We have investigated the DNA substrate specificity of BACH1 (BRCA1-associated C-terminal helicase). The importance of various DNA structural elements for efficient unwinding by purified recombinant BACH1 helicase was examined. The results indicated that BACH1 preferentially binds and unwinds a forked duplex substrate compared with a duplex flanked by only one single-stranded DNA (ssDNA) tail. In support of its DNA substrate preference, helicase sequestration studies revealed that BACH1 can be preferentially trapped by forked duplex molecules. BACH1 helicase requires a minimal 5’ ssDNA tail of 15 nucleotides for unwinding of conventional duplex DNA substrates; however, the enzyme is able to catalytically release the third strand of the homologous recombination intermediate D-loop structure irrespective of DNA tail status. In contrast, BACH1 completely fails to unwind a synthetic Holliday junction structure. Moreover, BACH1 requires nucleic acid continuity in the 5’ ssDNA tail of the forked duplex substrate within six nucleotides of the ssDNA-dsDNA junction to initiate efficiently DNA unwinding. These studies provide the first detailed information on the DNA substrate specificity of BACH1 helicase and provide insight to the types of DNA structures the enzyme is likely to act upon to perform its functions in DNA repair or recombination.

Germ line mutations in BRCA1 lead to an increased lifetime risk of breast and/or ovarian cancer. Cellular studies have revealed that the BRCA1 tumor suppressor gene is required for the maintenance of genomic integrity and a normal level of resistance to DNA damage (for review see Refs. 1–3). The nuclear phosphoprotein BRCA1 contains tandem C-terminal BRCT motifs, a conserved protein sequence found in a large number of DNA damage-response proteins (4). The integrity of the BRCT motifs is required for the role of BRCA1 in double strand break repair (DSBR)\(^1\) and homologous recombination (5–8). Tumor-predisposing missense and deletion mutations in the BRCA1 BRCT domain, all of which render BRCA1 defective in its DSBR function, also disrupt the ability of BRCA1 to bind BACH1. BACH1 is a member of the DEAH subfamily of superfamily 2 helicases (9). Consistent with its predicted helicase domain, BACH1 was recently shown to catalyze DNA unwinding of M13 partial duplex substrates and have a 5’ to 3’ polarity on a linearized M13 directionality substrate (10). A role of BACH1 helicase in DSBR was suggested by the observation that overexpression of a BACH1 allele (K52R) carrying a mutation in its ATP-binding pocket that inactivates its ATPase/helicase function (10) resulted in a marked decrease in the ability of cells to repair DSBs, and that this dominant negative phenotype depended on a specific interaction between BACH1 and BRCA1 (9). More recently, it was shown that the interaction between BRCA1 and BACH1 depends on the phosphorylation status of BACH1 (11–13), and that this phosphorylation-dependent interaction is required for DNA damage-induced checkpoint control during the G2/M phase of the cell cycle (14). Thus, BACH1 likely plays a critical role in DSBR in a manner dependent on its association with BRCA1.

Genetic importance of BACH1 helicase deficiency in the promotion of breast cancer was suggested by the identification of two females among a cohort of 65 women with early-onset breast cancer who carried germ line sequence changes in the BACH1 coding region and normal genotypes for BRCA1 and BRCA2 (9). The clinically relevant BACH1 mutations were shown to interfere with its DNA unwinding function by either completely eliminating catalytic activity (P47A) or significantly reducing the ability of mutant BACH1 enzyme (M299I) to unwind a long (100 bp) M13 partial duplex substrate (10). Taken together, the evidence linking BACH1 with the hereditary breast cancer gene BRCA1 and the identification of patients with mutations in the BACH1 gene itself are consistent with the hypothesis that BACH1 helicase functions in tumor suppression.

Although BACH1 was shown previously to unwind partial duplex DNA substrates with very long ssDNA tails (10), little was known about BACH1 DNA interactions or the substrate preference of the enzyme. To determine which features of the DNA substrates are important for BACH1 to initiate the unwinding reaction efficiently, we systematically evaluated the ability of purified recombinant BACH1 to unwind a panel of related DNA substrates with distinct tail variations including single-stranded versus double-stranded character, tail length, or backbone continuity. In addition, we have assessed the ability of BACH1 to catalytically unwind DNA structures proposed to be key intermediates of cellular DNA metabolism. The results from these unwinding studies provide a platform to investigate the molecular interactions of...
the BACH1 helicase with its protein partners in DSBR by homologous recombination.

**MATERIALS AND METHODS**

**Proteins**—Baculovirus encoding BACH1 (wild-type or mutant K52R) with a C-terminal FLAG tag was used to infect High Five insect cells, and the recombinant BACH1 protein was purified as described previously (10). Briefly, High Five insect cells were infected with baculovirus encoding recombinant BACH1 with a C-terminal FLAG tag. After cell lysis, recombinant BACH1 was bound to FLAG antibody resin and eluted with FLAG peptide. Purified recombinant BACH1 protein predominantly migrated as a single band of the predicted size (130 kDa) on an SDS-polyacrylamide gel (supplemental Fig. 1), as reported previously (10). Protein from cells infected with baculovirus containing BACH1-K52R, a point mutant in motif I of the conserved ATPase domain of BACH1 (10), was purified in an identical manner and co-migrated with the wild-type BACH1 (supplemental Fig. 1), as expected (10).

**DNA Substrates**—PAGE-purified oligonucleotides used for the preparation of DNA substrates were purchased from Midland Certified Reagent Co. and are listed in Table I. DNA duplex substrates were 5′-32P-end-labeled and prepared as described previously (15). DNA substrates are shown in Tables II–IV. Synthetic Holliday junction substrate with a 12-nucleotide (nt) homologous core, designated HJ(X12), was made by annealing four 50-mer oligonucleotides (X12-1, X12-2, X12-3, and X12-4) as described previously (16). D-loop substrates were prepared as described previously (17).

**Helicase Assays**—Helicase assay reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 2% glycerol, 100 ng/μl bovine serum albumin, 2 mM ATP, 10 fmol of the specified duplex DNA substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of BACH1 helicase. Helicase reactions were initiated by the addition of BACH1 and then incubated at 30 °C for 15 min unless otherwise indicated. Reactions were quenched with the addition of 20 μl of 2× Stop buffer (17.5 mM EDTA, 0.3% SDS, 12.5% glycerol, 0.02% bromphenol blue, 0.02% xylene cyanol). For standard duplex DNA substrates, a 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand was included in the quench to prevent reannealing. Unlabeled oligonucleotide was omitted in the quench for helicase reactions with the HJ(X12) and D-loop substrates. The products of the helicase reactions were resolved on non-denaturing 12% (19:1 acrylamide:bisacrylamide) polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Amersham Biosciences).

The percent helicase substrate unwound was calculated by using the following formula: % unwinding = 100 × (P/(S - P)), where P is the product and S is the substrate. The values of P and S have been corrected after subtracting background values in the no enzyme and heat-denatured substrate controls, respectively. Helicase data (Figs. 1, 3–7) represent the mean of at least three independent experiments with standard deviations shown by error bars.

**Helicase Sequestration Experiments**—For helicase sequestration studies, BACH1 (0.5 nM) was preincubated with the indicated concentrations (0–25 nM) of the specified unlabeled competitor DNA molecule (forked duplex, 3′ ssDNA tailed duplex or 5′ ssDNA tailed duplex) in standard helicase reaction buffer (described above) in the absence of ATP for 3 min at 30 °C. 10 fmol of radiolabeled forked duplex molecules (tracker substrate 34, Table III) along with 2 mM ATP was subsequently added to the reaction mixture and incubated for 7 min at 30 °C. Reactions were quenched and resolved on native polyacrylamide gels as described above. Typically, ~60% of the tracker substrate was unwound in reactions lacking competitor DNA molecule. Helicase data (% control) was expressed relative to the control reactions lacking the competitor DNA.

**DNA Binding Assays**—Protein/DNA binding mixtures (20 μl) contained the indicated concentrations of BACH1 and 0.5 nM of the specified 32P-end-labeled DNA substrate in the same reaction buffer as that used for helicase assays (see above) containing 2 mM ATP.
or no nucleotide. The binding mixtures were incubated at 24 °C for 30 min after the addition of BACH1. After incubation, 3 μl of loading dye (74% glycerol, 0.01% xylene cyanol, 0.01% bromphenol blue) was added to each mixture, and samples were loaded onto native 5% (19:1 acrylamide:bisacrylamide) polyacrylamide gels and electrophoresed at 200 V for 2 h at 4 °C using 1× TBE as the Running buffer. The resolved radiolabeled species were visualized using a PhosphorImager and analyzed with ImageQuant software (Amersham Biosciences).

RESULTS

Although BACH1 helicase was shown previously to unwind linearized M13 partial duplex DNA with a 5' to 3' directionality (10), the BACH1 helicase DNA substrate preference had not been examined previously. To determine the DNA substrate requirements for efficient unwinding of double-stranded DNA by BACH1 helicase, we have tested a series of related DNA substrates. Moreover, we tested several key intermediates of recombination as well as standard B-form duplex DNA substrates that represent intermediates of DNA replication and repair.

BACH1 Preferentially Unwinds a Forked Duplex DNA Substrate—Initially, we tested BACH1 on a simple duplex DNA substrate of 19 bp flanked by a 5' ssDNA tail of 26 nt (Table II, substrate 6). BACH1 was able to unwind an increasing percentage of the substrate with greater concentrations of BACH1 helicase (Fig. 1A). However, only 40% substrate was unwound at the highest BACH1 concentration tested (Fig. 1D). DNA unwinding studies indicate that certain DNA helicases preferentially unwind forked duplex substrates (15, 18, 19); therefore, we examined BACH1 helicase activity on a related forked duplex substrate that was flanked by 5' and 3' ssDNA tails of 26 and 25 nt, respectively (Table II, substrate 15). BACH1 efficiently unwound the forked duplex substrate, achieving 50% substrate unwinding at a BACH1 concentration of 0.6 nM (Fig. 1B and D). In contrast, only 13% substrate was unwound with 0.6 nM BACH1 on the 5'-tailed duplex substrate (Table II, substrate 6) (Fig. 1D). At 4.8 nM BACH1, ~80% of the forked duplex substrate was unwound compared with 40% of the 5'-tailed duplex substrate. These results indicate that BACH1 preferentially unwinds a forked duplex substrate with 3' and 5' ssDNA arms compared with a substrate flanked by only a 5' ssDNA tail.

To confirm that the unwinding activity detected was truly
dependent on BACH1 and not because of contaminants in the preparation of the BACH1 protein, helicase assays were conducted on the wild-type and mutant BACH1-K52R proteins purified in an identical manner. In the BACH1-K52R mutant protein, the invariant lysine residue of the conserved motif I (Walker A ATPase site) of DNA helicases was replaced with an arginine (9). On the forked duplex (Table II, substrate 15), wild-type BACH1 unwound ~80% of the substrate molecules in 15 min (Fig. 1, B and D), whereas no unwinding of the forked duplex by BACH1-K52R was detected (supplemental Fig. 2). The inability of the K52R BACH1 mutant to unwind the forked duplex is consistent with those data previously published for a 19-bp M13 partial duplex substrate (10), indicating that the purified recombinant BACH1 used for the helicase analyses was enzymatically pure.

Recently the human DNA helicase CHL1 was shown to unwind with both a 5′ to 3′ and 3′ to 5′ directionality of movement with respect to the single-stranded DNA residing between the duplexes (20). BACH1 shares 48% sequence homology with CHL1 (10, 21); therefore, we addressed the possibility that BACH1 might unwind with a 3′ to 5′ directionality. However, we found that BACH1 poorly unwinds the substrate with a 3′ ssDNA tail of 25 nt (Table II, substrate 10) (Fig. 1, C and D), and BACH1 unwinding efficiency was not improved with increasing BACH1 concentration. These results confirm that BACH1 helicase unwinds with a 5′ to 3′ directionality (10). The inability of BACH1 to unwind with a 3′ to 5′ directionality suggests that this activity does not contribute to the preferential unwinding of the forked duplex substrate by BACH1.

BACH1 Preferentially Binds a Forked Duplex—The preference of BACH1 to unwind the forked duplex substrate raised the possibility that BACH1 might bind this DNA substrate more stably compared with the less efficiently unwound DNA substrate with only a 5′ ssDNA tail. To address this, we performed gel mobility shift assays with BACH1 and the corresponding radiolabeled DNA substrates used for the helicase assays. A shift of the forked duplex substrate (Table II, substrate 15) to a more slowly migrating species was detected on a native 5% polyacrylamide gel in a BACH1 concentration-dependent manner. BACH1 retained its ability to shift the forked duplex in the presence of ATPγS (Fig. 2A) and also in the absence of nucleotides (data not shown). In contrast, no shift of the duplex DNA substrate with only a 5′ ssDNA tail (Table II, substrate 6) was detected in the presence of BACH1 (Fig. 2B, lane 6). Moreover, a reduced shift of the 5′-tailed substrate compared with the forked duplex was observed at lower BACH1 concentrations (data not shown). The DNA substrate with only a 3′ ssDNA (Table II, substrate 10) was bound least efficiently by BACH1 (Fig. 2B, lane 4). These results suggest that BACH1 preferentially binds forked duplex compared with simpler substrates with either a 5′ or 3′ ssDNA tail, and that the binding preference may contribute to the helicase substrate preference of BACH1.

BACH1 Helicase Sequestration Studies—The results from gel mobility shift assays suggested that BACH1 more stably binds the forked duplex substrate compared with the DNA molecules flanked by either a 5′ or 3′ ssDNA tail. If BACH1 preferentially binds the forked duplex, preincubation of BACH1 helicase with unlabeled forked duplex should sequester BACH1 and prevent it from unwinding a labeled forked duplex tracker substrate. To test this possibility, we performed helicase sequestration studies with DNA molecules containing a 3′ ssDNA, 5′ ssDNA, or both 3′ and 5′ ssDNA arms. When BACH1 was preincubated with the unlabeled forked duplex as a competitor, significant inhibition of BACH1-catalyzed unwinding of the tracker substrate (Table III, substrate 34) was observed (Fig. 3A). At all concentrations of forked duplex molecules tested, BACH1 helicase activity on the tracker substrate was inversely related to the amount of competitor DNA present in the preincubation step. Preincubation of BACH1 with 1.5 nM
forked duplex DNA resulted in nearly a 50% reduction of BACH1 helicase activity on the tracker substrate. At 6.25 nM forked duplex, BACH1 helicase activity was reduced by 80% of the control reactions lacking unlabeled forked duplex competitor (Fig. 3, A and D). Preincubation of BACH1 with the DNA duplex flanked by only a 3' ssDNA tail as a competitor exerted a minor effect on BACH1 unwinding of the tracker substrate with only 15% inhibition at 3.12 nM 3'-tailed duplex compared with 70% inhibition at the same concentration of forked duplex (Fig. 3, A and D). Preincubation of BACH1 with the 5'-tailed duplex resulted in an intermediate level of inhibition of BACH1 helicase activity on the tracker substrate. With a 5'-tailed duplex concentration of 3.12 nM, a 36% inhibition was detected. With a 5'-tailed duplex concentration of 12.3 nM, a 69% inhibition was detected (Fig. 3, B and D). Through-
out the range of 0.75–25 nM competitor DNA, the forked duplex more effectively sequestered BACH1 than the 5’H11032-tailed duplex. However, the 5’H11032-tailed duplex was able to trap BACH1 to some extent as it was more effective than the 3’H11032-tailed duplex throughout the competitor DNA titration (Fig. 3D). These results suggest that BACH1 was preferentially sequestered by the forked duplex followed by the 5’H11032-tailed duplex, and to a lesser extent by the 3’H11032-tailed duplex. In addition, the sequestration assays are consistent with the results from the gel mobility shift assays, suggesting that BACH1 preferentially binds the forked duplex structure.

BACH1 Helicase Activity on Duplex DNA Substrates with Increasing 5’ or 3’ ssDNA Tail Lengths—Given that suboptimal unwinding was obtained on a duplex substrate with a 26-nt 5’H11032 ssDNA tail (substrate 6, Table II) at the highest BACH1 concentration tested, we sought to determine whether the length of 5’H11032 tail might influence the ability of BACH1 to unwind the flanking duplex. To evaluate this issue and determine the minimal loading dock requirements by BACH1 to catalyze efficient DNA unwinding, we performed BACH1 helicase assays on duplex DNA substrates flanked by a 5’ ssDNA tail of increasing lengths (Fig. 4A). Minor unwinding of the 19-bp duplex by BACH1 (2.4 nM) was detected on substrates flanked by 5’ tails of 5 or 10 nt. An increase of the 5’ tail length to 15 nt

FIG. 3. BACH1 is preferentially sequestered on forked duplex DNA molecules. Reaction mixtures (20 μl) containing 0.5 nM BACH1 (lanes 2–9) and the indicated concentrations of unlabeled forked duplex (A), 5’ ssDNA tailed duplex (B), or 3’ ssDNA tailed duplex (C) were incubated at 30 °C for 3 min as described under “Materials and Methods.” After 3 min, 0.5 nM radiolabeled forked duplex tracker (substrate 34) and 2 mM ATP were added to each reaction and incubated for an additional 7 min at 30 °C and subsequently quenched. Helicase reaction products were resolved on 12% polyacrylamide gels. Lane 1, no enzyme control (NE); lane 10, heat-denatured DNA substrate control. D, quantitative analyses of BACH1 helicase data are shown. Filled square, forked duplex; open diamond, 5’ ssDNA tailed duplex; filled triangle, 3’ ssDNA tailed duplex. Helicase data represent the mean of at least three independent experiments with S.D. indicated by error bars.

FIG. 4. BACH1 helicase activity on duplex DNA substrates with increasing 5’ or 3’ ssDNA tail lengths. A, substrates 1–18 (Table II) were incubated with 2.4 nM BACH1 at 30 °C for 15 min under standard helicase assay conditions as described under “Materials and Methods.” Quantitative analyses of BACH1 helicase data are shown. Open squares, helicase data for 5’-tailed duplex substrates 1–9 with increasing 5’ ssDNA tail lengths. Filled squares, helicase data for forked duplex substrates 10–18 with increasing 5’ ssDNA tail lengths. B, substrate 7 and substrates 19–25 (Table II) were incubated with 0.6 nM BACH1 at 30 °C for 15 min under standard helicase assay conditions as described under “Materials and Methods.” Quantitative analyses of BACH1 helicase data are shown. Helicase data represent the mean of at least three independent experiments with S.D. indicated by error bars.
resulted in ~12% of the substrate unwound by BACH1. As the 5’ ssDNA tail was increased from 15 to 35 nt, a proportionally greater percentage of the DNA substrate was unwound. Approximately 60% of the duplex substrate flanked by a 5’ tail of 35 nt was unwound by BACH1. Increasing the 5’ tail length to 40 or 50 nt resulted in ~15% reduction of the substrate unwound. From these results, we can conclude that for a simple 5’-tailed duplex substrate, BACH1 requires a 5’ tail of ~15 nt to catalyze appreciable unwinding and that a 5’ ssDNA tail of ~35 nt is optimal.

Because the forked duplex is a preferred substrate for BACH1 helicase, we next addressed the importance of the length of the non-complementary 5’ ssDNA tail of a DNA duplex that is also flanked by a fixed 3’ ssDNA tail of 25 nt (Fig. 4A). A forked duplex possessing up to 10 nt of 5’ tail was not unwound by BACH1. However, a forked duplex with a 15-nt 5’ tail was unwound by BACH1 resulting in 40% displacement. Proportionally greater percentages of the forked duplex were unwound as the 5’ tail was increased from 15 to 35 nt, where an optimum was reached. Further increase of the 5’ tail to 40 or 50 nt did not yield greater unwinding of the forked duplex. These unwinding experiments indicate that a 5’ ssDNA tail of 15 nt is the minimal length for BACH1 to unwind the forked duplex substrate and that a 35-nt 5’ ssDNA tail can be judged to be optimal. In addition, the presence of the 3’ ssDNA tail does not relax the minimal 5’ ssDNA tail length requirement for BACH1 unwinding of the forked duplex substrate.

We also addressed the importance of the length of the non-complementary 3’ ssDNA tail of a forked DNA duplex that was flanked by a fixed 5’ ssDNA tail of optimal length, 35 nt (Fig. 4B). Proportionally greater percentages of the forked duplex were unwound by BACH1 helicase as the 3’ tail was increased from 0 to 26 nt. An optimum was reached for the forked duplex helicase substrate with a 35-nt 3’ tail. Further increase of the 3’ tail to 40 or 50 nt resulted in reduced unwinding of the forked duplex. These results are consistent with our observations that forked duplex substrates are preferentially unwound by BACH1 compared with simple 5’-tailed substrates, and indicate that increasing the 3’ ssDNA tail of the forked duplex up to 35 nt enhances the ability of BACH1 to unwind the forked substrate.

**BACH1 Requires a 5’ ssDNA tail for Unwinding of Standard Duplex DNA Substrates**—Having demonstrated that BACH1 preferentially unwinds forked DNA duplexes, we were interested in the possibility that the BACH1 unwinding preference may stem from a requirement for preexisting ssDNA tracts flanking the duplex region. Thus, we tested whether BACH1 could unwind a 5’ flap substrate. This substrate resembles a fork substrate but also contains an upstream 25-mer hybridized to the upstream ssDNA region that resides below the flap, creating a nick between the adjacent oligonucleotides (Table III, substrate 26). The results from BACH1 helicase assays with the 5’ flap substrate (Fig. 5A) demonstrate that BACH1 efficiently unwound the 5’ flap substrate, releasing the labeled 5’ flap oligonucleotide as evidenced by its co-migration with the heat-denatured substrate control (lane 8). A very small percentage of the upstream primer was displaced from the 5’ flap substrate at higher concentrations of BACH1 (2.4 and 4.8 nM) to yield the forked duplex migrating slightly faster than the intact three-stranded 5’ flap substrate. A quantitative analysis of the BACH1 helicase activity revealed that the 5’ flap substrate is unwound as efficiently as the forked duplex substrate that contains both 5’ and 3’ ssDNA arms (Fig. 5B). In fact, slightly greater unwinding of the 5’ flap was observed at the highest BACH1 concentration, 4.8 nM.

**Fig. 5. BACH1 helicase activity on forked DNA substrates with dsDNA 5’ and/or 3’ tails.** Helicase reactions (20 μl) were performed by incubating the indicated BACH1 concentrations with 0.5 nM of the 5’ flap (Table III, substrate 26), 3’ flap (Table III, substrate 27), or synthetic replication fork (Table III, substrate 28) at 30 °C for 15 min under standard helicase assay conditions as described under “Materials and Methods.” A, phosphorimagery of a typical gel for helicase assays with the 5’ flap (substrate 27) is shown. Lane 1, no enzyme control; lanes 2–7, indicated concentrations of BACH1; lane 8, heat-denatured DNA substrate control. B, quantitative helicase data for substrates 26–28 represent the mean of at least three independent experiments with S.D. indicated by error bars.

The observation that BACH1 efficiently unwound the 5’ flap substrate raised the possibility that BACH1 may have preference for a forked junction. The results from BACH1 helicase assays, however, demonstrated that BACH1 completely failed to unwind the 3’ flap substrate that also has a forked junction (Table III, substrate 27) (Fig. 5B). Similarly, BACH1 failed to unwind a forked duplex in which both the 5’ and 3’ tails were double-stranded, a synthetic replication fork structure (Table III, substrate 28) (Fig. 5B). From these results, we can conclude that BACH1 requires a 5’ ssDNA tail to initiate unwinding of conventional B-form duplex DNA substrates.

**BACH1 Helicase Activity on DNA Substrates with a Backbone Perturbation**—The requirement of a 5’ ssDNA tail flanking the duplex region for BACH1 to unwind DNA substrates suggests two possible mechanisms for BACH1 loading and/or unwinding of the duplex DNA. First, BACH1 displays a defined DNA binding polarity in which it specifically recognizes ssDNA-dsDNA junctions that are flanked by a 5’ ssDNA tail. Second, BACH1 translocates 5’ to 3’ along the strand where it is bound. To investigate the importance of BACH1 helicase contacts with the DNA tails of the helicase substrate during the loading step required for productive initiation of DNA unwinding, we engineered chemically modified oligonucleotide substrates. Such modified substrates are used extensively to in-
vestigate the importance of nucleic acid elements for substrate recognition/initiation of DNA unwinding (22, 23) as well as translocation during the elongation phase of helicase-catalyzed strand separation (24).

To assess the importance of the ssDNA tail for BACH1 to efficiently load onto a DNA substrate, we tested BACH1 helicase activity on a series of DNA substrates in which a triethylene glycol linker was incorporated at different positions into the phosphodiester backbone within the 3’ or 5’ ssDNA tail flanking the duplex. The 18-atom polyglycol linker that we used is estimated to maximally extend up to 6 nt in length for ssDNA and effectively disrupts nucleic acid continuity. Two classes of DNA substrates were tested, the 5’-tailed duplex (Table IV, substrates 35–38) or the forked duplex flanked by both 5’ and 3’ ssDNA tails (Table IV, substrates 39–45). For both substrates, the 5’ tail backbone modification was placed 1, 6, or 25 nt away from the ssDNA-dsDNA junction in the 5’ tail. In addition, the polyglycol linkage was also inserted 1, 6, or 25 nt away from the ssDNA-dsDNA junction in the 3’ tail of the forked duplex.

For the 5’-tailed substrates, BACH1 helicase activity was abolished by a polyglycol linker placed 1 nt away from the ssDNA-dsDNA junction (Fig. 6A). When the modification was moved 6 nt away from the junction, a low but detectable level of unwinding (5%) was observed; however, this was ~6-fold less than either the control (unmodified) substrate or the substrate that had a polyglycol linkage 25 nt away from the ssDNA-dsDNA junction (Fig. 6A). These results suggest that BACH1 requires nucleic acid continuity in the 5’ ssDNA tail to initiate efficiently DNA unwinding of the 5’-tailed DNA substrate. The significantly improved ability of BACH1 to unwind a 5’-tailed duplex substrate with the synthetic linkage 6 nt away from the junction compared with 1 nt away from the ssDNA-dsDNA junction indicates that the position of the phosphate backbone modification differentially affects the ability of BACH1 to initiate DNA unwinding of the helicase substrate.

We next examined BACH1 helicase activity on the forked duplex substrates with the polyglycol backbone modification. As before, the presence of a polyglycol linker in the 5’ ssDNA tail 1 nt away from the ssDNA-dsDNA junction dramatically inhibited BACH1 unwinding of the forked duplex (Fig. 6B). Weak but improved BACH1 unwinding was achieved with positioning the polyglycol linkage in the 5’ tail 6 nt away from the junction (Fig. 6B). Despite the significant inhibition of BACH1 helicase activity by the polyglycol linkage inserted in the 5’ tail either 1 or 6 nt away from the duplex, gel mobility shift assays indicated that BACH1 was able to bind efficiently these modified forked duplex substrates (data not shown). These results suggest that BACH1 requires nucleic acid continuity in the 5’ ssDNA tail of the forked duplex substrate within 6 nt of the ssDNA-dsDNA junction to initiate efficiently DNA unwinding despite the apparent lack of an effect on DNA binding. In contrast, modifications to the 3’ tail do not substantially perturb unwinding of the forked duplex by BACH1 (Fig. 6C).

BACH1 Helicase Activity on DNA Recombination Intermediates—BRCA1 was shown to associate with Bloom syndrome helicase, which has been proposed to function in recombinational repair (25). Although the role of BACH1 in DSBR is not well understood, we began by evaluating whether BACH1 could unwind recombination intermediates. We tested BACH1 helicase activity on a four-stranded Holliday junction structure and a three-stranded D-loop structure, two key intermediates of homologous recombination. At all concentrations tested, BACH1 failed to unwind a Holliday junction structure (Table III, substrate 29) under conditions where the helicase efficiently unwound recombination intermediates—RecQ5 of Drosophila melanogaster (26), RecG of Escherichia coli (27), and Saccharomyces cerevisiae Mer3 (28). To address this possibility, we tested BACH1 for unwinding of the Holliday junction at lower MgCl2 concentrations (1–4 mM), but we did not detect unwinding by BACH1 (data not shown). Because the BACH1 helicase reactions contained 2 mM ATP and 5 mM MgCl2, the free Mg2+ concentration (~3 mM) may have inhibited BACH1 unwinding of the synthetic Holliday junction, as reported previously for several other helicases, including Drosophila RecQ5β (26), Escherichia coli RecG (27), and Saccharomyces cerevisiae Mer3 (28). To address this possibility, we tested BACH1 for unwinding of the Holliday junction at lower MgCl2 concentrations (1–4 mM), but we did not detect unwinding by BACH1 (data not shown). However, BACH1 was able to unwind the forked duplex (Table II, substrate 15) at these lower Mg2+ concentrations (data not shown). As shown previously, WRN helicase efficiently unwound this
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BACH1 to unwind D-loop substrates (Table III, substrates 30–32). BACH1 was able to unwind all three D-loop substrates in a manner dependent on the concentration of BACH1 in the reaction; however, the percent of substrate unwound was dependent on the nature of the third strand tail (Fig. 7, A–C). As expected, the D-loop substrate with a 5’ ssDNA tail was most efficiently unwound, resulting in ~50% displacement of the third strand at a BACH1 concentration of 4.8 nM (Fig. 7D). The D-loop substrates with a 3’ tail or no tail were unwound less efficiently. Most interestingly, BACH1 could unwind (~35%) the D-loop substrate that lacked a tail (Table III, substrate 33) (Fig. 7D). The resolved products from the BACH1 helicase assays with all three D-loop substrates co-migrated with the heat-denatured DNA substrate control with no intermediate structures (Fig. 7, A–C, lane 8). This result indicates that BACH1 specifically released the invading third strand of the D-loop structure only. The inability of BACH1 to unwind a duplex bubble structure (Table III, substrate 33) (data not shown) is consistent with the absence of a two-stranded intermediate product from the BACH1 helicase assays with the three D-loop structures (Fig. 7, A–C). WRN helicase, on the other hand, unwound this bubble substrate in the BACH1 reaction conditions (data not shown). These results suggest that BACH1 poorly unwinds bubble structures under the reaction conditions tested and that BACH1 specifically recognizes the three-stranded D-loop structures and unwinds the invading strand of the D-loop. Unlike standard duplex DNA substrates, a 5’ ssDNA tail is not required for displacement of the invading strand of the D-loop structure by BACH1 helicase.

DISCUSSION

The BACH1 DNA helicase was identified as a direct binding partner of the tumor suppressor BRCA1. Recent structural evidence (11–13) has highlighted the finding that two intact BRCA1 BRCT repeats as well as phosphorylation of BACH1 are critical for BRCA1 binding to BACH1. This highly regulated and specific interaction suggests that BRCA1 binding to BACH1 is significant for BRCA1 function. Along these lines, an interaction between BRCA1 and BACH1 is proposed to be essential for normal repair of DSBs (9). The importance of BACH1 helicase activity in the BRCA1-dependent DNA damage-response was suggested by the observation that the expression of a site-directed BACH1 mutant that inactivated its ATPase/helicase activity (10) exerted a dominant negative effect on BRCA1-dependent DSBR (9). In addition, naturally occurring BACH1 missense mutations that impair the DNA unwinding activity of the enzyme (10) have been genetically linked to breast cancer (9). Although these studies implicate the importance of BACH1 helicase function in double strand break repair, the precise biological function of BACH1-catalyzed unwinding of a cellular DNA intermediate(s) in the pathway is not known.

Presently, the substrate preference and requirements for efficient BACH1 unwinding of DNA have been minimally defined (10). Thus, we have examined the ability of BACH1 to unwind a panel of defined DNA substrates. Our biochemical studies indicate that BACH1 preferentially unwinds a forked duplex substrate compared with a DNA duplex substrate with only a 5’ ssDNA tail. The results from gel mobility shift assays indicate that BACH1 more stably bound the forked duplex substrate compared with the 5’-tailed duplex substrate, suggesting that DNA binding is important in the DNA substrate specificity of BACH1. Furthermore, we found that BACH1 was sequestered by a competitor forked duplex that was present during a preincubation step with BACH1. However, BACH1 was not sequestered by either a 5’- or 3’-tailed duplex competitor molecule. These results provide further evidence that

Holliday junction structure (16) and was active in the BACH1 reaction conditions (data not shown). The inability of BACH1 to unwind the synthetic Holliday junction structure suggests that the enzyme is not likely to act in branch migration of Holliday junctions as proposed for the RecQ helicases (for review see Ref. 25).

Because an early step of homologous recombination that can occur at DSBs is the invasion of ssDNA into a homologous duplex to form a D-loop structure, we examined the ability of
BACH1 has the ability to recognize certain DNA structural elements that are likely to be important for efficient loading of the enzyme during the initiation of DNA unwinding.

Although the assembly state or tertiary structure of BACH1 is not known, the 3' tail may stimulate BACH1 unwinding by sterically determining whether one or two DNA strands pass through a central channel of BACH1, as proposed for DnaB (19). In support of the notion that BACH1 may exist as a multimer, it was observed by gel exclusion chromatography that recombinant wild-type FLAG-tagged BACH1 (an ~130-kDa polypeptide) migrates as an ~500-kDa, enzymatically active species (10). Future studies will likely address the potential importance of BACH1 subunit interactions for its catalytic activity as a helicase in DNA repair pathways.

Consistent with previous results (10), we report that BACH1 requires a 5' ssDNA tail to efficiently unwind standard duplex DNA substrates flanked by either one or two non-complementary tails, single-stranded or double-stranded in nature. The minimal loading site of 15 nt was shown to be required for BACH1 to appreciably unwind multiple substrates. The requirement for a single-stranded 5' tail might be expected for a 5' to 3' DNA helicase; however, a number of DNA helicases have relaxed DNA substrate specificities and are more promiscuous in nature. For example, the human 3' to 5' RecQ helicase WRN does not require a pre-existing ssDNA tail of defined polarity to catalyze efficient unwinding of B-form duplex DNA substrates (15). Although BACH1 requires a 5' ssDNA flanking sequence to unwind conventional DNA substrates, the enzyme is able to unwind efficiently a 5' flap substrate, a key intermediate of DNA replication/repair as well as a substructure of the D-loop recombination intermediate. The ability of BACH1 to unwind robustly the 5' flap substrate suggests that the helicase is tolerant of single-stranded or double-stranded DNA in the non-translocating tail of the forked duplex substrate. This property of BACH1 is distinct from certain other well characterized 5' to 3' helicases such as the T7 gene 4 helicase that requires that the 3' arm of the forked duplex substrate be single-stranded (18).

If the role of BACH1 in BRCA1-dependent DSBR is to unwind dsDNA flanked by ssDNA near the site of DNA damage, then in the absence of other DNA substrate discriminatory factors, BACH1 may only act upon DNA intermediates that have been processed to provide 5' ssDNA tails of sufficient length (~15 nt). Alternatively, BACH1 helicase may target D-loop intermediates that arise as intermediates in homologous recombination as a result of DSBs. Unlike the requirement for a 5' ssDNA region flanking the duplex of a simple tailed DNA substrate, BACH1 is able to displace catalytically the invading strand of a D-loop substrate even if it lacks a 5' ssDNA tail. However, BACH1 does not unwind Holliday junction structures, suggesting that BACH1 does not catalyze Holliday junction branch fork migration as proposed for the RecQ helicases that participate in homologous recombination and/or replication fork restart (25).

Because very little was known about the nature of the helicase substrate recognition or DNA tracking by BACH1, we examined the importance of helicase contacts with the sugar phosphate backbone in the loading region, i.e. DNA tail of the helicase substrate. These studies revealed that a continuous strand of nucleic acid backbone in the 5' tail is important for BACH1 unwinding of the DNA substrate whether it was

![Image](https://example.com/image.png)

**Fig. 7.** BACH1 releases the invading strand of a D-loop structure. Helicase reactions (20 μl) were performed by incubating the indicated BACH1 concentrations with 0.5 mM D-loop DNA substrate that contained an invading strand with a 20-nt 5' ssDNA tail (substrate 31, A), 20-nt 3' ssDNA tail (substrate 30, B), or no tail (substrate 32, C) at 30 °C for 15 min under standard helicase assay conditions as described under “Materials and Methods.” Lane 1, no enzyme control; lanes 2–7, indicated concentrations of BACH1; lane 8, heat-denatured DNA substrate control. A phosphorimage of a typical gel for helicase assays with each substrate is shown. D, quantitative helicase data represent the mean of at least three independent experiments with S.D. indicated by error bars. Filled square, D-loop with 3' ssDNA tail; open diamond, D-loop with no tail; filled diamond, D-loop with 5' ssDNA tail.
flanked by 5’ and 3’ ssDNA arms (forked duplex) or just a 5’ ssDNA arm. In contrast, the Superfamily 1 DNA helicase Rep efficiently unwound a DNA substrate harboring a polyglycol linkage in the single strand loading region adjacent to the duplex, suggesting a mechanism for initiation of DNA unwinding that is not passive and is consistent with a rolling mechanism during which Rep binds to ssDNA and dsDNA simultaneously (22).

The results from the helicase substrate characterization studies support the conclusion that BACH1 loads onto the 5’ ssDNA flanking a duplex to initiate unwinding. Longer 5’ ssDNA tails, up to 35 nt, provided an optimal loading site for BACH1; however, longer 5’ tails decreased the efficiency of unwinding. Continuity of the sugar phosphate backbone in the 5’ ssDNA tail, particularly near the ssDNA-dsDNA junction, is important for BACH1 to efficiently initiate unwinding of the duplex. BACH1 can tolerate duplex character in the non-translocating strand of the preferred forked duplex substrate, suggesting that the enzyme can unwind 5’ flap intermediates that may arise during normal DNA metabolism.

By having characterized the DNA substrate specificity of BACH1 helicase, this information may be useful for studying how the activity of the enzyme on its preferred helicase substrates may be regulated by its post-translational modifications or protein interactions. The role of the phosphorylation-dependent interaction between BACH1 and BRCA1 in DSBR (9) and DNA damage-induced checkpoint control during the G2/M phase of the cell cycle (29) is likely to involve BACH1-catalyzed unwinding of its cellular DNA substrate. It will be insightful to investigate the effects of BACH1 phosphorylation in the absence or presence of its interacting partner BRCA1 on the ability of BACH1 to unwind its helicase substrates. The ability of BRCA1 to preferentially bind branched DNA structures (30) may be important to facilitate the efficient loading of BACH1 on its helicase substrate to perform its DNA repair function.

Although we have focused our initial efforts on the biochemical characterization of BACH1 helicase activity on more conventional duplex DNA structures, the potential importance of BACH1 action on alternate DNA structures cannot be overlooked. Genetic evidence indicated that mutations in a BACH1 homologue in Caenorhabditis elegans, dog-1 (for deletions of guanine-rich DNA), led to germ line as well as somatic deletions in genes containing polyguanine tracts (31). It was proposed that dog-1 is required to resolve the special secondary structures (e.g. tetraplexes) that occasionally form in guanine-rich DNA during lagging strand DNA synthesis. This raises the issue whether BACH1, or a related putative helicase (RTEL) (32), performs a similar function in mammalian cells. Failure to resolve alternate DNA structures during replication could result in the types of genomic instability (intragenic deletions and regions of loss of heterozygosity), known to be associated with cancer cell development.

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