Pol II in Human and Other Cells—Because of suitable antibody cross-reactivity, we were able to show that both human and bovine BRCA1 proteins interacted with RNA pol II holoenzyme complexes that contained hyperphosphorylated (IIO) p220 (above and data not shown). To demonstrate that this interaction was conserved more generally and to study structural features of BRCA1 required for p220 association, we tested unphosphorylated BRCA1-CTDs (human, mouse, cow, and dog) for their ability to bind RNA pol II complexes from various cells. To do this, GST fusions with the BRCA1-CTDs were purified from bacterial cells, and the purified proteins were incubated with nuclear extracts. Specifically bound proteins were subjected to immunoblot analysis with antisera recognizing the 220-kDa catalytic subunit of RNA pol II (Fig. 4B). All four BRCA1-CTDs interacted with the hyperphosphorylated RNA pol II (IIO). The presence of hyperphosphorylated p220 in the complexes was confirmed by incubation of the blots with antibodies specific for phosphorylated forms of p220, including serine 2 (S2) and serine 5 (S5). In the same experiment, treatment of bound proteins with calf intestinal phosphatase resulted in a reduction in the molecular weight of the p220 subunit to that of the predicted size for the unphosphorylated p220 (IIA).

Importantly, unphosphorylated BRCA1-CTDs from all species tested behaved equivalently in binding the hyperphosphorylated RNA pol II (Fig. 4B). In similar types of GST pull-down assays, the human and mouse BRCA1-CTDs bound specifically and preferentially with hyperphosphorylated RNA pol II from mouse mammary epithelial cells (C57MG), and the human and cow BRCA1-CTDs interacted specifically and preferentially with hyperphosphorylated RNA pol II from Madin-Darby bovine kidney cells (data not shown).

BRCA1-associated RNA Pol II Represents a Large Proportion of the Catalytically Active Pol II Activity in Cycling Cells—To specifically demonstrate that the BRCA1-RNA pol II complex...
Minimal Transcriptional Activation Activity in Mammalian BRCA1

BRCA1 interacts with a large proportion of functionally processive RNA polymerase II holoenzyme. A, schematic representation of the modified RNA polymerase run-off transcription assay (C-tailed assay) used in this study. Double-stranded DNA templates were designed to generate a full-length (FL) RNA transcript of 45 nucleotides in the presence of active RNA pol II. B, run-off transcription assays were conducted with increasing amounts of immunoprecipitated pol II catalytic subunit (p220) or subunit that co-precipitated with BRCA1. Immunoprecipitations were carried out from equivalent pol II catalytic subunit (p220) or subunit that co-precipitated with BRCA1. Assays were conducted with increasing amounts of immunoprecipitated (C-tailed assay) used in this study. Double-stranded DNA templates.

Interestingly, BRCA1-immunoprecipitated fractions consistently demonstrated greater run-off transcription activity than was found in p220 precipitates from the same number of cells (Fig. 5B, compare lane 3, with lane 6; Fig. 5D, compare lanes 1 and 2). To test this further, an enzyme-linked immunosorbent assay was developed to quantify the amount of p220 found in cells (Fig. 5C). Briefly, anti-BRCA1 or anti-p220 immune complexes were immobilized on immunosorbent chambers, and each complex was subsequently probed with a distinct p220-specific antisemur (8WG16). These assays confirmed that BRCA1 immune complexes contained only 4–6% of cellular p220. When these values were used to normalize the run-off transcription assays (Fig. 5D), the RNA pol II found in BRCA1 precipitates was 6-fold more active than the total pool of p220 in the same volume of extract (Fig. 5D, compare lanes 3 and 4). These results indicate that BRCA1 interacted with a large percentage of the transcriptionally active RNA pol II in these extracts.

Represented functional RNA pol II, we performed transcription run-off assays with proteins that co-precipitated with BRCA1 from human cells. These assays utilize a modification of the C-tail template assay known to require functional RNA pol II (43, 44). The diagram (Fig. 5A) shows that a 45-nucleotide RNA transcript is generated when RNA pol II is added to a short double-stranded DNA template containing a 3' poly(dC) tail. As shown in Fig. 5B, the RNA pol II that associates with BRCA1 is capable of transcribing full-length transcripts from this template.

Discussion

From these studies, we propose that minimally phosphorylated BRCA1 interacts with postinitiation forms of RNA pol II and that this interaction diminishes following phosphorylation of BRCA1 as a result of genotoxic stress. In binding this specific form of the RNA pol II holoenzyme, BRCA1 is unlikely to play a direct or specific role in “transcriptional activation” (promoter activation) per se. However, interaction with processive RNA pol II places BRCA1 in a position where it can play a significant role in transcription, through roles in postinitiation events. In addition, association with the processive RNA pol II holoenzyme places BRCA1 in position to act as a sensor or regulator of multiple cellular functions, including chromatin remodeling, transcription-coupled repair, and genomic surveillance for DNA damage, all of which are perturbed in BRCA1-deficient cells (62, 63, 69). Preferential interaction of unphosphorylated BRCA1 with the processive RNA pol II complex allows a close linkage of transcription and genomic repair processes in eukaryotic cells.

Several models of BRCA1 interaction with the transcriptional complex proposed that BRCA1 functioned in preinitiation roles to regulate expression of specific genes, potentially including genes involved with DNA repair or the genomic stress response (20, 64, 65) and that the interactions affected promoter-associated complexes (19, 23, 25). A key element to this line of reasoning was the observation that CTD of human BRCA1 could act as a transcriptional activator in one-hybrid type assays (14–18). We present seven pieces of data that call aspects of the preinitiation models into question: 1) full-length BRCA1 proteins (and Δ11 splice variants) isolated from several species, including humans, lack one-hybrid transcriptional activation activity when tethered to a promoter element in GAL4- UAS one-hybrid assays; 2) one-hybrid transcriptional activation activity of BRCA1-CTDs is poorly conserved and correlates, in part, with negative charge; 3) closely related BRCA1-CTDs from mice and cows lack significant one-hybrid transcriptional activation activity when tested in multiple cell types and under multiple conditions; 4) in-frame fusion of the mouse CTD onto portions of the human CTD sequence severely dissipates CTD-associated one-hybrid activity; 5) in vivo, BRCA1 proteins from all four species tested interact specifically with hyperphosphorylated (IIA) RNA pol II, widely considered to represent postpromoter forms of the pol II complex; 6) in vivo, BRCA1 proteins fail to interact with hypophosphorylated (IIB) forms of RNA pol II that would be expected in preinitiation stages of the transcriptional apparatus; 7) in vivo, BRCA1 interacts specifically with a very large percentage of the catalytically active RNA pol II while binding to a very small percentage of the total RNA pol II found in these cells, suggesting that BRCA1 interacts preferentially with postpromoter (late) forms of the transcriptional machinery.

Human BRCA1 rescues homologous recombination, viability, gametogenesis, and tumor-free survival in Brcal-null mice and murine cell lines (11, 12, 48). If transcriptional activation were an intrinsic function of BRCA1, one would expect to see conservation of this activity in other species, and association of BRCA1 with promoter-bound forms of RNA pol II. In the present study, the interaction of BRCA1 proteins has been tested in a variety of assays aimed at assessing functional activity of the BRCA1-RNA pol II complex. Both full-length proteins and BRCA1-CTDs have been tested in protein-protein interaction studies, co-immunoprecipitation assays, and two types of functional assays (one-hybrid transcription studies and run-off transcription assay). Whereas CTD-associated transcriptional activation activity was poorly conserved and correlated in part with charge in the four BRCA1 orthologs tested, all BRCA1 proteins interacted with the hyperphosphorylated form of RNA pol II.

RNA pol II is sequentially phosphorylated during elongation of the mRNA transcript (66). As part of promoter initiation,
serine 5 residues within the heptamer repeats of p220 are phosphorylated by Cdk7, a subunit of TFIIH (67). Subsequently, Cdk9/TFIIEb phosphorylates serine 2 of the heptad to create highly processive postpromoter forms of RNA pol II holoenzyme (68). There are 52 repeats of this heptad sequence in mammalian p220 catalytic subunits, and phosphorylation of multiple heptads is required to generate the hyperphosphorylated p220 form associated with elongation (51–53). In the present study, unphosphorylated full-length BRCA1 and BRCA1-derived CTDs were shown to interact with hyperphosphorylated RNA pol II containing multiply phosphorylated heptad repeats. Efficient detection of hyperphosphorylated IIO RNA pol II required rapid analysis of fresh nuclear extracts, which contained significant levels of endogenous RNA pol II phosphatases, including FCP1.

Based on the present study, we conclude that unphosphorylated BRCA1 associates with processive RNA pol II and that the interaction is diminished following genomic stress. We suggest that effects observed on transcription, in cells engineered to constitutively overexpress BRCA1, may result from complex regulation of transcription as opposed to direct binding and recruitment of RNA pol II to specific promoters. It is clear that ultimate resolution of this issue will require analysis of BRCA1 interactions with chromatin from multiple regions within genes and across chromosomes. Interactions of BRCA1 with processive RNA pol II would probably position the protein to interact with chromatin at sites downstream of the promoter. Whereas such interactions could contribute to postinitiation functions of RNA pol II (33–35), they would also position BRCA1 for other functions such as DNA damage surveillance, which correlate well with other studies (5, 54, 69).

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