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

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Published experiments suggest that BRCA1 interaction with RAPNII 
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 mecha-
nism 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 fac-
tors 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 activ-
ity, but the BRCA1-binding partner, BARD1, is not. Our data support a 
model whereby BRCA1 stabilizes productive preinitiation complexes and 
thus stimulates transcription.

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 (RNAP II) holoenzyme (3, 4), and reporter assays and microarray studies 
show that it regulates the expression of a range of p53-dependent and -in-
dependent 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 Ga4 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 Ga4 fusions to full-length 
BRCA1 could not activate transcription in transfected cells and that the degree 
of transcriptional activation conferred by Ga4 fusions to the carboxyl termi-

m of bovine BRCA1 was much lower than human BRCA1 (10). Since the 
human carboxyl terminus is more acidic than the bovine version, the trans-
scriptional activity may simply be a function of its acidity. Regardless, in vivo 
reporter assays using BRCA1 without a Ga4 fusion indicate that transcrip-
tional 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 trans-
scription, 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 transcrip-
tional activity by full-length BRCA1.

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 
cells (16).

Plasmid Templates—G-less cassette templates were based upon the 
p(CAZT)Ap 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 
nm 32P-O-methyl-GTP, 0.003 mm CTP, 1 mm diethiothreitol, 0.15 mg/ml 
bovine serum albumin, 2 mm MgCl2, 0.003 mm ZnSO4, 1.2 µg/ml plasmid 
template (1 nm), 100 µCi of [α-32P]CTP (800 Ci/mmol; PerkinElmer Life Sci-
ences) 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 TFII D (containing ~4 ng of TBP), 60 ng of TFII F (60 
µm), 100 ng of TFII G (60 µm), 100 ng of calf thymus RNA polymerase II, 100 ng 
of TFII I (40 nm), 4 ng of TFII H (1.8 nm), and 0.5 µl of TFII H fraction. Tran-
scriptional 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. 

Results 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 mM NaOH, QAC)-phenol/chloroform 
extracted, ethanol-precipitated, and resolved on 6% polyacrylamide gels con-
taining 8.3 µm urea. Gels were dried and exposed to film with an intensifying 
screen. PhosphorImager analysis was performed using an Amersham Bio-
sciences PhosphorImager and ImageQuant software.

RESULTS AND DISCUSSION

Based on the prior evidence that BRCA1 is a coactivator of p53 transcrip-
tional 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 Ga4 fusion, sequence specific p53 binding might serve to localize BRCA1/
BARD1 to the promoter region. Transcription reactions were performed with 
purified TFII D, TFII F, TFII A, RAPNII, TFII E, TFII F, TFII H, 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 (19). 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 
avtivated/basal transcription, demonstrating that a modest amount of coacti-
vation can occur with these purified factors (lane 4). We were intrigued that 
BRCA1/BARD1 could stimulate transcription in the absence of p53 or a Ga4 
fusion. In the following experiments we characterized the mechanism by 
which BRCA1 directly stimulates basal transcription.

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 sig-
ificantly higher and the stimulatory effect on transcription by BRCA1 was 
apparent (Fig. 1A, lane 1). 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 syn-
thesis by BRCA1/BARD1 differed among templates, indicating that the effect 
of BRCA1/BARD1 varied dependent on core promoter sequences (Fig. 1B). 
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 TFII E/TFII H, indicated that these factors were not 
required for transcriptional stimulation. Indeed, removal of TFII E and TFII H 
from the reaction and substitution of TBP for TFII D did not affect the stimu-
lation of RNA synthesis by BRCA1/BARD1 (Fig. 1C).

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

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2 The abbreviations used are: BRCA1, breast cancer gene 1; BARD1, BRCA1-associated 
RING domain protein 1; RAPNII, RNA polymerase II; TBP, TATA-binding protein; TFII, 
transcription factor II.
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. 1D). Transcriptions were conducted using a linearized ΔML template that requires TBP, TFIIA, RNAPII, TFIIH, and TFIIIF. 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 transcription initiation from elongation (Fig. 2A). In the pulse phase, only ATP and [α-32P]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 TFIIH 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. 2B). These results indicated that BRCA1/BARD1 stimulate basal transcription by promoting transcription 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. 2C).

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. 3A). 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 amino-terminal residues or the 336 carboxyl-terminal residues of BRCA1 abolished stimulation of transcription (Fig. 3B). 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. 3C). 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. 3D).

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. 4A). 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 TFIIH (23, 24), so repression was unexpected.
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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 disassociate 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.

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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. 4C). 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 TFIIA and TFB, 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.

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REFERENCES

1. Chapman, M. S., and Verma, I. M. (1996) Nature 382, 678–679
2. Monteiro, A. N., August, A., and Hanafusa, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13595–13599
3. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5605–5610
4. Neubauer, S. E., Anderson, S. F., Schlegel, B. P., Wei, W., and Parvin, J. D. (1998) Nucleic Acids Res. 26, 847–853
5. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., and El-Deiry, W. S. (1998) Oncogene 16, 1713–1721
6. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2302–2306
7. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997) Nature 389, 187–190
8. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Engler, C., Christians, F. C., Ellisen, L. W., Maheshwaran, S., Oliner, J. D., and Haber, D. A. (1999) Cell 97, 575–586
9. Haile, D. T., and Parvin, J. D. (1999) J. Biol. Chem. 274, 2113–2117
10. Krum, S. A., Womack, J. E., and Lane, T. F. (2003) Oncogene 22, 6032–6044
11. Jin, S., Zhao, H., Fan, F., Blanken, P., Fan, W., Colacchio, A., B. F., Fornace, A. J., Jr., and Zhan, Q. (2000) Oncogene 19, 4050–4057
12. Mondal, N., Zhang, Y., Jonsson, Z., Dhar, S. K., Kannapiran, M., and Parvin, J. D. (2003) Nucleic Acids Res. 31, 5016–5024
13. Schlegel, B. P., Green, V. J., Ladas, J. A., and Parvin, J. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 97, 3148–3153
14. Starita, L. M., Machida, Y., Sankaran, S., Elias, J. E., Griffin, K., Schlegel, B. P., Gygi, S. P., and Parvin, J. D. (2004) Mol. Cell. Biol. 24, 8457–8466
15. Starita, L. M., Horowitz, A. A., Keogh, M. C., Ishioka, C., Parvin, J. D., and Chiba, N. (2005) J. Biol. Chem. 280, 24498–24505
16. Mondal, N., and Parvin, J. D. (2005) Cancer Biol. Ther. 4, 411–418
17. Sawadogo, M., and Roeder, R. G. (1985) Cell 39, 165–175
18. Parvin, J. D., Skykint, B. M., Meyers, R. E., Kim, J., and Sharp, P. A. (1994) J. Biol. Chem. 269, 18414–18421
19. Parvin, J. D., and Sharp, P. A. (1993) Cell 73, 533–546
20. Kaiser, K., Stelzer, G., and Mestecky, M. (1995) EMBO J. 14, 3520–3527
21. Chiba, N., and Parvin, J. D. (2002) Cancer Res. 62, 4222–4228
22. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001) J. Biol. Chem. 276, 14537–14540
23. Ma, D., Watanabe, H., Mermelstein, F., Admon, A., Ogar, K., Sun, X., Wada, T., Imai, T., Shiroya, T., and Reinberg, D. (1993) Genes Dev. 7, 2246–2257
24. Ma, D., Olave, I., Merino, A., and Reinberg, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6583–6588
25. Asso, T., Serizawa, H., Conaway, R. C., and Conaway, J. W. (1994) EMBO J. 13, 435–445
26. Aube, D. T., and Hahn, S. (1993) Genes Dev. 7, 844–856
27. Aube, D. T., Hansen, K. E., Mueller, C. G., Lane, W. S., Thorner, J., and Hahn, S. (1994) Genes Dev. 8, 1920–1934
28. Collart, M. A. (1996) Mol. Cell. Biol. 16, 6668–6676
29. Madison, J. M., and Winston, F. (1997) Mol. Cell. Biol. 17, 287–295
30. Prelich, G. (1997) Mol. Cell. Biol. 17, 2057–2065
31. Muldrow, T. A., Campbell, A. M., Weil, P. A., and Aube, D. T. (1999) Mol. Cell. Biol. 19, 2835–2845