AD_____

Award Number: W81XWH-04-1-0371

TITLE: BRCA1 Protein Complexes: Dynamic Changes and Functions Important in Breast Cancer

PRINCIPAL INVESTIGATOR: Andrew Horwitz

CONTRACTING ORGANIZATION: Harvard Medical School
Boston, MA 02115

REPORT DATE: April 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2006	Annual Summary	15 Mar 2005 - 14 Mar 2006

4. TITLE AND SUBTITLE
BRCA1 Protein Complexes: Dynamic Changes and Functions Important in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0371

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Andrew Horwitz

E-Mail: Andrew_horwitz@student.hms.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Harvard Medical School
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

In the past year, I have made substantial progress towards completion of the tasks outlined in my Statement of Work. I have continued to study the intrinsic transcriptional activity of BRCA1 in the fully purified system, and I have described distinct mechanisms for transcriptional stimulation and repression. These activities recapitulate the *in vivo* transcriptional functions of BRCA1.

15. SUBJECT TERMS
BRCA1, Tumor Suppressors

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

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Intro

In the past year, I have continued to study the intrinsic transcriptional activity of BRCA1 in the fully purified system, and I have described distinct mechanisms for transcriptional stimulation and repression. These activities recapitulate the *in vivo* transcriptional functions of BRCA1.

Body

Task 1: To purify and characterize BRCA1 complexes from cultured mammalian cell lines.

A) Subcloning to assemble a retroviral zz-TEV fusion vector.
B) Infection and selection of stable cell lines
C) Purification by chromatography and affinity steps.
D) Identification of protein subunits by mass spectrometry and comparison between breast and non-breast cell line.

Several stable cell lines were previously established (as reported in year 1). We are currently focusing on the interaction between BRCA1/BARD1 and the basal transcription machinery.

Task 2: To describe the dynamics of BRCA1 complex formation and redistribution.

A) Purify BRCA1 complexes from cells synchronized at different points in the cell cycle or following DNA damage.
B) Compare distribution of complexes by Native Blue PAGE.

See Task 1.

Task 3: To test the function of purified BRCA1 complexes by *in vitro* assays.

Since the last report, I have devoted my time to characterization of the BRCA1/BARD1 interaction with RNA Polymerase II, and we have made great progress in this area. As reported previously, BRCA1/BARD1 ubiquitinate the large subunit of RNA Polymerase II (Rpb1), and we developed a fully-purified transcription/ubiquitination assay to ask whether the enzymatic activity of RNA Polymerase II was regulated by this modification. This assay demonstrates that ubiquitination of the pre-initiation-complex (PIC) by BRCA1/BARD1 induces dissociation of TFIIE, leading to a failure of initiation. BRCA1 also can stimulate transcription, independent of its E3 ubiquitin ligase activity. By stabilizing properly initiated PICs, BRCA1 promotes productive transcriptional initiation. In the cell, these opposing activities are likely regulated by interacting transcription factors that participate in combinatorial regulation of specific gene targets with BRCA1.

Key Research Accomplishments

• Established cell lines for affinity purification of BRCA1 complexes (completed).

• Characterized the *in vitro* ubiquitination of Pol II by BRCA1/BARD1; confirmed the results *in vivo* (completed).

• Developed an *in vitro* system to assay the transcriptional activity of BRCA1. Described distinct mechanisms for transcriptional repression and stimulation by BRCA1 (in progress)

Reportable Outcomes

1) Stable cell lines expressing affinity-tagged BRCA1, Pol II components.

2) BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. Lea M. Starita[*], Andrew A. Horwitz[*], Michael-Christopher Keogh, Chikashi Ishioka, Jeffrey D. Parvin, Natsuko Chiba. Submitted. [*]*These authors contributed equally.*

3) Horwitz, AH, Sankaran, S, Parvin JD. (2006) Direct stimulation of transcription initation by BRCA1 requires both its amino and carboxy termini. J Biol Chem 281(13) 8317-8320. (see Appendix).

4) Horwitz, AH and Parvin JD. The E3 ubiquitin ligase activity of BRCA1/BARD1 represses transcription initiation. In preparation. See Figures.

Conclusions

The studies described here are aimed at understanding how BRCA1 functions as a tumor suppressor. I have made progress in several areas of my fellowship proposal. The primary area of investigation in the past year has focused on BRCA1 complex function *in vitro,* in particular as it relates to the interaction between BRCA1/BARD1 and the Pol II complex. We previously confirmed that Pol II was a substrate for BRCA1/BARD1 *in vitro* and *in vivo*. First, using a fully purified *in vitro* system, we showed that ubiquitination of RNA Polymerase II by BRCA1/BARD1 represses transcription initiation through dissociation of TFIIE/TFIIH (See Figures). Second, we have shown that BRCA1 also activates transcription, independent of its E3 ligase activity, and working at the initiation stage (See attached .pdf). The purified system reveals that BRCA1 regulates localization of the pre-initiation complex, stabilizing properly initiated complexes. To examine these mechanisms in the cell, we are using gene profiling techniques to compare the transcriptomes of cells expressing wild type BRCA1 and an E3 ligase-defective mutant. Based on our results from the *in* vitro system, we predict that this mutation will affect repression, but not stimulation targets of BRCA1. Regulation of Pol II by BRCA1/BARD1 could effect tumor suppression in two ways. First, in the acute response to DNA damage, the repressive action of BRCA1/BARD1 may contribute to the

global, transient repression of transcription. Second, by regulation of specific gene targets, BRCA1 may control a tumor-suppressive transcription program.

FIGURES

1. For BRCA1 stimulation of transcription, see Appendix.

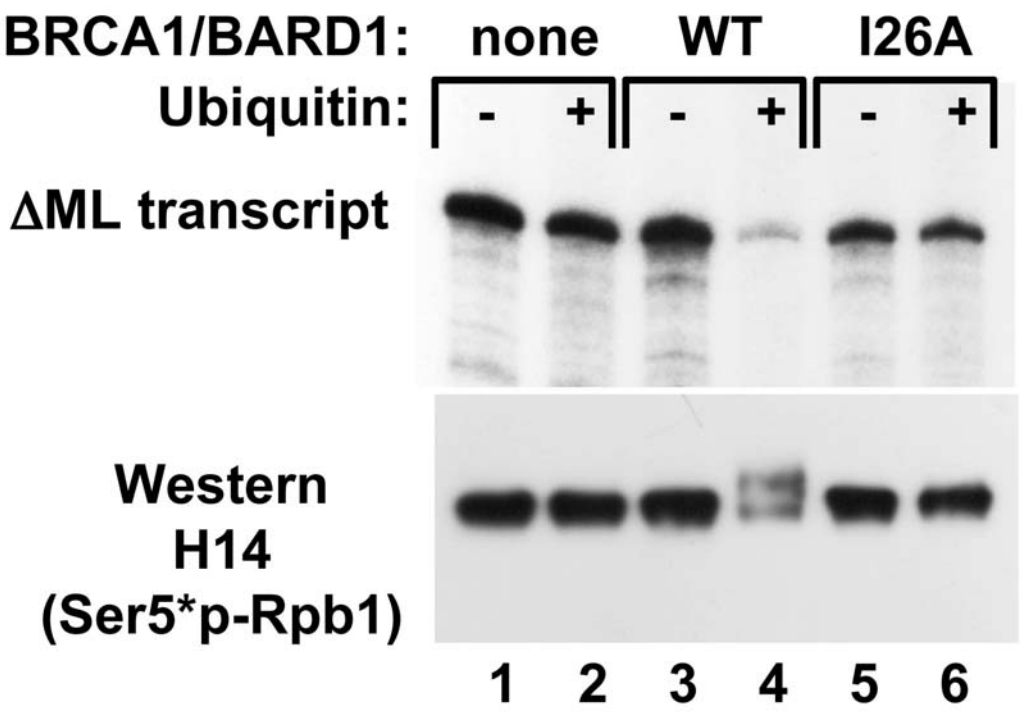


Figure 1A: The ubiquitin ligase activity of BRCA1/BARD1 represses transcription in a purified system. Upper Panel: *In vitro* transcription/ubiquitination reactions (TBP, TFIIB, RNA Polymerase II, TFIIF, TFIIE, TFIIH, E1 and E2). Ubiquitin was added to even-numbered lanes. Bottom Panel: Western blot with H14 antibody (Ser5*p-Rpb1) on identical reactions.

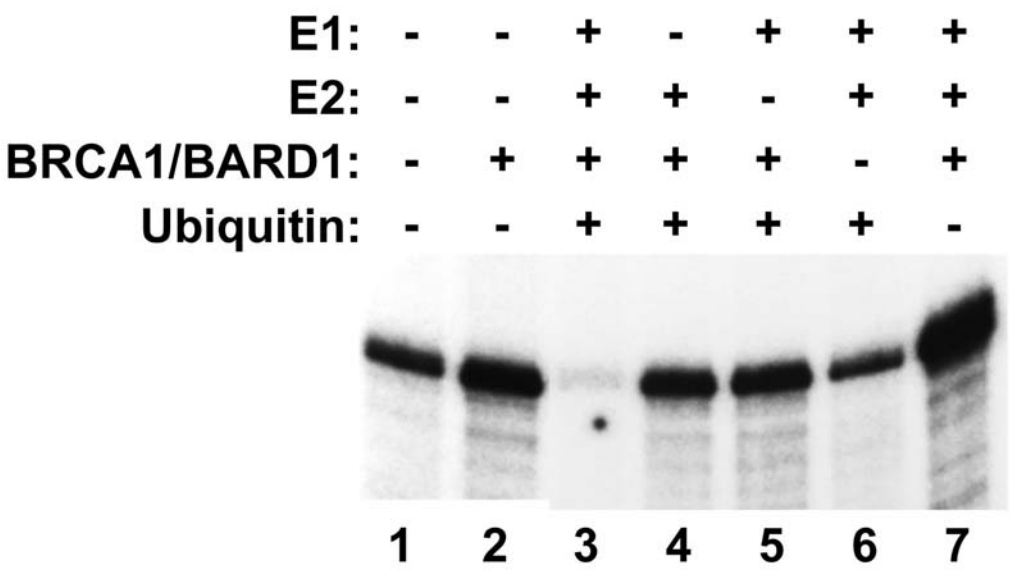


Figure 1B: Repression of transcription requires E1, E2, BRCA1/BARD1 and ubiquitin. Transcription reactions were assembled as in Figure 1A with omission of single or multiple ubiquitination factors.

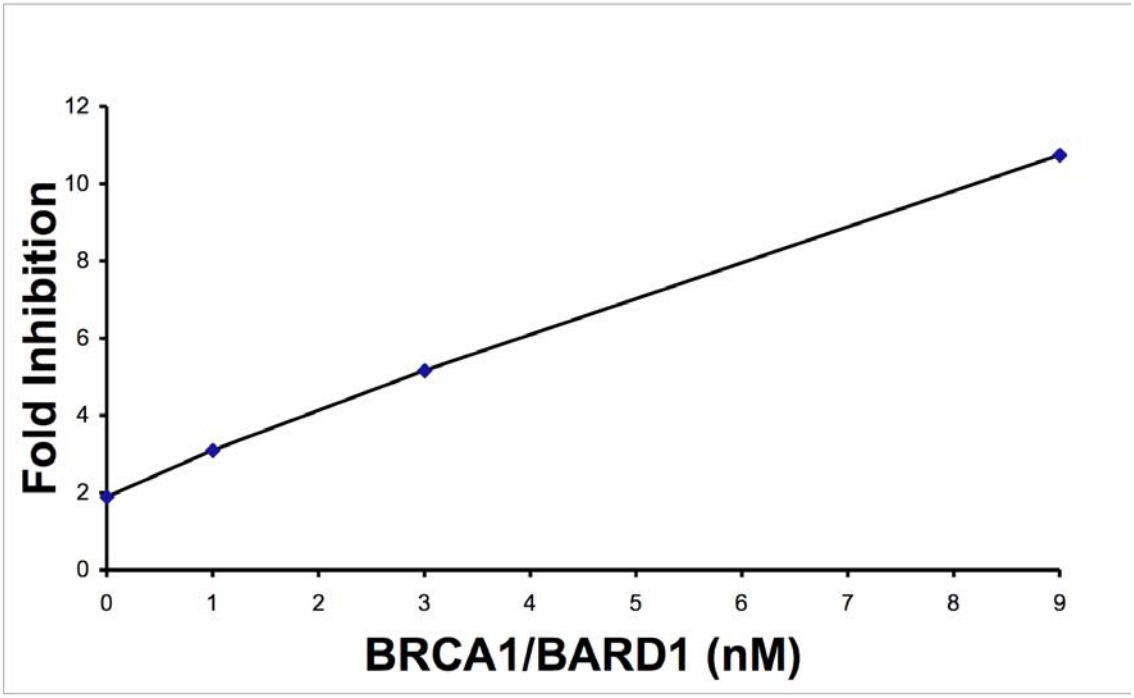


Figure 1C: BRCA1/BARD1 repression of transcription is dose-dependent. BRCA1/BARD1 was titrated (0-9 nM) into transcription/ubiquitination reactions containing all transcription factors, E1 and E2. "Fold Inhibition" is the quantity of transcript produced in reactions without ubiquitin divided by the quantity produced in reactions containing ubiquitin, as determined by phosphorimager analysis.

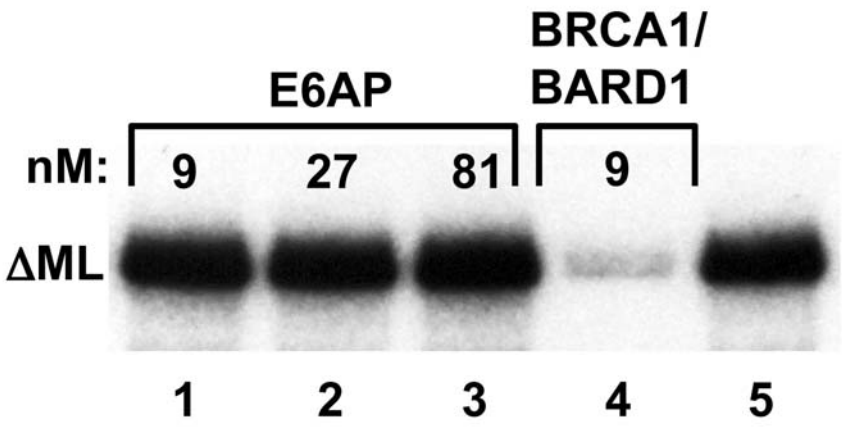


Figure 1D: The HECT E3 ligase E6AP does not repress transcription. E6AP (lanes 1-3) or BRCA1/BARD1 (lane 4) or no E3 were added to transcription reactions containing E1, E2 and ubiquitin.

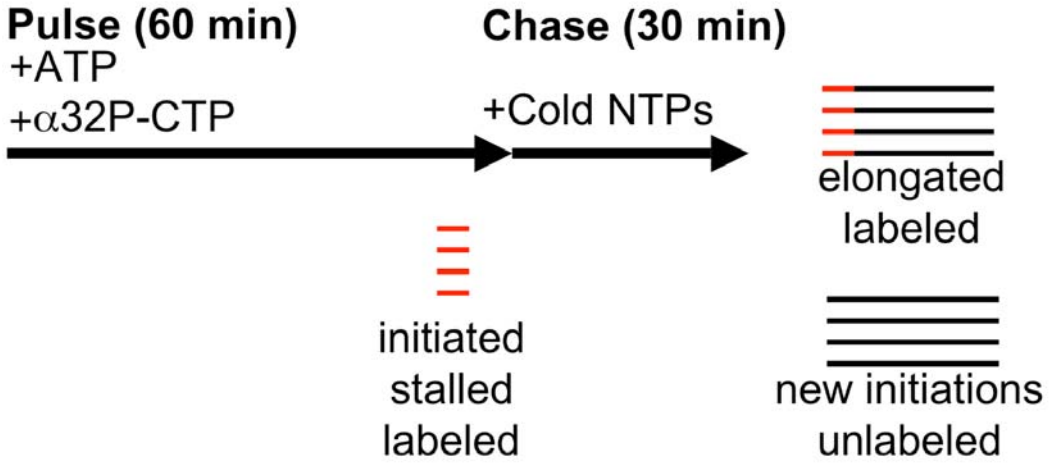


Figure 2A: Schematic of pulse-chase experiment for separating initiation and elongation effects. Transcription reactions containing E1, E2 and BRCA1/BARD1 were assembled with a limiting nucleotide mixture (pulse). Elongation continued with addition of complete, cold NTPs (chase).

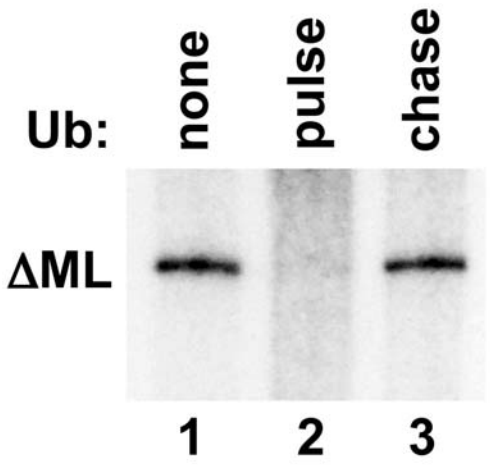


Figure 2B: Transcriptional repression targets the initiation phase. Pulse-chase transcription reaction (see Figure 2A for experimental details) without ubiquitin (lane 1), with ubiquitin added before the pulse (lane 2) or before the chase (lane 3).

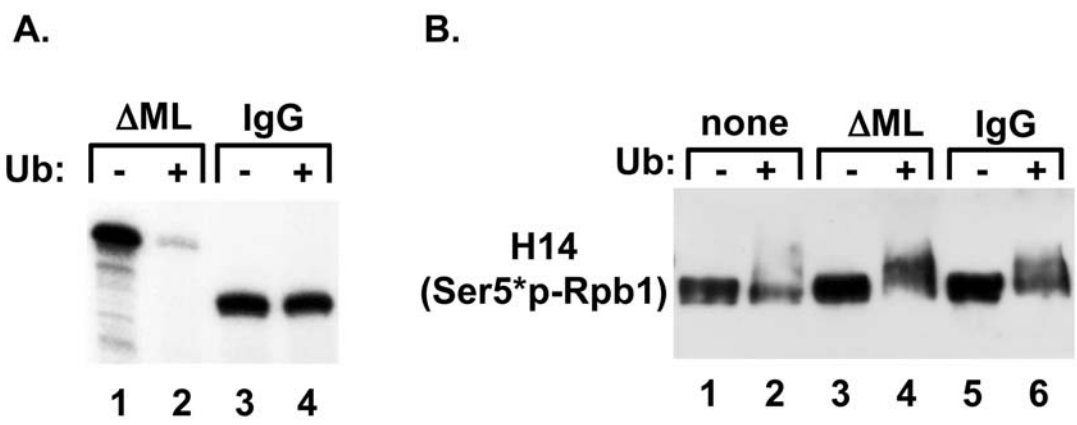
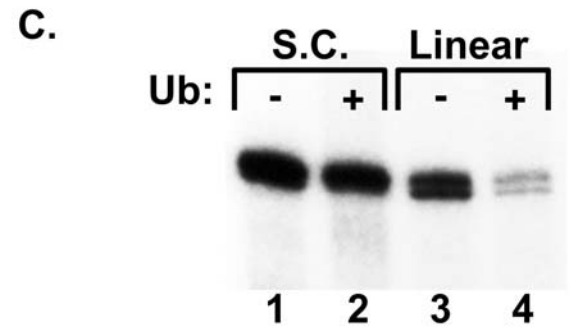


Figure 3A: BRCA1/BARD1 E3 ubiquitin ligase does not repress transcription from the IgG promoter. Transcription reactions containing BRCA1/BARD1, E1 and E2 were assembled with ΔML or IgG promoter templates and ubiquitin was added in even lanes. **Figure 3B: Rpb1 is ubiquitinated by BRCA1/BARD1 regardless of template.** Transcription reactions were assembled as in 3A with no template (lanes 1 and 2), the ΔML template (lanes 3 and 4) or the IgG template (lanes 5 and 6).



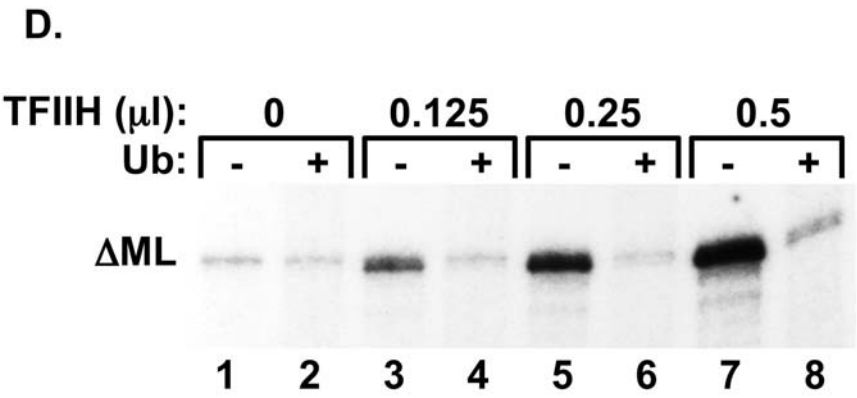


Figure 3C: Linear topology of the IgG promoter-containing template renders it susceptible to repression by BRCA1/BARD1 E3 ubiquitin ligase. Transcription/ubiquitination reactions containing all factors but ubiquitin. The IgG promoter template was on a supercoiled (lanes 1 and 2) or linear (lanes 3 and 4) plasmid. **Figure 3D: BRCA1/BARD1 E3 ubiquitin ligase blocks stimulatory activity of TFIIH on transcriptions from the ΔML promoter.** Transcription/ubiquitination reactions were assembled without TFIIH or ubiquitin. Variable amounts of TFIIH were added to the reaction (0-0.5 μl).

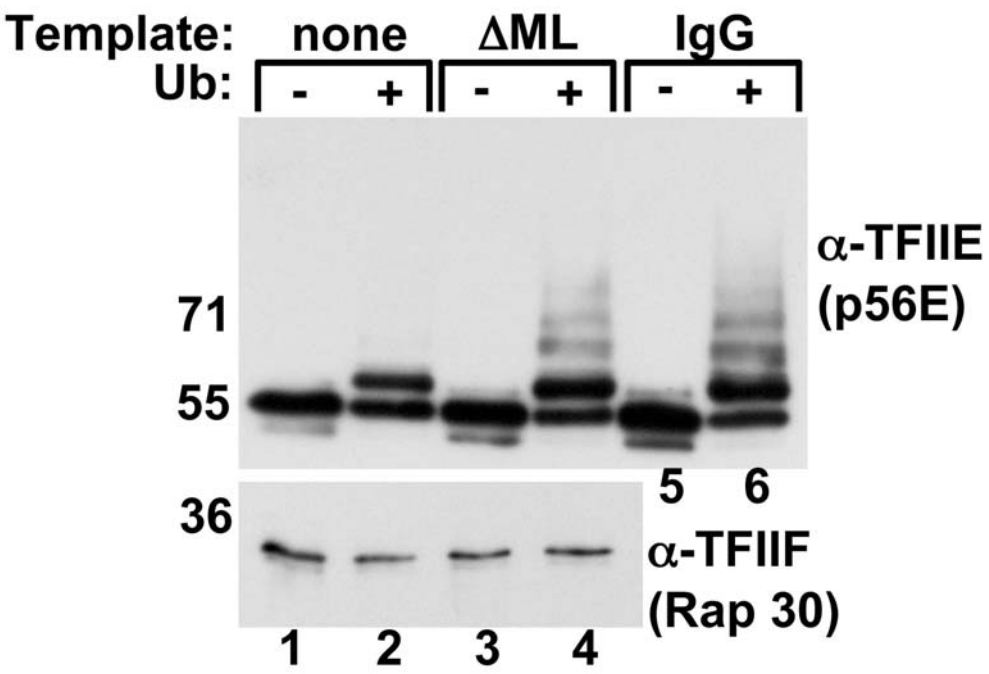


Figure 4A: TFIIE (p56E) is co-transcriptionally ubiquitinated by by BRCA1/BARD1; TFIIF (Rap 30) is not. Transcription/ubiquitination reactions were assembled without a promoter (lanes 1 and 2), with the ΔML promoter (lanes 3 and 4), or with the IgG promoter (lanes 5 and 6). Ubiquitin was present in even numbered lanes. Western blots were probed with antibodies to TFIIE (p56E) or TFIIF (Rap 30).

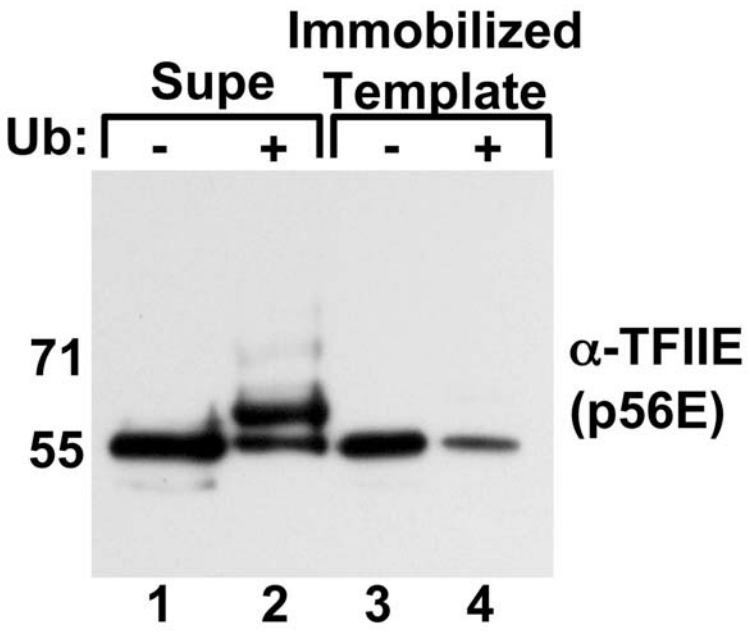


Figure 4B: Ubiquitination of the PIC by BRCA1/BARD1 dissociates TFIIE from an immobilized transcription template. Linear ΔML template was immobilized on agarose beads. Pre-initiation complexes (transcription factors plus E1, E2 and BRCA1/BARD1) were assembled on the templates in buffer containing ATP only. Ubiquitin was added to even lanes. After 60 minutes incubation, reactions were separated into supernatant (supe) and template fractions and western blots were probed for p56E.

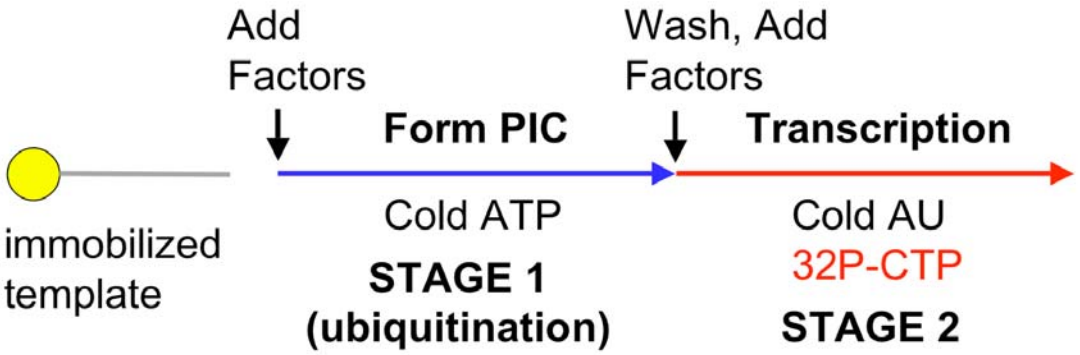


Figure 5A: Schematic of staged transcription reaction. To test whether ubiquitination of TFIIE is required for the repression of transcription.

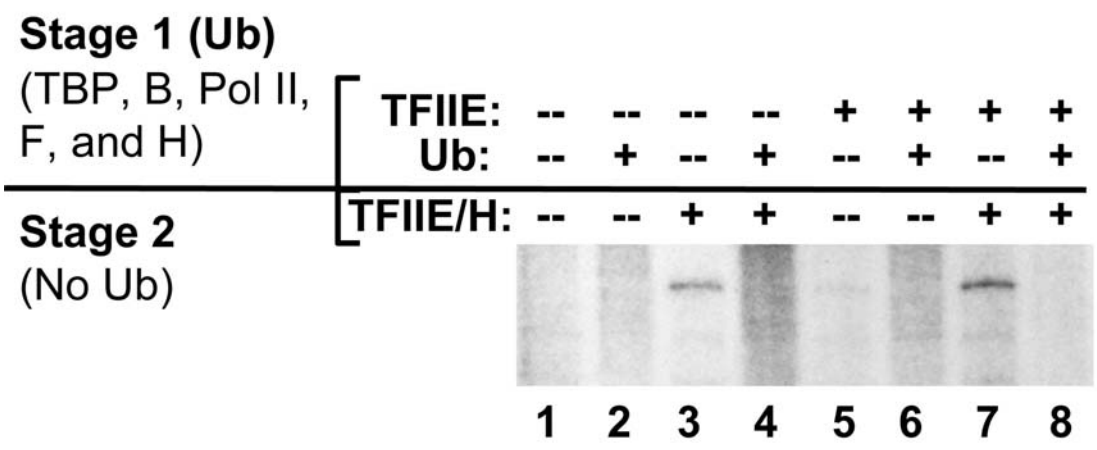


Figure 5B: Staged transcription reactions demonstrate that unmodified TFIIE cannot rescue transcription from an ubiquitinated PIC. Staged transcription scheme is outlined in Figure 5A. Transcriptions were assembled on an immobilized template in Stage 1 with the noted factors (including all ubiquitination factors). TFIIE was added in lanes 5-8. After incubation, the template was washed to remove unbound factors, and transcriptions were continued in new buffer, with the addition of TFIIE/TFIIH (lanes 3, 4, 7 and 8).

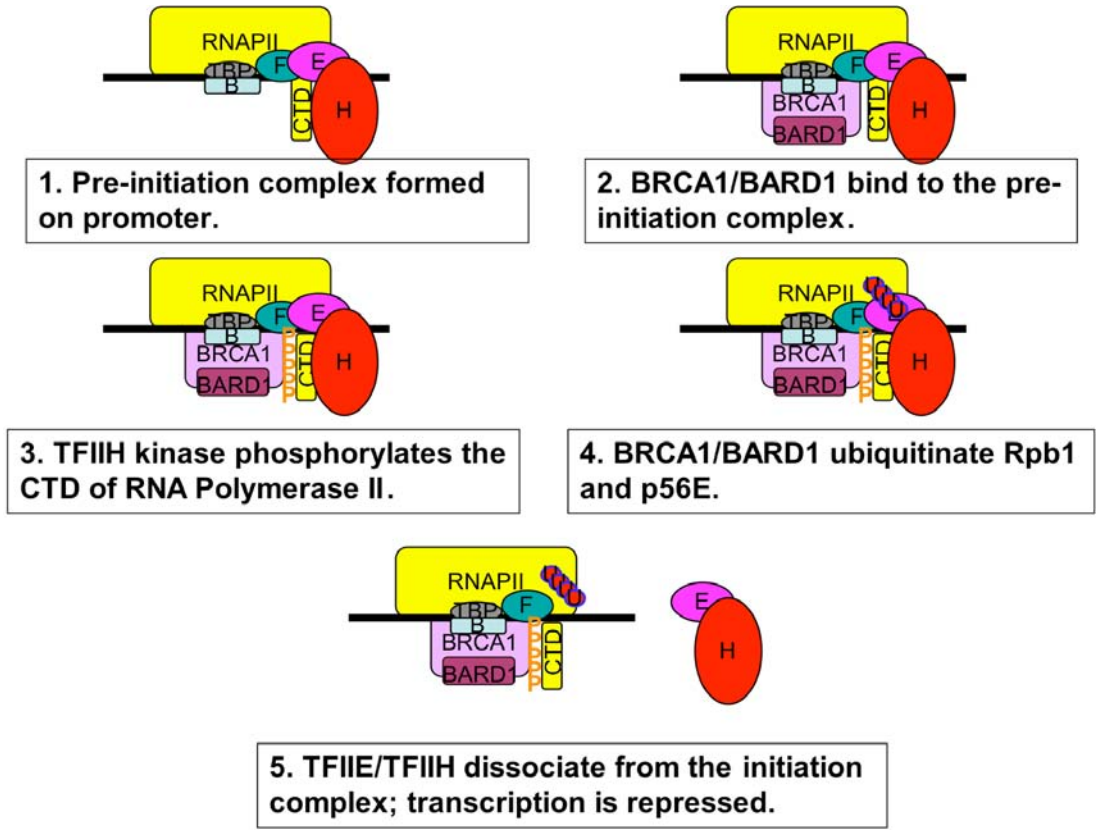


Figure 6: Model for repression of transcription by BRCA1/BARD1.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. ??, pp. 1–xxx, ???? ??, 2006

Direct Stimulation of Transcription Initiation by BRCA1 Requires Both Its Amino and Carboxyl Termini*

Received for publication, December 21, 2005, and in revised form, January 27, 2006
Published, JBC Papers in Press, February 10, 2006, DOI 10.1074/jbc.C500475200

Andrew A. Horwitz[‡], Satish Sankaran[§], and Jeffrey D. Parvin[§1]

From the [‡]Program in Biology and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02115 and the [§]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Published experiments suggest that BRCA1 interaction with RNAPII and regulation of a number of target genes may be central to its role as a tumor suppressor. Previous *in vivo* and *in vitro* work has implicated the carboxyl terminus of BRCA1 in transcriptional stimulation, but the mechanism of action remains unknown, and whether the full-length protein stimulates transcription is controversial. BRCA1 interacts with a number of enhancer-binding transcriptional activators, suggesting that these factors recruit BRCA1 to promoters, where it stimulates RNA synthesis. To investigate whether BRCA1 has intrinsic transcriptional activity, we established a fully purified transcription assay. We demonstrate here that BRCA1 stimulates transcription initiation across a range of promoters. Both the amino and carboxyl termini of BRCA1 are required for this activity, but the BRCA1-binding partner, BARD1, is not. Our data support a model whereby BRCA1 stabilizes productive preinitiation complexes and thus stimulates transcription.

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Of the many functions attributed to BRCA1,[2] one of the first identified was transcriptional stimulation (1, 2). BRCA1 copurifies with the RNA polymerase II (RNAPII) holoenzyme (3, 4), and reporter assays and microarray studies show that it regulates the expression of a range of p53-dependent and -independent targets (5, 6). Thus, one way in which BRCA1 may serve as a tumor suppressor is through up-regulation of growth-suppressive targets (7, 8). While the mechanism of stimulation is unknown, the transcriptional activity of BRCA1 most likely depends in part on its reported interactions with a wide range of transcriptional activators. However, in a defined system assayed *in vitro*, a Gal4 fusion to the carboxyl terminus of BRCA1 activates transcription, independent of other activators (9), suggesting an intrinsic transcriptional activity for BRCA1. A subsequent study found that Gal4 fusions to full-length BRCA1 could not activate transcription in transfected cells and that the degree of transcriptional activation conferred by Gal4 fusions to the carboxyl terminus of bovine BRCA1 was much lower than human BRCA1 (10). Since the human carboxyl terminus is more acidic than the bovine version, the transcriptional activity may simply be a function of its acidity. Regardless, *in vivo* reporter assays using BRCA1 without a Gal4 fusion indicate that transcriptional stimulation by BRCA1 is dependent on its carboxyl terminus (6, 11). To better understand whether BRCA1 might directly regulate transcription, we developed an assay to test the function of full-length human BRCA1 in transcription, independent of an artificial DNA-binding domain protein fusion. We demonstrate here that BRCA1 stimulates basal transcription by promoting initiation of RNA synthesis. This is the first demonstration of direct transcriptional activity by full-length BRCA1.

* This work was supported by a predoctoral fellowship from the Department of Defense Breast Cancer Research Program (to A. A. H.), a postdoctoral fellowship from the Komen Foundation (to S. S.), and National Institutes of Health Research Grant CA90281 (to J. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] To whom correspondence should be addressed. E-mail: jparvin@rics.bwh.harvard.edu.

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[2] The abbreviations used are: BRCA1, breast cancer gene 1; BARD1, BRCA1-associated RING domain protein 1; RNAPII, RNA polymerase II; TBP, TATA-binding protein; TFII, transcription factor II.

MATERIALS AND METHODS

Transcription Factors—The transcription factors used in these assays were purified using established techniques (9, 12, 13). BRCA1/BARD1, BRCA1, and the truncation mutants were purified from baculovirus infected insect cells as described previously (14, 15). p53 was also purified from baculovirus infected insect cells (16).

Plasmid Templates—G-less cassette templates were based upon the $p(C2AT)_{19}$ vector (17) and have been described previously (18).

Transcription Assay—Transcription assays were based on reactions described by Parvin and Sharp (19). Reactions contained 20 mM Hepes-NaOH, pH 7.9, 20% glycerol, 1 mM EDTA, 60 mM KCl, 0.1 mM each ATP and UTP, 0.05 mM 3′-*O*-methyl-GTP, 0.003 mM CTP, 1 mM dithiothreitol, 0.15 mg/ml bovine serum albumin, 2 mM $MgCl_2$, 0.003 mM $ZnSO_4$, 1.2 μg/ml plasmid template (1 nM), 10 μCi of [α-^{32}P]CTP (800 Ci/mmol; PerkinElmer Life Sciences) and transcription factors. Unless otherwise noted, the amount of each factor used per 25-μl reaction was: 8 ng of yeast TBP (16 nM) or 1 μl of immunoaffinity-purified TFIID (containing ~4 ng of TBP), 60 ng of TFIIB (60 nM), 100 ng of TFIIA (60 nM), 100 ng of calf thymus RNA polymerase II, 100 ng of TFIIF (40 nM), 4 ng of TFIIE (1.8 nM), and 0.5 μl of TFIIH fraction. Transcriptional activation reactions with p53 contained 100 ng of PC4 (270 nM). Reactions were assembled on ice and then incubated at 30 °C for 120 min. Reactions were terminated by addition of 200 μl of transcription stop mix (7 M urea, 0.5% SDS, 2 mM EDTA, 0.1 M LiCl, 0.35 M NH_4OAc), phenol/chloroform-extracted, ethanol-precipitated, and resolved on 6% polyacrylamide gels containing 8.3 M urea. Gels were dried and exposed to film with an intensifying screen. PhosphorImager analysis was performed using an Amersham Biosciences PhosphorImager and ImageQuant software.

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RESULTS AND DISCUSSION

Based on the prior evidence that BRCA1 is a coactivator of p53 transcriptional targets (5, 6), we first attempted to reconstitute coactivation by purified full-length BRCA1/BARD1 and p53 *in vitro*. We reasoned that in the absence of a Gal4 fusion, sequence specific p53 binding might serve to localize BRCA1/BARD1 to the promoter region. Transcription reactions were performed with purified TFIID, TFIIB, TFIIA, RNAPII, TFIIE, TFIIF, TFIIH, and PC4. To detect transcriptional activation, a modified adenoviral E4 promoter with upstream p53 response elements (p53 G5E4) linked to a 384-base pair G-less cassette was used (16). As an internal control template for basal transcription, the adenoviral major late promoter (ΔML) linked to a 210-base pair G-less cassette was used. Transcription from both templates was low in the absence of BRCA1/BARD1 and p53 (Fig. 1*A*, *lane 1*). To our surprise, addition of BRCA1/BARD1 alone stimulated transcription from both templates (*lane 2*). Addition of p53 specifically activated transcription of the p53 G5E4 template (*lane 3*). Addition of both p53 and BRCA1/BARD1 resulted in the highest ratio of activated/basal transcription, demonstrating that a modest amount of coactivation can occur with these purified factors (*lane 4*). We were intrigued that BRCA1/BARD1 could stimulate transcription in the absence of p53 or a Gal4 fusion. In the following experiments we characterized the mechanism by which BRCA1 directly stimulates basal transcription.

F1

In addition to leaving out p53, we found that by omitting PC4, a factor required for activated transcription (20), the level of RNA synthesis was significantly higher and the stimulatory effect on transcription by BRCA1 was apparent (Fig. 1*B*). We tested several promoters for effects by BRCA1/BARD1 on RNA synthesis. All of these templates were identical with the exception of the 50 base pairs of sequence in the core promoter immediately upstream of the G-less cassette sequence. The magnitude of the stimulation of RNA synthesis by BRCA1/BARD1 differed among templates, indicating that the effect of BRCA1/BARD1 varied dependent on core promoter sequences (Fig. 1*B*). Stimulation was highest (~10-fold) for the p53 G5E4 promoter template (*lanes 5* and *6*), and we chose that template for subsequent experiments. The fact that BRCA1/BARD1 stimulated transcription from the IgG template, which does not require TFIIE/TFIIH, indicated that these factors were not required for transcriptional stimulation. Indeed, removal of TFIIE and TFIIH from the reaction and substitution of TBP for TFIID did not affect the stimulation of RNA synthesis by BRCA1/BARD1 (Fig. 1*C*).

One trivial explanation for these results would be if the BRCA1/BARD1 preparation used in our assay contained a contaminating general transcription

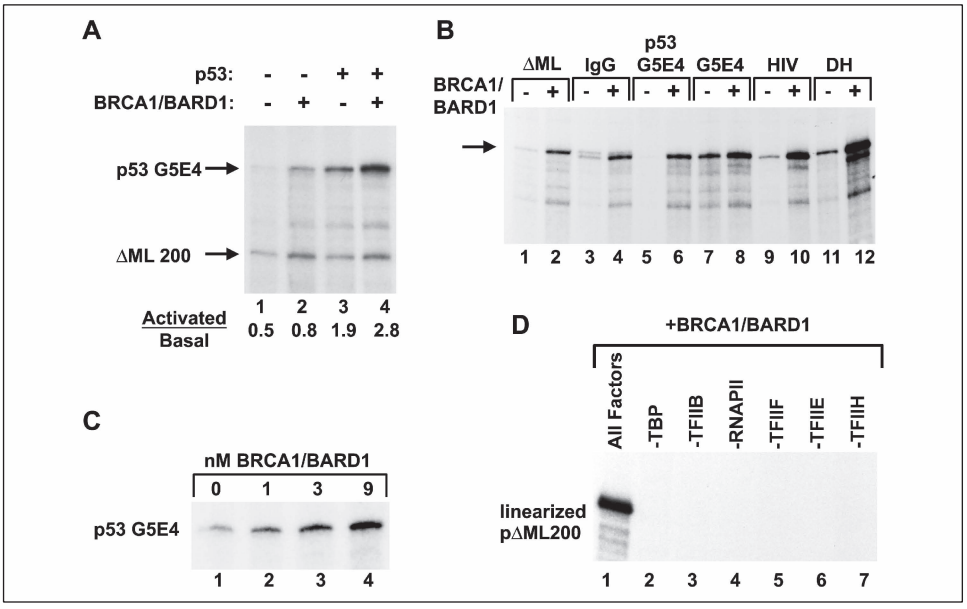

FIGURE 1. **BRCA1/BARD1 stimulates basal transcription.** *A*, BRCA1/BARD1 (10 nM) or p53 (40 nM) were added to *in vitro* transcription reactions containing purified factors (TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH) and the coactivator PC4. Transcription from the p53 G5E4 plasmid, which contains a p53 response element, yields a 390-nucleotide RNA. Transcription from the basal control template, ΔML200, which lacks p53 response elements, yields a 210-nucleotide RNA. Basal transcription in the absence of activators (*lane 1*) or stimulation of transcription by p53 (*lanes 3* and *4*) and BRCA1/BARD1 (*lanes 2* and *4*) were assayed. The ratio of stimulated/basal transcription was determined by PhosphorImager analysis of the accumulated RNA from the p53 G5E4 template relative to the ΔML200 template. *B*, stimulation of transcription by BRCA1/BARD1 was tested in reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH using a variety of single ~400-base pair G-less cassette templates. Full-length transcript is noted by an *arrow*. The following promoters linked to G-less cassette templates were used: ΔML, the core adenoviral major late promoter (*lanes 1* and *2*); IgG, the immunoglobulin heavy chain promoter (*lanes 3* and *4*); p53 G5E4, the adenoviral E4 promoter with a 20-bp p53 response element upstream of the TATA box (*lanes 5* and *6*); G5E4, the adenoviral E4 promoter without p53 response elements (*lanes 7* and *8*); HIV, the human immunodeficiency virus promoter (*lanes 9* and *10*); DH, the *Drosophila* heat shock promoter (*lanes 11* and *12*). ***C.*** Transcriptional stimulation by BRCA1/BARD1 in a minimal system, including TBP, TFIIA, TFIIB, RNAPII, TFIIF and the p53 G5E4 template. BRCA1/BARD1 was omitted (*lane 1*) or added at 1–9 nM concentrations, as indicated (*lanes 2–4*). *D*, the BRCA1/BARD1 preparation does not complement transcription reactions lacking a single factor. Transcriptions from a linearized ΔML200 template were assembled with BRCA1/BARD1 and TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH (*lane 1*). *Lanes 2–7* were assembled in the same way but with a single transcription factor omitted: TBP (*lane 2*), TFIIB (*lane 3*), RNAPII (*lane 4*), TFIIF (*lane 5*), TFIIE (*lane 6*), and TFIIH (*lane 7*).

factor that was limiting in the assay. The BRCA1/BARD1 protein was purified from insect cells and judged free of major contaminants by silver stained protein gels (15). However, to rule out this possibility, we tested whether the BRCA1/BARD1 preparation could complement transcription reactions lacking a single factor (Fig. 1*D*). Transcriptions were conducted using a linearized ΔML template that requires TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH. BRCA1/BARD1 was present in all reactions at a 9 nM concentration. Transcription was observed only when all factors were present, and thus we exclude the possibility that the BRCA1/BARD1 preparation contained a general transcription factor.

Having established that BRCA1/BARD1 stimulated basal transcription in a minimal RNAPII transcription system, we next asked what stage of transcription BRCA1/BARD1 enhanced. We used a pulse/chase strategy to separate transcriptional initiation from elongation (Fig. 2*A*). In the pulse phase, only ATP and [α-^{32}P]CTP were added to the reaction mixture. The lack of UTP prevented elongation from occurring beyond four nucleotides, resulting in a stalled RNAPII complex. In the chase phase, a complete, unlabeled nucleotide mixture was added with excess CTP, allowing elongation of the labeled nascent transcripts. Any new initiations that occurred during the chase phase were unlabeled and thus not detected. Regardless of whether TFIID or TBP was used for TATA binding activity, inclusion of BRCA1/BARD1 during the pulse stimulated transcription, while addition during the chase had no effect (Fig. 2*B*). These results indicated that BRCA1/BARD1 stimulate basal transcription by promoting initiation. However, it was also possible that BRCA1/BARD1 load during the initiation phase but then promote transcriptional elongation. To determine whether this might be true, we examined transcription from very short templates (40–50 nucleotides), reasoning that the importance of an elongation factor over such a short template would be greatly reduced. A similar level of stimulation of RNA synthesis was observed for these mini-templates (~10-fold) as was seen for the ~400-base pair templates, thus supporting the idea that BRCA1/BARD1 promote the initiation of transcription (Fig. 2*C*).

Both BRCA1 and BARD1 copurify with the RNAPII holoenzyme (21), and thus we used the heterodimer in experiments to this point. The major functional outcome of the BRCA1/BARD1 interaction is to potentiate the E3 ubiquitin ligase activity of BRCA1 (22). We had no reason to believe this enzymatic function had a role in transcriptional stimulation because E1 and E2 enzymes and ubiquitin were omitted from the reactions. Therefore, we tested whether BARD1 was required for transcriptional stimulation by BRCA1. When comparing BRCA1/BARD1 to BRCA1 alone, we observed similar levels of stimulation of RNA synthesis, evident in each case at concentrations as low as 1 nM (Fig. 3*A*). We conclude that BARD1 is not required for transcriptional stimulation by BRCA1. Next we examined truncations of BRCA1 to determine what portion of the protein contains the stimulatory activity. Deletion of either the 300 amino-terminal residues or the 336 carboxyl-terminal residues of BRCA1 abolished stimulation of transcription (Fig. 3*B*). Both the amino and carboxyl termini of BRCA1 are known to interact with RNAPII (21), and these truncations may reduce association with RNAPII in our assay. In addition, previous reports localize transcriptional activity to the carboxyl terminus of BRCA1 (2, 9). Since truncation of either terminus did not support transcriptional stimulation, we tested an additional two internal deletions spanning most of the intervening sequence (Fig. 3*C*). Both BRCA1-(Δ303–770)/BARD1 and BRCA1-(Δ770–1290)/BARD1 stimulated transcription as well as or better than BRCA1/BARD1. At the highest concentration tested (9 nM), the BRCA1-(Δ770–1290)/BARD1 actually repressed transcription, possibly reflecting a transcriptional squelching effect. In summary, the amino and carboxyl termini, but not internal domains of BRCA1, are required for transcriptional stimulation (Fig. 3*D*).

Our data to this point suggested that BRCA1 might be promoting formation of the initiation complex through contacts mediated by its amino and carboxyl termini. To determine which transcription factors might be affected by these contacts, we attempted to titrate factors downward in concentration, reasoning that the stimulatory activity should be enhanced by limiting conditions for the relevant factors. To our surprise, downward titration of TFIIA resulted in higher levels of basal transcription and a reduction in the stimulatory effect of BRCA1 (Fig. 4*A*). Without TFIIA (*lanes 1* and *2*), we observed a negligible stimulatory effect of BRCA1, but the inhibitory activity of TFIIA on basal transcription was relieved by addition of BRCA1. TFIIA is known to act as an anti-repressor for TBP-binding inhibitors and is a required factor in activated transcription systems utilizing TFIID (23, 24), so repression was unexpected.

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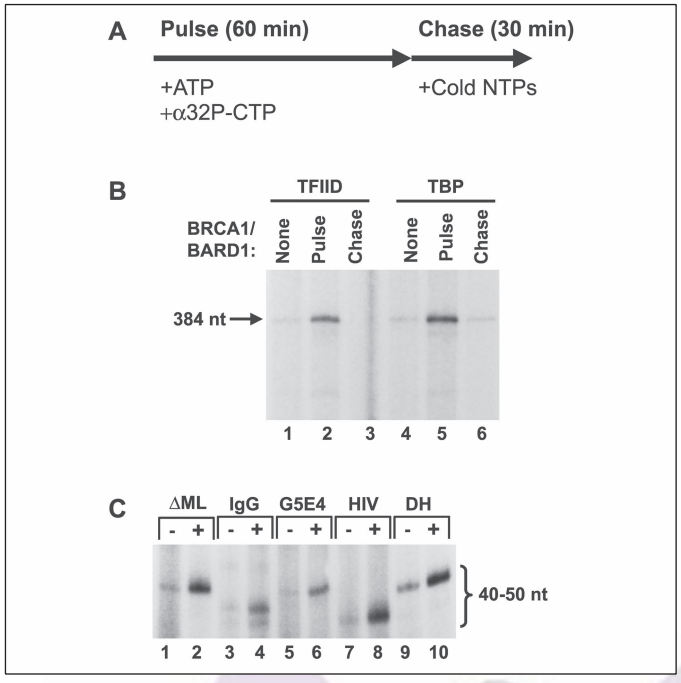

FIGURE 2. **BRCA1/BARD1 stimulate transcriptional initiation.** *A*, schematic of pulse/chase experiment used to separate transcriptional initiation from elongation. Transcription reactions were assembled without UTP and incubated for 60 min (pulse). Complete cold nucleotides, with excess CTP, were added for an additional 30-min incubation (chase). B, transcription reactions containing TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and TFIID (*lanes 1–3*) or TBP (*lanes 4–6*) were assembled as described above. BRCA1/BARD1 (10 nM) was omitted (*lanes 1* and *4*), added during the pulse (*lanes 2* and *5*), or added during the chase (*lanes 3* and *6*). *C*, transcription reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH and mini-templates (~50 base pairs) were performed with (*even lanes*) or without (*odd lanes*) BRCA1/BARD1 (10 nM). The promoters correspond to those used with the ~400-base pair templates described in the legend to Fig. 1*B*.

However, this was not the first observation of basal repressive action by TFIIA. Prior to the cloning and recombinant expression of TFIIA, researchers reported on a repressive activity that purified closely with TFIIA (25). This activity repressed basal transcription from consensus TATA box promoters but stimulated transcription from non-consensus promoters. The authors (25) suggested a model whereby TFIIA interacts with TBP, altering its conformation and association with the promoter. If this conformational change altered the preference of TBP for the TATA box, then it could interfere with formation of the preinitiation complex on the correct DNA site and repress transcription (25).

Based on these previous findings, we speculated that BRCA1 might prevent improper TBP localization, either by disrupting non-TATA bound TBP or by stabilizing complex formation on *bona fide* TATA boxes. Precedent for regulation of TBP binding exists in the ATPase Mot1, which can dissociate TBP from DNA. Initial *in vitro* work cast Mot1 as a transcriptional inhibitor (26, 27), but examination *in vivo* also demonstrated activation of several targets (28–30). Subsequent *in vitro* work using lower concentrations of Mot1 recapitulated transcriptional stimulation, especially under conditions where excess non-promoter DNA was present (31). The authors (31) concluded that Mot1 acts by promoting dissociation of TBP from non-TATA DNA sequences and thereby raising the effective TBP concentration.

The plasmid templates used in our experiments have ~3000 base pairs of sequence, of which about 50 base pairs serve as promoter. Many suboptimal TATA boxes exist in the extraneous DNA, and we infer that TFIIA stabilizes TBP on these non-promoter sites, thus reducing the effective concentration of TBP. Our results show that BRCA1 counters TFIIA repression, and our results are consistent with this rescue occurring during preinitiation or initiation. To test whether BRCA1 could stimulate basal transcription in the absence of TFIIA, but under conditions that were unfavorable for initiation, we limited the general transcription factors involved in nucleation of the preinitiation complex, TBP and TFIIB. By limiting TBP 10-fold (from 16 to 1.6 nM), a modest stimulation of transcription by BRCA1 was revealed (Fig. 4*B*, *lanes 1* and *2*, compare with Fig. 4*A*, *lanes 1* and *2*). Under conditions where both TBP and TFIIB are limiting, the effect of BRCA1 was further enhanced (Fig. 4*B*, *lanes 3* and *4*). This result demonstrates that the stimulatory activity of BRCA1

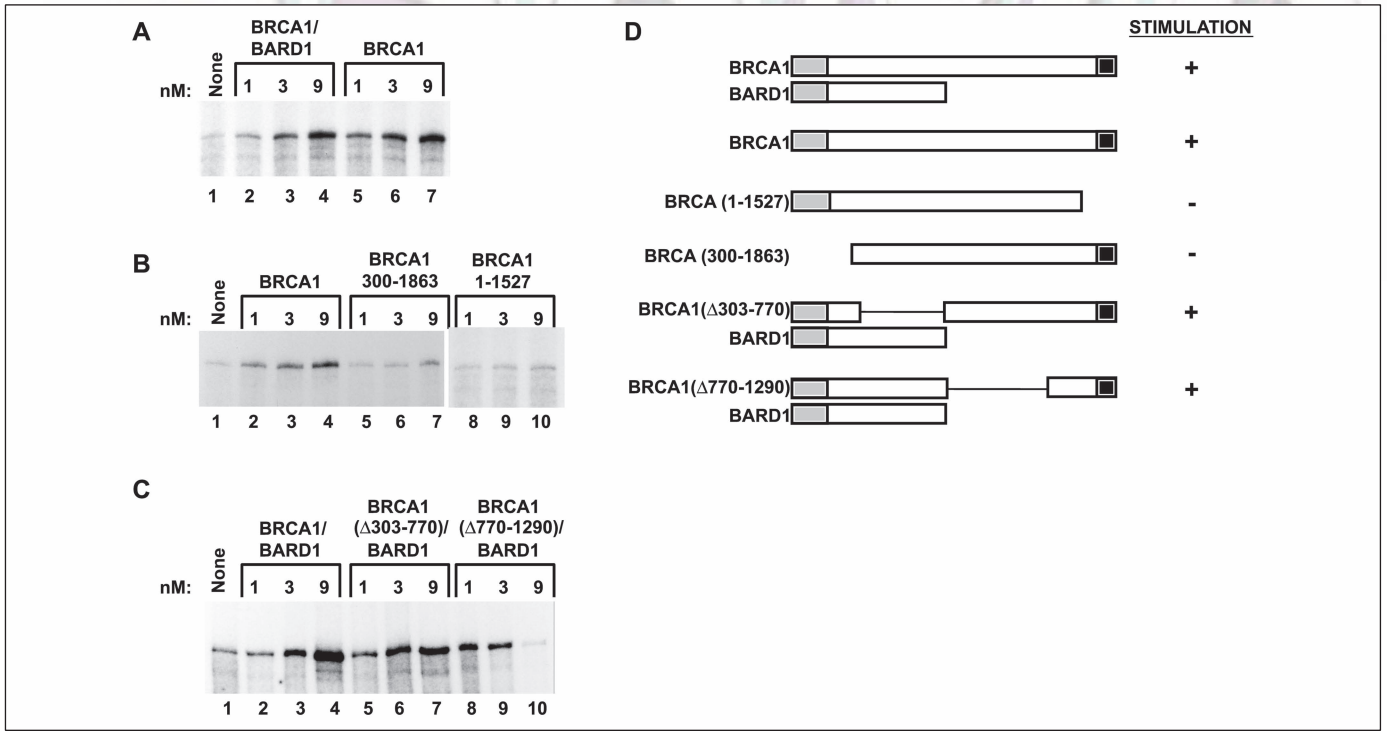

FIGURE 3. **BRCA1 amino and carboxyl termini are required for transcriptional stimulation.** *A*, transcriptional stimulation by BRCA1/BARD1 was compared with BRCA1 alone in reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and the p53 G5E4 template. The BRCA1 preparations were balanced by BRCA1 content and titrated into the reactions at 1 nM (*lanes 2* and *5*), 3 nM (*lanes 3* and *6*), and 9 nM (*lanes 4* and *7*). *B*, transcriptional stimulation by BRCA1 and the truncations BRCA1-(300–1863) and BRCA1-(1–1527) was tested as in *A*. *C*, transcriptional stimulation by BRCA1/BARD1 and internal deletions BRCA1-(Δ303–770)/BARD1 and BRCA1-(Δ770–1290)/BARD1 was tested as in *A*. *D*, summary of transcriptional stimulation by BRCA1 variants. Amino-terminal domain (*gray*) denotes RING finger domains in BRCA1 and BARD1, and the carboxyl-terminal domain (*black*) corresponds to BRCT repeats in BRCA1.

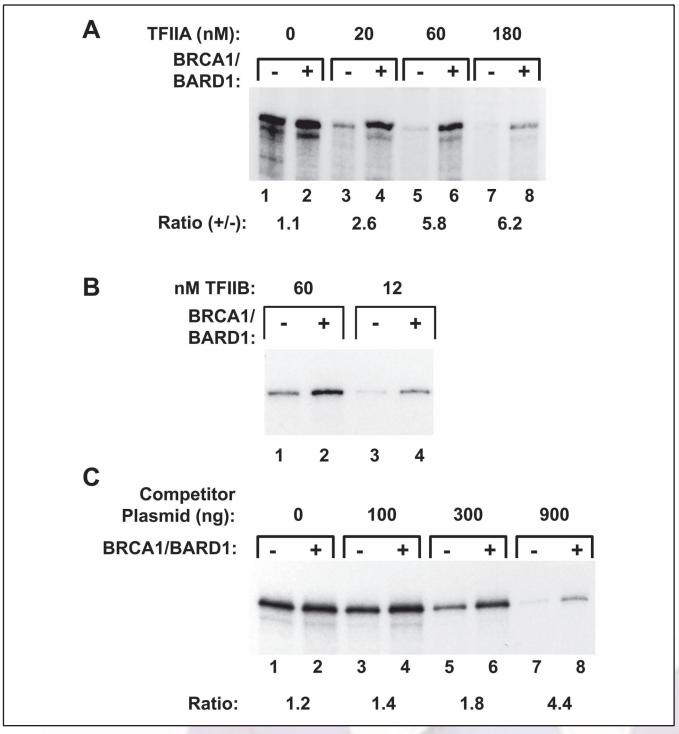

FIGURE 4. **BRCA1 promotes productive preinitiation complex formation.** *A*, TFIIA was titrated into transcription reactions containing TBP, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and p53 G5E4 template, with BRCA1/BARD1 (10 nM) included in the *even-numbered lanes*. TFIIA concentrations were 0 nM (*lanes 1* and *2*), 20 nM (*lanes 3* and *4*), 60 nM (*lanes 5* and *6*), and 180 nM (*lanes 7* and *8*). For each concentration of TFIIA, the ratio of RNA products in transcription reactions containing BRCA1/BARD1 to without BRCA1/BARD1 was determined by PhosphorImager analysis. *B*, transcription reactions were assembled without TFIIA, containing TBP (1.6 nM), RNAPII, TFIIF, TFIIE, TFIIH, and the p53 G5E4 template. TFIIB was added at 60 nM (*lanes 1* and *2*) or 12 nM (*lanes 3* and *4*), with BRCA1/BARD1 added in the *even-numbered lanes*. *C*, competitor plasmid DNA lacking eukaryotic promoter sequences was titrated into transcription reactions utilizing p53 G5E4 template and containing TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH. BRCA1/BARD1 was added to the *even-numbered lanes*. Lanes contained 0 ng of competitor plasmid (*lanes 1* and *2*), 100 ng (*lanes 3* and *4*), 300 ng (*lanes 5* and *6*), and 900 ng (*lanes 7* and *8*). The fold stimulation of transcription by BRCA1/BARD1 at each amount of plasmid addition was determined using a PhosphorImager.

is not limited to reversal of TFIIA basal repression but applies more generally to situations under which preinitiation complex assembly is a limiting step.

The challenges to proper initiation in our transcription assay likely underestimate the difficulties *in vivo*, where correct promoters must be discriminated from total genomic DNA. To test whether the presence of excess plasmid DNA could inhibit transcription, we titrated a competitor plasmid lacking promoter sequences into transcription reactions that were conducted in the presence or absence of BRCA1/BARD1 (Fig. 4*C*). With addition of 300 ng or more of competitor DNA, transcription levels were reduced, confirming that excess DNA can inhibit transcription (compare *lanes 1* and *2* with *lanes 5–8*). The most likely explanation for this effect was that the competitor DNA titrated initiation factors away from the bona fide TATA box. Although transcription levels were lower overall, we observed an increasing degree of transcriptional stimulation by BRCA1/BARD1 with increasing competitor plasmid. Without competitor DNA, the addition of BRCA1/BARD1 stimulated transcription only 1.2-fold (*lanes 1* and *2*). At the highest level of competitor plasmid tested (900 ng), RNA synthesis was stimulated by BRCA1/BARD1 over 4-fold (*lanes 7* and *8*). Therefore, the presence of excess competitor DNA inhibits transcription but increases the potential for stimulation by BRCA1.

We find that limiting the initiation factors TFIIB and TBP, either directly or by addition of excess competitor DNA, increases the stimulatory effect of BRCA1. This outcome could be explained by BRCA1 stabilization of productive initiation complexes or conversely by destabilization of non-productive complexes. Based on the known interaction between BRCA1 and RNAPII, the former possibility is, in our opinion, more likely. Taken together, our data support a model where BRCA1 stabilizes productive transcription initiation complexes, and this may be one mechanism by which it coactivates the transcription of gene targets. Stimulation by BRCA1 was observed in our assays with purified components and a range of promoters at concentrations as low as 1 nM BRCA1. However, in the cell, where BRCA1 concentration is likely even lower, it could be recruited to specific promoters by enhancer-binding factors. Once bound to a specific promoter, BRCA1 could stimulate assembly of the preinitiation complex through its interactions with RNAPII and perhaps other general transcription factors.

Acknowledgments—We thank Karen Griffin for protein purification, Lea Starita and Amanda Simons for BRCA1 truncation constructs, and Steve Buratowski and Karl Munger for helpful discussion.

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