The 14-3-3 Protein Homologues from *Saccharomyces cerevisiae*, Bmh1p and Bmh2p, Have Cruciform DNA-binding Activity and Associate *in Vivo* with ARS307*

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We have previously shown that, in human cells, cruciform DNA-binding activity is due to 14-3-3 proteins (Todd, A., Cossons, N., Aitken, A., Price, G. B., and Zannis-Hadjopoulos, M. (1998) *Biochemistry* 37, 14317-14325). Here, wild-type and single- and double-knockout nuclear extracts from the 14-3-3 *Saccharomyces cerevisiae* homologues Bmh1p and Bmh2p were analyzed for similar cruciform-binding activities in relation to these proteins. The Bmh1p-Bmh2p heterodimer, present in the wild-type strain, bound efficiently to cruciform-containing DNA in a structure-specific manner because cruciform DNA efficiently competed with the formation of the complex, whereas linear DNA did not. In contrast, the band-shift ability of the Bmh1p-Bmh1p and Bmh2p-Bmh2p homodimers present in the *bmh2Δ* and *bmh1Δ* single-knockout cells, respectively, was reduced by ~93 and 82%, respectively. The 14-3-3 plant homologue GF14 was also able to bind to cruciform DNA, suggesting that cruciform-binding activity is a common feature of the family of 14-3-3 proteins across species. Bmh1p and Bmh2p were found to associate *in vivo* with the yeast autonomous replication sequence ARS307, as assayed by formaldehyde cross-linking, followed by immunoprecipitation with anti-Bmh1p/Bmh2p antibody and conventional PCR. In agreement with the finding of an association of Bmh1p and Bmh2p with ARS307, another immunoprecipitation experiment using 2D3, an anti-cruciform DNA monoclonal antibody, revealed the presence of cruciform-containing DNA in ARS307.

Although 14-3-3 protein was primarily isolated and characterized as an abundant brain protein (1), during the last decade, it has become evident that members of the 14-3-3 protein family are present in all types of eukaryotic cells. The family is characterized in it by comparison with the fission yeast *S. pombe*. 14-3-3 proteins function as homo- or heterodimers, and each monomer (30–35 kDa) is able to bind a phosphorylated target protein to its amphipathic binding groove (16). Involvement of 14-3-3 proteins in important biological processes such as apoptosis, signal transduction, and the cell cycle has been well documented (for review, see Ref. 17). Although there is a large list of binding partners (currently >50 signaling proteins) (17), functional studies of 14-3-3 proteins are incomplete.

Previously, we identified and characterized a cruciform DNA-binding protein (CBP) in HeLa cells (18, 19). Microsequence analysis of three tryptic peptides of CBP revealed 100% homology to the 14-3-3 family of proteins (15), which in turn revealed a novel activity associated with 14-3-3 proteins, namely the binding to cruciform-containing DNA. Because cruciforms have been implicated in the initiation of DNA replication in prokaryotic plasmids (20–22), eukaryotic viruses (23), and mammalian cells (24), the cruciform-binding activity of 14-3-3 proteins represents a new direction in the study of these multifunctional proteins. Furthermore, in recent studies, we have found that several 14-3-3 isoforms (ε, β, γ, and ζ) associate *in vivo* with origins of DNA replication in a cell cycle-dependent manner and are involved in DNA replication (25).

In view of the strong conservation of the 14-3-3 proteins not only with respect to sequence, but also to function, data obtained in yeast would have great relevance for the higher eukaryotic systems. The use of the powerful genetic and biochemical techniques available for *S. cerevisiae* should advance our understanding of the role of 14-3-3 proteins in cruciform binding and eukaryotic DNA replication. To investigate whether the cruciform-binding activity of 14-3-3 found in mammalian cells is also present in yeast, we analyzed the 14-3-3 *S. cerevisiae* homologues Bmh1p and Bmh2p by electrophoretic mobility shift assays or band-shift assays. *S. cerevisiae* was used as a model yeast system because DNA replication is better characterized in it by comparison with the fission yeast *S. pombe*. Bmh1p and Bmh2p, like their mammalian counterparts, are involved in interactions with a large number of proteins that are important in intracellular regulatory processes (for review, see Ref. 26). These two proteins are ~60% identical to the mammalian 14-3-3ε isoform, and except for the C-terminal amino acids, Bmh1p (30.1 kDa) and Bmh2p (31.1 kDa) are 91 and 97% identical, respectively, to it, with the highest homology residing in the conserved domains of these proteins (26).
Single deletion of the BMH1 and BMH2 genes had a modest effect on the physiology of the cells, but the bmh1/bmh2 double knockout resulted in lethality for the budding yeast (6, 7). This lethal disruption has been complemented by at least four different Arabidopsis isofoms (27).

This study demonstrates the interaction of Bmh1p and Bmh2p with cruciform-containing DNA in vitro and their in vivo association with the S. cerevisiae autonomous replication sequence ARS307. A cruciform-specific association of these yeast homologues of 14-3-3 is also illustrated, akin to their human counterpart. Furthermore, the presence of cruciform structure at ARS307 is revealed by anti-cruciform antibody immunoprecipitation and conventional PCR.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions

The yeast strains used in this study were generous gifts of Dr. G. P. H. van Heusden (Institute of Molecular Plant Sciences, Leiden University, Leiden, The Netherlands): GG582-5D (wild-type, haploid leu2-3,112 ura3-52 trp1-92 his4Δ14-3-3), GG583-24A (haploid leu2-3,112 ura3-52 trp1-92 his4Δ14-3-3), GG585-24D (haploid leu2-3,112 ura3-52 trp1-92 his4Δ14-3-3 LEU2), GG1259 (haploid leu2-3,112 ura3-52 trp1-92 his4Δ14-3-3 LEU2), ARS352 (YEp-pG13, pYEp-TRS(GF14)), S. cerevisiae rich (yeast extract/peptone/dextrose (YEPD)) and complete minimal dropout mediums were prepared as previously described (28) and maintained as previously described (30).

In Vivo Cell Cycle Synchronization

S. cerevisiae wild-type cells (strain GG582-5D) were cultured in YEPD medium at 30 °C until they reached exponential phase (A_{595} ~ 0.5). For synchronization to the G2/M phase of the cell cycle, the cells were transferred to (NH_4)_2SO_4-free minimal medium for 48 h. For synchronization to G1/S, the cells were placed in YEPD medium containing 10 μM hydroxyurea (Sigma) for 36 h. Synchronization to G1/S was achieved by releasing the cells from the hydroxyurea block into YEPD medium containing 10 μg/ml nocardazole (Sigma) for 24 h. Cell synchronization was monitored by flow cytometry.

Isolation of Yeast Nuclei

Preparation of nuclei from S. cerevisiae was carried out by differential centrifugation as previously described (28).

Electrophoretic Mobility Shift Analysis

Band-shift Assays—Cruciform-containing DNA (pRGM21-pRGM29) was constructed and end-labeled as described previously (15). 5 μg of GG582-5D, GG583-24A, GG585-24D, or GG1259 nuclear extract as well as 3 μg of human CBP1/4-3-3 (15, 18) were used as a positive control and were incubated with ~3 ng of labeled cruciform DNA for 20 min on ice in binding buffer as previously described (18). The mixtures were subjected to 4% polyacrylamide gel electrophoresis at 180 V for 2 h. The gel was dried and exposed for autoradiography. The same protocol was followed with increasing amounts (3, 6, and 12 μg) of wild-type, bmh1Δ, or bmh2Δ S. cerevisiae nuclear extracts.

Competition Binding Assays—0.1 μg of wild-type S. cerevisiae nuclear extract was incubated with 1 ng of labeled cruciform DNA (~8 × 10^-14 mol) and increasing molar amounts (100, 500, 1000, 1500, and 2000-fold) of either unlabeled cruciform DNA or unlabeled linear DNA (18), used as a nonspecific competitor, for 30 min on ice in binding buffer (18). The samples were subjected to 4% polyacrylamide gel electrophoresis at 180 V for 2 h. The gel was dried and exposed for autoradiography.

Band-shift Elution of Bmh1p, Bmh2p, and GF14 and Immunoblotting

Bmh1p, Bmh2p, and GF14 were purified as previously described (15) with some modifications. In brief, 300 ng of labeled cruciform DNA (18) was used for binding either Bmh1p or Bmh2p from 100 μg of wild-type S. cerevisiae nuclear extract or GF14 from the same amount of bmh1Δ bmh2Δ double-knockout (grown in the presence of galactose) nuclear extract. Cruciform-Bmh1p/Bmh2p and cruciform-GF14 complexes were loaded onto a 4% polyacrylamide gel and subjected to electrophoresis at 180 V for 1.5 h. Each complex was eluted from the gel by isochophoresis as previously described (15). The eluates were concentrated to ~3 μl using a Microcon YM-10 concentrator (Millipore Corp.). Then, 30 μg of each band-shift-eluted preparation and the corresponding nuclear extracts (total of 30 μg) were mixed with 1× SDS sample buffer containing 100 mM dithiothreitol, and the samples were loaded onto a 12% SDS-polyacrylamide gel. Electrophoresis was carried out at 200 V for 40 min, and the gels were subsequently transferred to Immobilon-P transfer membrane at 100 V for 1 h at 4 °C. Membranes were probed with rabbit anti-Bmh1p/Bmh2p antibody (7) or with anti-GF14 monoclonal antibody (kindly supplied by Dr. R. J. Ferl) (29).

Chromatin Immunoprecipitation Assay

In Vivo Cross-linking—In vivo cross-linking was carried out essentially as previously described (30). In brief, 100 ml of strain GG582-5D in YEPD medium containing 2% glucose was grown to A_{595} ~ 0.8–1.1. 0.1 volume of 11% formaldehyde solution was added to the culture (final concentration of 1%) and incubated for 10 min at 26 °C with gentle shaking. The cell culture was then chilled in an ice-water bath for 50 min with occasional shaking. Cells were harvested by centrifugation at 1000 × g × 4 °C for 15 min. The pellet was washed three times with ice-cold buffer I (50 mM Hepes/KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 7.5), 1% (v/v) Triton X-100, and 0.1% (v/v) sodium deoxycholate).

Preparation of Whole Cell Extracts—Pelelets from either cross-linked or uncross-linked cells were resuspended in 500 μl of ice-cold buffer I containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and one capsule of protease inhibitors (Roche Molecular Biochemicals)). The samples (5 μl; 500 μl of g) of g onto the cells were added to the reaction and the mixture was vigorously vortexed for 30 s and then chilled on ice for another 30 s for eight consecutive cycles until >80% of the cells were lysed. After washing the pellets, the supernatants were recovered and subjected to sonication to shear the chromatin DNA to sizes ranging between 0.5 and 1.0 kb. The suspensions were sonicated for 30 s four times on ice, leaving 1-min intervals on ice between each pulse. Chill Ultra-turrax (IKA) was used to keep the samples chilled at 180 V for 1.5 h. Each complex was eluted from the gel by isotachophoresis.

Immunoprecipitation and DNA Isolation—200 μl of the WCE was incubated with 50 μl of protein G-agarose (Roche Molecular Biochemicals) on a rotating wheel for 1 h at 4 °C to reduce the background caused by nonspecific adsorption of irrelevant proteins/DNAs to protein G-agarose beads. The cleared chromatin lysates were then incubated at 4 °C for 12 h with 10 μl of either preimmune serum or rabbit anti-Bmh1p/Bmh2p polyclonal antibody (7). 50 μl of protein G-agarose was then added, and the incubation was continued for 2 h. The precipitates were then pelleted by centrifugation at 14,000 rpm for 15 min at 4 °C, the supernatants were transferred to fresh Eppendorf tubes on ice. The protein concentration of each supematant was adjusted to 20 μg/ml by dilution with ice-cold buffer I. An aliquot of this supernatant served as the whole cell extract (WCE).

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PCR Amplification of the Co-immunoprecipitated DNA

The immunoprecipitated material (WCE and genomic DNA) was used as template in conventional PCRs with Ready-To-Go PCR beads (Amersham Biosciences). Primers ARS307A (5'-ATATTGCAATCTTCTCTCATGCAC-3') and ARS307B (5'-GGTAGGGATAATAATCTG-3') and ARS307A (5'-GGTAGGGATAATAATCTG-3') and ARS307B (5'-GGTAGGGATAATAATCTG-3') were used to amplify a 370-bp DNA fragment from a yeast genomic library sequence ARS307 (GenBank™/EMBL accession number AF087952). An initial denaturation for 5 min at 94 °C was followed by 35 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 50 °C, polymerization for 1 min at 72 °C, and a final extension for 10 min at 72 °C. PCR products were separated on 1.5% agarose gels, visualized with ethidium bromide, and photographed with an Eagle Eye apparatus (Speed Light/BT Scientech-LT1000).
**RESULTS**

**Cruciform-binding activity in S. cerevisiae Nuclear Extracts**—As shown in Fig. 1A (comparing lanes 1 and 3), nuclear proteins from *S. cerevisiae* wild-type cells (strain GG582-5D) were able to retard the migration of a cruciform-containing DNA molecule (see “Experimental Procedures”). As a positive control, human CBP/14-3-3 was used (Fig. 1A, lane 2), which produced the expected hand-shift pattern (15, 18). Nuclear extracts from the *bmh1* (strain GG583-24A) or *bmh2* (strain GG583-24D) single-knockout cells failed to produce large amounts of band shifts (Fig. 1A, lanes 4 and 5), although 18 and 7% retardation was observed when extracts from the GG583-24A and GG583-24D strains were used, respectively (lane 4). However, the latter band shift was not always reproducible. Band-shift assays were also carried out using nuclear extracts from the *bmh1/bmh2* double-knockout cells (strain GG1259) (Fig. 1A, lanes 6–9). Viability of this *S. cerevisiae* strain, containing a genetic deletion of the Bmh1p and Bmh2p proteins (7), was supported by the 14-3-3 *Arabidopsis* homologue GF14 (7). The cDNA encoding the GF14 protein is under the control of the GAL1 promoter (7). Induction of GF14 expression occurs in the presence of galactose, whereas repression occurs in medium containing glucose. Nuclear extracts from the GG1259 cells grown in 2% galactose showed a retardation in the migration of the cruciform DNA (Fig. 1A, lane 6). However, replacement with medium containing 2% glucose showed that, after 24 h in this new condition, the band shift was reduced (Fig. 1A, lane 7) and was completely lost after 48 h of repression of GF14 in the presence of glucose (lane 8). Although the *S. cerevisiae* double-knockout cells did not grow on medium with glucose (7), they remained viable for at least 48 h. Viability of 48-h cultures was confirmed by dilution transfer of the cells into new medium containing 2% galactose, which led to the recovery of the cruciform-binding activity (Fig. 1A, lane...
9). Furthermore, when these cells were plated on solid medium, their morphology was normal, excluding the possibility of mutant selection (data not shown).

The Bmh1p-Bmh2p Heterodimer Is More Efficient in Binding to the Cruciform than the Homodimers—14-3-3 proteins can associate with their partners either as monomers or as protein dimers. However, the cruciform-binding activity is found solely in 14-3-3 dimers (15). The data in Fig. 1A show that the Bmh1p-Bmh2p heterodimer (lane 3, nuclear extract from GG582-5D cells) is more efficient in binding to cruciform DNA than either of the homodimers, Bmh2p-Bmh2p (lane 4, nuclear extracts from GG583-24A cells) and Bmh1p-Bmh1p (lane 5, nuclear extract from GG583-24D cells). This conclusion was confirmed in a set of electrophoretic mobility shift assays in which increasing amounts (3, 6, and 12 µg) of nuclear extract from the three strains were used (Fig. 1B). A comparison of the band shift for each of the nuclear extract amounts indicated that Bmh2p-Bmh2p (strain GG583-24A) was able to retard the migration of the cruciform DNA (Fig. 1B, lanes 3, 6, and 9), although, even at the highest concentration (lane 9), its band shift was less efficient and weaker than the one obtained with the wild-type nuclear extract (lanes 2, 5, and 8), even at the lowest amount used (lane 2). No band shift was detectable when nuclear extracts from strain GG583-24D (bmh2−) were used (Fig. 1B, lanes 4, 7, and 10), even at the highest concentration (lane 10), indicating that the Bmh1p-Bmh1p homodimer is not able to bind to the cruciform DNA.

Structure-specific Binding of Bmh1p and Bmh2p to Cruciform-containing DNA—Human CBP/14-3-3 has been shown to recognize both S. cerevisiae isoforms, Bmh1p and Bmh2p. The nuclear extract from strain GG582-5D was used as a positive control, and as expected, both proteins were present in it (Fig. 3A, lane 3). Both Bmh1p and Bmh2p were also detected in the sample from the band-shifted complex (Fig. 3A, lane 4). The presence of both proteins in the band-shifted complex was further supported by the fact that both isoforms were present in the total cell extract from wild-type cells (strain GG582-5D) (Fig. 3A, lane 5), whereas only Bmh2p (lane 6) or Bmh1p (lane 7) was present in the total cell extract from bmh1− (strain GG583-24A) or bmh2− (strain GG583-24D) cells, respectively. The same analysis was performed with the complex obtained with the nuclear extract from bmh1/bmh2 double-knockout cells (strain GG1259) cultured in the presence of galactose (Fig. 3B, lane 2). Using a monoclonal antibody against GF14 (Mob-19, a gift from the laboratory of Dr. R. J. Ferl), the 14-3-3 plant protein was detected in both the nuclear extract (Fig. 3B, lane 3) and the band-shifted complex (lane 4).

Cruciform-containing DNA at ARS307—DNA replication origins have been well characterized in the budding yeast S. cerevisiae owing to the isolation and characterization of autonomous replication sequences (ARSs), which were recognized by their ability to promote the autonomous replication of plasmids into which they were cloned (33, 34). To investigate the presence of cruciform-containing DNA at S. cerevisiae replication origins, anti-cruciform DNA antibody immunoprecipitations were carried out (see “Experimental Procedures”) using mAb 2D3, which is specific for cruciform-containing DNA (31, 35). The immunoprecipitated DNA was subsequently amplified by conventional PCR using primers specific for amplifying a 370-bp fragment of ARS307 (GenBank™/EBI accession number AF087952) (36, 37) (see “Experimental Procedures”). ARS307 is the only one of four chromosome III ARS elements sequenced by Palzkill et al. (38) that contains a perfect (11 of 11) match with the core consensus sequence. A 370-bp fragment was specifically amplified by these primers when DNA immunoprecipitated by mAb 2D3 (Fig. 4A, lane 1) was used as template. This amplification was specific because the same primers failed to amplify the 370-bp fragment when the DNA used as template in the PCR was that immunoprecipitated by P3 (Fig. 4A, lane 2), a mAb secreted by the parental myeloma line of 2D3 (32), used here as a negative control. The identity of the 370-bp fragment was confirmed by PCR in which S. cerevisiae genomic DNA was used as template (Fig. 4A, lane 3). An additional control was carried out by amplifying the 2D3-immunoprecipitated DNA with a specific set of primers for a...
FIG. 3. Cruciform complexes contain Bmh1p, Bmh2p, and GF14. A and B: panel i, preparative band-shift assays were carried out as described under "Experimental Procedures." A: panel ii, shown is the anti-Bmh1p/Bmh2p antibody Western blot of total proteins from wild-type nuclear extracts (lane 3) and proteins purified by elution (see "Experimental Procedures") from a protein-cruciform DNA complex (lane 4). The membrane was probed with a 1:1000 dilution of anti-Bmh1p/Bmh2p polyclonal antibody (7) and a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology). A: panel iii, shown is the anti-Bmh1p/Bmh2p antibody Western blot of proteins of wild-type (lane 5), bmh1- (lane 6), and bmh2- (lane 7) total cell extracts. The molecular mass of Bmh1p is 30,176 Da, and that of Bmh2p is 31,167 Da. B: panel ii, shown is the anti-GF14 antibody Western blot of nuclear extracts from bmh1- bmh2- double-knockout cells grown in the presence of 2% galactose (lane 3) and proteins purified by elution from the protein-cruciform complex (lane 4). The membrane was probed with a 1:1000 dilution of anti-GF14 monoclonal antibody (29) and a 1:2500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology). Free Cruciform DNA denotes the position of the probe alone. Cruciform-Bmh1p/Bmh2p and Cruciform-GF14p represent the complexes obtained with the wild-type nuclear extracts.

FIG. 4. Cruciform-containing DNA at ARS307. A: immunoprecipitation using anti-cruciform DNA mAb 2D3 (lane 1) or the unrelated mAb P3 (lane 2) was carried out as described under "Experimental Procedures." The immunoprecipitated material was then used as template for conventional PCRs using primers ARS307A and ARS307B. 10 ng of yeast genomic DNA (lane 3) was used as a positive control for amplification of the 370-bp fragment (arrow) in ARS307. MW represents the molecular mass marker pX174 replicative form DNA digested with HaeIII. B: 2D3-immunoprecipitated material was used as template for conventional PCRs using either the YCL010C (lane 1) or ARS307 (lane 3) primers. 10 ng of yeast genomic DNA was used as a positive control for amplification of the 500-bp (lane 2) and 370-bp (lane 4) fragments. C: panel i, ARS307 sequence. The open box represents the ARS consensus sequence (complementary strand). Panel ii, the best stem-loop structure within ARS307, containing 10 bp in the stem and 10 bp in the loop, no mismatches or gaps, and only two G-T base pairs.
500-bp fragment of the YCL010C region, which is located upstream of the ARS307 region (Fig. 4B, lane 1) (EcoRI digestion excluded the entire 500-bp fragment from the one amplified by the ARS307 primers). The results show that these primers failed to produce any amplification when the 2D3-immunoprecipitated DNA was used as template, whereas they efficiently amplified the 500-bp fragment from genomic DNA (Fig. 4B, lane 2). In contrast and as previously observed, the ARS307 primers amplified the specific 370-bp fragment from both the 2D3-immunoprecipitated and genomic DNAs (Fig. 4B, lanes 3 and 4, respectively).

To determine whether the 370-bp fragment contains inverted repeat sequences with the potential to form stem-loop (cruciform) structures, the ARS307 sequence was subjected to analysis using Stem-loop software (Genetics Computer Group, Inc., Madison, WI). Several potential stem-loop structures were identified in this manner (data not shown), but the one included between nucleotides 143 and 182 of ARS307 (Fig. 4C, panels i and ii), containing 10 bp in the stem and 10 bp in the loop, was the best in that it contained no mismatches or gaps and only two G-T base pairings.

Using BestFit software (Genetics Computer Group, Inc.), the ARS consensus sequence (TTTGTGTATTATA) in the 370-bp ARS307 fragment is located adjacent to this inverted repeat sequence, between nucleotides 182 and 192 (Fig. 4C, panel i). A similar analysis of ARS305, ARS309, and ARS310 revealed potential stem-loop structures existing within a maximum of 110 bp from the ARS elements (data not shown).

In Vivo Association of Bmh1p and Bmh2p with ARS307

Detected by Chromatin Immunoprecipitation Assay—The in vivo association of Bmh1p and Bmh2p with ARS307 was analyzed using formaldehyde cross-linking and immunoprecipitation with anti-Bmh1p/Bmh2p antibody as previously described (39), followed by PCR (see “Experimental Procedures”). Formaldehyde is an easily reversible cross-linking agent that efficiently produces both DNA-protein and protein-protein cross-links in vivo by inducing covalent coupling of endogenous proteins bound to DNA or to each other. Antibodies are then used to immunoprecipitate specific proteins coupled to their target DNA (DNA fragments ranged in size from 0.5 to 1.0 kb; see “Experimental Procedures”). When the immunoprecipitated protein-DNA cross-link was reversed by incubation at 65 °C overnight (39), the 370-bp fragment was successfully amplified (Fig. 5A, lane 1). In contrast, the immunoprecipitated material from either untreated (not cross-linked) cells or cross-linked but not reversed cells did not result in PCR amplification (Fig. 5A, lanes 2 and 3, respectively), indicating that formaldehyde cross-linking is necessary before Bmh1p/Bmh2p immunoprecipitation and that the PCR amplification is blocked if the cross-linking is not reversed, as previously demonstrated (39). PCR amplification using material that was immunoprecipitated by the preimmune serum did not produce the 370-bp ARS307 fragment (Fig. 5A, lane 4), confirming the specificity of the amplification, whereas the use of S. cerevisiae WCE (Fig. 5A, lane 5) or genomic DNA (lane 6) as template produced the expected 370-bp fragment amplification.

To examine whether the absence of amplification in untreated (not cross-linked) cells was due to inefficient immunoprecipitation, the same material (without proteinase K treatment) was immunoblotted using rabbit anti-Bmh1p/Bmh2p polyclonal antibody. Both Bmh1p and Bmh2p were detected with the immunoprecipitated material from either the cross-linked or untreated cells (Fig. 5B, lanes 1 and 2, respectively). In contrast, these two proteins were not detected in the sample that was immunoprecipitated by the preimmune serum (Fig. 5B, lane 3).

Cell Cycle Profile of the Association of Bmh1p and Bmh2p—Real-time PCR was used to examine the quantity of Bmh1p and Bmh2p associated with ARS307 through the cell cycle. S. cerevisiae wild-type cells (strain G582-5D) were synchronized to G0, G1/S, and G2/M (see “Experimental Procedures”), and the synchronization was monitored by fluorescence-activated cell sorting analysis (Fig. 6A). After synchronization, cells at different phases of the cell cycle were subjected to in vivo formaldehyde cross-linking and immunoprecipitation with either anti-Bmh1p/Bmh2p antibody or preimmune serum. The immunoprecipitated material was then amplified by real-time PCR using the ARS307 primers. The association of the 14-3-3 yeast homologues Bmh1p and Bmh2p was found to be ~1.7-fold higher at the G1/S boundary by comparison with the G0 and G2/M phases (Fig. 6B). By contrast, the materials that were immunoprecipitated by the preimmune serum were of consistently low abundance.

DISCUSSION

This study provides evidence for the binding of the 14-3-3 S. cerevisiae homologues to cruciform-containing DNA. The data show that similar to their human CBP14-3-3 counterpart (15, 25), Bmh1p and Bmh2p have cruciform-binding activity both in vitro and in vivo. Comparison of the band shifts produced with the nuclear extracts from the G582-5D (wild-type), G583-24A (bmh1P−), and G583-24D (bmh2P−) strains indicated the function of these yeast isoforms as cruciform-
binding proteins, which was further confirmed in vivo by the chromatin immunoprecipitation assays. The fact that amplification was not observed with the non-reversed immunoprecipitated material suggests that the 14-3-3 S. cerevisiae homologues Bmh1p and Bmh2p are directly associated with or near ARS307, an active origin of DNA replication in S. cerevisiae (36, 37). Furthermore, the cell cycle studies indicate that the association of Bmh1p and Bmh2p with ARS307 is higher at the boundary of G1/S, akin to what has been observed for some mammalian 14-3-3 isoforms (25). Additional characterizations of the cell cycle-dependent association of the 14-3-3 yeast homologues with other active ARSs should allow a more generalized conclusion to be reached in this respect. The 14-3-3 plant homologue GF14 was also able to produce a band shift with cruciform DNA, supporting the notion that 14-3-3 proteins from different organisms can complement each other's function. The loss of band shift after 48 h in the presence of glucose in the GG1259 strain confirmed that cruciform-binding activity is a function of the two 14-3-3 yeast homologues.

CBP/14-3-3 binds to the cruciform-containing DNA only as a dimer (15). The dimer can be composed of either the same isoforms (homodimer) or different ones (heterodimer). Our group has recently demonstrated that the mammalian CBP/14-3-3 isoforms have a differential effect on DNA replication in vitro (25). Here, we have shown by the cruciform DNA retardation assay and using different S. cerevisiae genetic backgrounds that the Bmh1p-Bmh2p heterodimer was more efficient in binding to cruciform DNA than the Bmh2p-Bmh2p homodimer, there must be some contribution from Bmh1p. It is also possible that the association with Bmh2p confers cruciform-binding activity to the Bmh1p isoform. The association of Bmh1p and Bmh2p with cruciform-containing DNA was structure-specific because only unlabeled cruciform competitor was able to dissociate the protein-DNA complex, revealing a binding behavior similar to the one observed with human CBP/14-3-3 (18).

The implications of cruciform structures in the initiation of prokaryotic and eukaryotic DNA replication have been supported by numerous publications (20–25). In this work, the issue of the presence of cruciform structure at yeast origins of DNA replication has been addressed. Using anti-cruciform DNA antibody immunoprecipitation and conventional PCR, we demonstrated, for the first time, the presence of cruciform structure at the yeast origin of DNA replication ARS307. Mutational analysis of the ARS307 consensus sequence and its flanking regions demonstrated that substitutions of A to C and G to A at positions 176 and 173, respectively, did not affect the stability (efficiency) of this ARS, but that substitution of G to T at position 181 did affect the ARS function, causing a 2-fold increase in plasmid loss rate (40). The most likely explanation for this differential effect of the mutations on ARS stability relates to the process of cruciform formation. The kinetics of cruciform extrusion require melting and nucleation to occur at the center of symmetry before cruciform extrusion can occur (41). The A at position 176 and the G at position 173, which are located near the end of the potential stem structure, would not

**Fig. 6.** Profile of association of Bmh1p and Bmh2p with ARS307 through the cell cycle. A, shown are the results from fluorescence-activated cell sorting analysis of DNA contained in logarithmically growing or synchronized wild-type cells (strain GG582-5D) at the G0, G1/S, or G2/M phase of the cell cycle. B, the amount of Bmh1p or Bmh2p on ARS307 was measured by real-time PCR using, as template, the anti-Bmh1p/Bmh2p antibody-immunoprecipitated material from synchronized (G0, G1/S, or G2/M) and formaldehyde-cross-linked cells.
be expected to greatly affect the nucleation and therefore stability of a cruciform forming at that location, whereas the G at position 181, which is located at the base of the stem, would. In fact, an A at position 173 would substitute the existing G-T pair with an A-T pair, thus contributing to greater stability of the stem (see Fig. 4C, panel ii). Furthermore, characterization of the binding specificity of two anti-cruciform DNA monoclonal antibodies demonstrated that these antibodies specifically recognize conformational determinants at the base of the stem-loop structure (31, 35, 42). Those mutations altering the structure at the base of the cruciform (as the one at 181 position) would affect the binding of these monoclonal antibodies to the cruciform. The finding of a potential cruciform structure adjacent to the ARS consensus sequence suggests that DNA cruciform structures may be elements of initiation within the minimal core elements of yeast origins.

Footprinting analyses revealed a novel mode of interaction between CBP/14-3-3 and the cruciform DNA whereby CBP interacts with the elbows of the cruciform junction in an asymmetric fashion (19). However, the regions of CBP/14-3-3 involved in cruciform association remain to be determined. Alignment of human, yeast, and Drosophila 14-3-3 isoforms illustrated five sequence blocks that are evolutionarily conserved across all species (43). The X-ray structures of mammalian 14-3-3 bound to two different phosphoserine peptides revealed that all peptide-interacting residues observed in the two complexes lie within four of the five conserved blocks (44). We are currently studying some mutants of the 14-3-3 yeast homologues that will improve the knowledge of this specific association.

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