Schizosaccharomyces pombe Bir1p, a Nuclear Protein That Localizes to Kinetochores and the Spindle Midzone, Is Essential for Chromosome Condensation and Spindle Elongation During Mitosis

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ABSTRACT

The inhibitor of apoptosis (IAP) family of proteins contains a subset of members characterized by the presence of highly conserved baculoviral IAP repeat (BIR) domains. Recent work has shown that some of these BIR-domain proteins play a prominent role in the regulation of cell division, in particular at the stage of chromosome segregation and cytokinesis. We and others have shown that the *Schizosaccharomyces pombe* BIR-domain protein, Bir1p/Pbh1p/Cut17p, is important for the regulation of mitosis. Here we further characterize *S. pombe* Bir1p using methods of cell biology and genetics. We show that Bir1p is dispersed throughout the nucleus during the cell cycle. In addition, a significant part of Bir1p is also detected at the kinetochores and the spindle midzone during mitosis and meiosis. Time-lapse microscopy studies suggest that Bir1p relocates from the kinetochores to the spindle at the end of anaphase A. Bir1p colocalizes with the *S. pombe* Aurora kinase homolog Aim1p, a protein essential for mitosis, at the kinetochores as well as the spindle midzone during mitosis, and functional Bir1p is essential for localization of Aim1p to the kinetochores and the spindle midzone. Analyses of *bir1* conditional mutants revealed that Bir1p is essential for chromosome condensation during mitosis. In addition, anaphase cells show the presence of lagging chromosomes and a defect in spindle elongation. We conclude that Bir1p is important for multiple processes that occur during mitosis in *S. pombe*.

BIR-DOMAINS were first identified in the inhibitor of apoptosis (IAP) family of proteins that are one of the major components of the apoptotic machinery in higher eukaryotes (Crook *et al.* 1993). Recent evidence has shown that some of these BIR-domain-containing proteins (BIRPs) not only function in apoptosis, but also are important for fundamental cellular processes (Reed and Bischoff 2000; Adams *et al.* 2001). Previous studies have shown that BIRPs, which are conserved in a wide range of eukaryotes ranging from yeasts to humans, are essential for cell cycle-related processes, in particular mitosis and cytokinesis (Li *et al.* 1998; Fraser *et al.* 1999; Rajagopalan and Balasubramanian 1999; Uren *et al.* 1999, 2000). The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for the study of cell cycle regulatory mechanisms including those that control mitosis and cytokinesis. The ease of cytological and genetic analyses has led to the identification of a large number of cell cycle regulatory molecules in *S. pombe* that are highly conserved across a wide range of phyla (Nurse 1990; Gould and Simanis 1997; Yanagida 1998). *S. pombe* mitosis is very similar to that of higher eukaryotes in that spindle is formed only when mitosis occurs and separation of the spindle pole body (SPB) occurs only at the onset of mitosis (McCully and Robinow 1971; Hiraoka *et al.* 1984; Hagan and Hyams 1988; Robinow and Hyams 1989). The presence of three chromosomes that distinctly condense at the onset of mitosis facilitates cytological analyses of chromosome behavior during mitosis.

Several kinds of cytological and genetic screens in *S. pombe* have identified a wide range of mutants that are defective in one or more stages of mitosis (Nurse *et al.* 1976; Nasmyth and Nurse 1981; Toda *et al.* 1983; Hirano *et al.* 1988; Ohkura *et al.* 1988; Yamamoto 1988; Samejima *et al.* 1993). A distinct class of such mutants, in which nuclear division is uncoupled from cell division, display a characteristic phenotype of the division septum "tearing" through the unsegregated chromosomes leading to the so-called "cut" phenotype (*c*ell *u*ntimely *t*orn; Hirano *et al.* 1986; Samejima *et al.* 1993). Many of the *cut* genes encode highly conserved proteins and their characterization has provided a great deal of information on the regulation of various aspects of mitosis (Yanagida 1998).

We and others have previously shown that the BIR-domain-containing protein in *S. pombe*, Bir1p/Pbh1p/Cut17p, is essential for chromosome segregation (Samejima *et al.* 1993; Rajagopalan and Balasubramanian 1999; Uren *et al.* 1999). Throughout the rest of this article this protein is referred to as Bir1p. In this study, we show that Bir1p localizes to the kinetochores and

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the midzone of the spindle in both mitotic and meiotic cells. Time-lapse microscopic analysis suggests that redistribution of Bir1p might occur from the kinetochores to the spindle when the kinetochores reach the spindle poles at the end of anaphase A. Bir1p colocalizes with the *S. pombe* aurora kinase-related protein, Aim1p, at kinetochores and the spindle midzone. Also, Bir1p function is required for kinetochore and spindle midzone localization of Aim1p. With the aid of conditional mutants of *bir1*$^+$, we show that Bir1p is essential for establishing chromosome condensation at the onset of mitosis and the proper elongation of the mitotic spindle. We discuss possible roles of Bir1p in chromosome condensation and mitotic spindle elongation.

MATERIALS AND METHODS

***S. pombe* strains and culture conditions:** Media for vegetative growth were as described in Moreno *et al.* 1991. Transcriptional repression by the highly attenuated version of the *nmt1* promoter (Basi *et al.* 1993), denoted as P*nmt1*-81, was achieved by the addition of thiamine to a final concentration of 2 μM. Synchronous meiosis of a homothallic strain expressing green fluorescent protein (GFP)-Bir1p was achieved by growing the strain to a cell density of 10^7 cells/ml in Edinburgh minimal medium (EMM) without glucose at 36°. The cells were then resuspended at an equal density in EMM + 0.2% glucose lacking nitrogen and grown at 26° to induce conjugation and meiosis (Beach *et al.* 1985). Chemical transformations of *S. pombe* were carried out using a lithium-acetate-mediated method (Keeney and Boeke 1994).

Temperature-sensitive mutant of *bir1*: A modification of the strategy of hydroxylamine mutagenesis described in Busby and Dreyfus 1983 was carried out to generate temperature-sensitive alleles of *bir1*. A 2.5-kb fragment, corresponding to 1 kb of the 5′ untranslated region (UTR) *bir1*$^+$ and 1.5 kb of the N-terminal region of *bir1*$^+$ genomic DNA, was cloned into the integration vector pJK210 (Keeney and Boeke 1994). This plasmid DNA (100 μg) was subjected to mutagenesis using 1 M hydroxylamine solution in sodium phosphate buffer, pH 6.0 at 75° and time points were taken at 0, 30, 60, 90, and 120 min after addition of hydroxylamine. Chemical competent *E. coli* MC1061 cells were transformed with "mutated" plasmids from each time point. The extent of mutagenesis was assessed by scoring the percentage survival of ampicillin-resistant bacterial colonies. Plasmid DNA that was mutagenized for a duration of 60 min yielded ~50% survival rate of transformed colonies on ampicillin-resistant plates. This DNA was linearized using *Pst*1 that cleaved the plasmid between nucleotide positions 436–441 within the *bir1*$^+$ gDNA region and was used to transform wild-type *S. pombe*. Uracil prototrophic integrants obtained by homologous recombination at 24° were replica plated to 36° to screen for sensitivity at high temperatures. Five out of 600 uracil prototrophic colonies screened showed lethality to varying levels at 36° and displayed a cut phenotype. Only one of the five mutants was chosen for further analysis because the other four mutants failed to display a "tight" phenotype at 36°. The mutant, *bir1*-1, was backcrossed three times to ensure 2:2 segregation of the *ts* phenotype and polymerase chain reaction (PCR) was used to verify integration of the mutated plasmid at the chromosomal locus of *bir1*$^+$.

Thiamine-dependent shutoff of Bir1p expression: A linear DNA fragment, marked for uracil prototrophy, in which *bir1*$^+$ was under the control of the highly attenuated version of the *nmt1* promoter (Basi *et al.* 1993), denoted as P*nmt1*-81, was integrated into the chromosomal locus of *bir1*$^+$ by homologous recombination. The uracil prototrophs were screened for lethality when replica plated to plates containing thiamine at 32°. One out of ~1000 uracil prototrophs that were screened yielded a strain that was defective for growth only in the presence of thiamine. The strain was backcrossed to ensure 2:2 segregation of the *ura4*$^+$ marker and integration of the P*nmt1*-81::*bir1*$^+$ linear DNA fragment at the chromosomal locus of *bir1*$^+$ was confirmed by PCR. The thiamine-dependent Bir1p depletion strain is denoted in this article as P*nmt1*-81::*bir1*$^+$. The cut phenotype was apparent when cells were grown in the presence of thiamine for at least two cell cycles (~5 hr) at 32°.

Construction of strain expressing GFP-Bir1p: A 3.2-kb fragment comprising the 5′ UTR of *bir1*$^+$ (1 kb), coding region of GFP (0.7 kb), and the N-terminal region of *bir1*$^+$ gDNA (1.5 kb) was cloned into the pJK210 vector. This plasmid was linearized with *Pst*1, which cleaved the plasmid between nucleotide positions 436–441 within the *bir1*$^+$ gDNA region. This linear piece of DNA was used to transform wild-type *S. pombe* cells by homologous recombination. The integrants were screened by PCR to ensure correct recombination of the plasmid at the *bir1* chromosomal locus. The GFP-Bir1p strain thus obtained expressed the functional fusion protein, the sole copy of Bir1p in the cell, under the control of the native *bir1*$^+$ regulatory elements, so as to achieve wild-type levels of GFP-Bir1p expression.

Fluorescence and time-lapse microscopy: Stainings with 4′,6-diamidino-2-phenylindole (DAPI) were performed as described by Balasubramanian *et al.* (1997). For immunostainings, cells were fixed with formaldehyde (Balasubramanian *et al.* 1997) and stained with primary antibodies. α-Tat1p, α-GFP (Molecular Probes, Eugene, OR), and α-myc (Sigma, St. Louis) were used at concentrations of 1:200, 1:500, and 1:100, respectively. Fluorescein isothiocyanate-conjugated secondary antibodies (Molecular Probes) were used at a concentration of 1:200 for detection of bound antibodies. Cells were viewed using a Leica DMLB microscope and images were captured using an Optronics DEI-750T cooled charge-coupled device (CCD) camera. Leica Qwin software was used to acquire images that were then assembled using Adobe Photoshop 6.0 and Canvas 5.0 programs. For time-lapse analysis, cells from logarithmic phase cultures were concentrated by centrifugation. Borosilicate glass slides and coverslips (Matsunami Trading, Tokyo) were used in all imaging experiments. One microliter of cells was spotted on a slide and the coverslip was immediately pressed down on the slide to get a good smear of cells. It was then sealed at the corners with 1:1:1 vaseline/lanolin/paraffin. This method effectively maintained the cells alive and prevented them from drying out for at least 1 hr. Time-lapse fluorescence microscopy on wild-type *S. pombe* cells was performed at room temperature (21°–24°) using the LEICA DM IRBE inverted microscope equipped with a LEICA N Plan 100×/1.25 oil objective and an OrcaII C4742-98 CCD camera (Hamamatsu, Bridgewater, NJ). Images were obtained with the Metaview (Universal Imaging Corporation, West Chester, PA). Time-lapse analysis on *cdc25*-22 cells expressing GFP-Bir1p, was performed using a Zeiss laser scanning microscope (LSM) 510, equipped with a 458-nm Argon laser at 25% transmission. Assembly of images was done using NIH Image 1.62 (Bethesda, MD).

RESULTS

Bir1p, a nuclear protein, localizes to the kinetochores and the spindle midzone during mitosis: Previous stud-

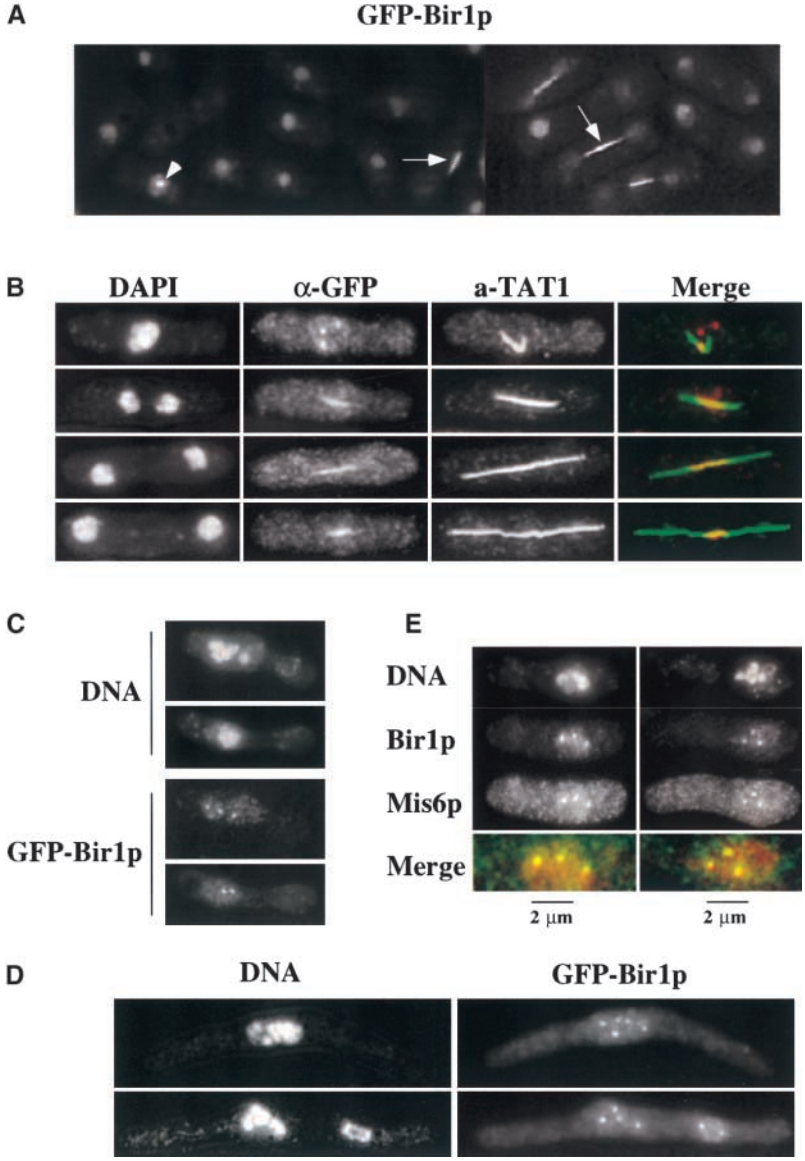

FIGURE 1.—GFP-Bir1p localizes to the nucleus, kinetochores, and the spindle midzone in vegetative cells. (A) GFP-Bir1p-expressing cells were grown to exponential growth phase at 32° in complete medium to visualize GFP epifluorescence. The arrowhead indicates the localization of Bir1p to spots and arrows indicate the localization of Bir1p to the spindle midzone. (B) Exponentially growing cells expressing GFP-Bir1p were fixed with formaldehyde and stained with DAPI to visualize chromosomes, with α-Tat1p to visualize microtubules, and with α-GFP to visualize GFP-Bir1p. Merge represents an overlap of GFP (red) and microtubule (green) staining. A haploid *nda3*-KM311 strain (C) and a diploid *nda3*-KM311/*nda3*-KM311 strain (D) expressing GFP-Bir1p were grown to exponential growth phase at 32° and shifted to 18° for 6 hr, fixed, stained with DAPI to visualize chromosomes, and with α-GFP to visualize GFP-Bir1p. (E) *nda3*-KM311 cells expressing GFP-Bir1p and Mis6p-13myc fusions were grown at 32° to exponential growth phase and shifted to 18° for 6 hr, fixed, stained with DAPI to visualize chromosomes, with α-GFP to visualize GFP-Bir1p, and with α-myc to visualize Mis6p-13myc. (Bottom) Enlarged merged images of GFP-Bir1p (red) and Mis6p-13myc (green).

ies have demonstrated that the *S. pombe* protein, Bir1p/Pbh1p/Cut17p, is essential for chromosome segregation (SAMEJIMA *et al.* 1993; RAJAGOPALAN and BALASUBRAMANIAN 1999; UREN *et al.* 1999). To gain further insight into the role of Bir1p in mitosis and cell division, we characterized its intracellular localization. To facilitate these studies, a strain was constructed in which the coding region of Bir1p was fused downstream of the jellyfish GFP. Expression of the functional fusion protein, the sole copy of Bir1p in the cell, was under the control of the native *bir1*$^{+}$ regulatory elements, so as to achieve wild-type levels of expression of GFP-Bir1p.

In this strain, GFP-Bir1p was detected at several distinct cellular locations in a cell cycle-dependent manner. Consistent with a previous study (UREN *et al.* 1999), Bir1p was found dispersed throughout the nucleus at all stages of the cell cycle in >300 cells (Figure 1A). Additionally, GFP-Bir1p was detected in spot-like structures in the vicinity of the chromosomes. The number and intensity of these spots were variable: usually one "spot" was observed in all interphase cells and during mitosis; one to three brightly stained spots were observed in ~19% of mitotic cells (117/600 mitotic cells scored). These cells typically contained a short mitotic spindle, suggesting that they were cells undergoing either metaphase or anaphase A. In ~81% of the mitotic cells (483/600 cells scored), GFP-Bir1p strongly stained the midzone of the elongating spindle (Figure 1A). Microtubule and nuclear staining confirmed that GFP-Bir1p localized to the spindle midzone in cells undergoing anaphase B (Figure 1B). The spot-like localization of Bir1p was reminiscent of the pattern of localization of kinetochore proteins, such as Mis6p and Mis12p (SAITOH *et al.* 1997; GOSHIMA *et al.* 1999). Previous studies have shown that kinetochores are prominent in metaphase-arrested cells (FUNABIKI *et al.* 1993). To test if the

GFP-Bir1p spot-like structures represented kinetochores, we utilized two approaches. In one approach, we assessed the localization of GFP-Bir1p in haploid and diploid cells carrying a mutant version of the essential β-tubulin gene, *nda3* (Hiraoka *et al.* 1984). In cold-arrested *nda3* cultures, cells arrest with highly condensed chromosomes that are unable to execute anaphase owing to the activation of the spindle assembly checkpoint in the absence of a mitotic spindle (Hiraoka *et al.* 1984; He *et al.* 1997). These cells have been particularly useful for visualizing kinetochore proteins (Saitoh *et al.* 1997; Goshima *et al.* 1999). Immunostaining with GFP antibodies in arrested *nda3*-KM311 haploid cells revealed the presence of up to three spots that colocalized with the highly condensed chromosomes. In arrested *nda3*-KM311/*nda3*-KM311 diploid cells, up to six spots were observed. The presence of three spots in haploid and six spots in diploid cells arrested at prometaphase reaffirmed the idea that Bir1p spots represented kinetochores (Figure 1, C and D). We then tested if Bir1p colocalized with the previously described kinetochore protein Mis6p (Saitoh *et al.* 1997). For this purpose, an *nda3*-KM311 strain expressing GFP-Bir1p and Mis6p-13myc fusion proteins was constructed and the localization of Bir1p in relation to Mis6p assessed upon cold arrest. As previously reported, Mis6p was detected in spot-like structures representing kinetochores (Saitoh *et al.* 1997). Interestingly, as shown in Figure 1E, Bir1p and Mis6p colocalized to a large extent, which strongly supports the idea that Bir1p localizes to the kinetochores. Thus, we conclude that *S. pombe* Bir1p is a nuclear protein that localizes to the kinetochores prominently in early mitosis and to the spindle midzone at the onset of anaphase B.

At anaphase B, Bir1p appears to spread from the spindle poles to the mitotic spindle: Since Bir1p localizes to two distinct structural locations in mitosis, first to the kinetochores and then to the midspindle, we investigated the transition, if any, of Bir1p localization from one structure to the other in more detail. Wild-type and *cdc25*-22 strains expressing GFP-Bir1p were used to carry out time-lapse analyses on mitotic cells. Examples of selected images from two different time-lapse series are shown in Figure 2, A and B. Each series consists of images that were captured at 30-sec intervals. Time-lapse images of GFP-Bir1p revealed the dynamic nature of kinetochores in metaphase before attachment to the spindle consistent with previous observations (Funabiki *et al.* 1993). A striking rearrangement of kinetochores presumably along the length of the short spindle was observed, which was suggestive of kinetochore movement to the spindle ends in anaphase A. Shortly thereafter, the kinetochore staining of Bir1p appeared to "spread" from the pole ends on to the entire length of the short spindle (Figure 2A). The duration of this transition, from the time of kinetochore rearrangements to Bir1p localization on the spindle, was ~4.5–5 min in wild-type cells. Once GFP-Bir1p relocated

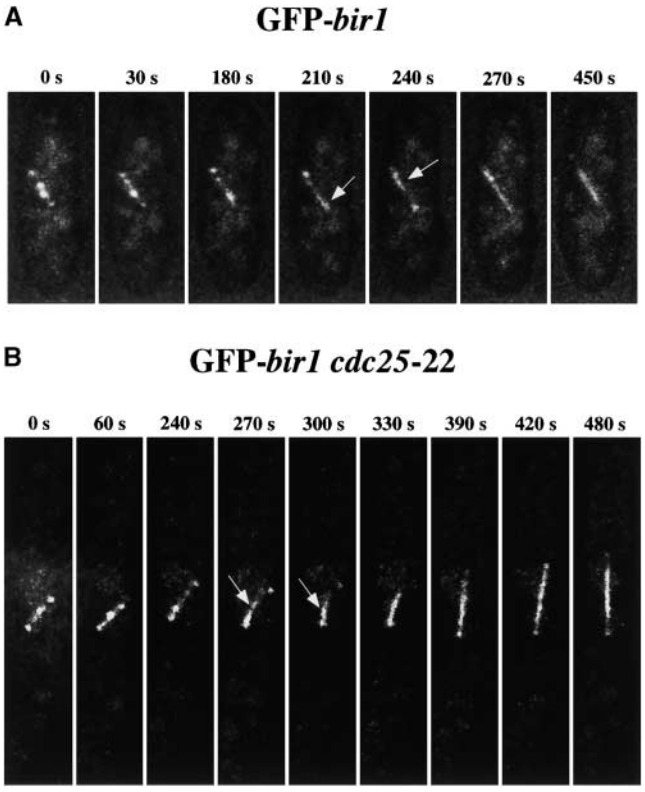

Figure 2.—Time-lapse analyses of GFP-Bir1p. Numbers refer to the time indicated in seconds (s). (A) GFP-Bir1p cells were grown to exponential growth phase and concentrated by centrifugation. One microliter of cells was spotted on a slide and immediately pressed down with a coverslip to get an even smear of cells. The edges were sealed to prevent drying. Slides were viewed using a Leica DMIRBE inverted microscope fitted with an ORCA 2 CCD camera. Images were captured at 30-sec intervals and processed using NIH image 1.62 and Adobe Photoshop software packages. (B) Exponentially growing GFP-Bir1p, *cdc25*-22 cells at 24° were shifted to 36° for 4 hr. The cells were then shifted down to 24° for 10 min before mounting for time-lapse analysis as described in the legend to Figure 2A. Arrows indicate the redistribution of GFP-Bir1p staining from the spindle poles to the spindle. Examples of individual images of time-lapse series performed at 24° are shown.

to the spindle, its localization was restricted to the midzone of the elongating spindle. The GFP-Bir1p staining persisted for ~20 min at the midzone of the spindle before gradually shortening to a spot in the middle of the spindle that eventually disappeared (data not shown). Similar analyses were carried out with *cdc25*-22 cells expressing GFP-Bir1p that were synchronized to enter mitosis following their release from G_2 arrest. Soon after release, a majority of cells showed the presence of GFP-Bir1p on kinetochores. A few other GFP spots were also seen at this stage, the nature of which is presently unknown. The highly dynamic rearrangement of kinetochores and the redistribution of Bir1p staining from the kinetochores to the spindle were similar to that observed in wild-type cells described earlier (Figure 2B). The duration of GFP-Bir1p localization transition from

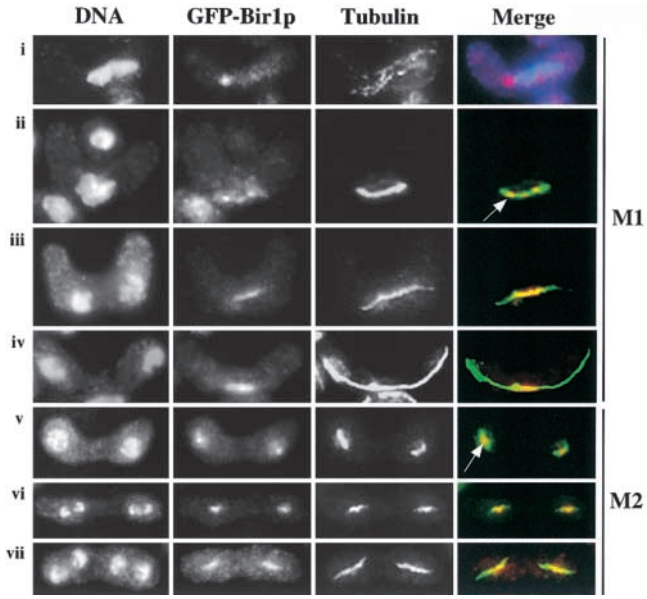

Figure 3.—Localization of GFP to the kinetochores and the spindle midzone in meiosis I and II. A homothallic GFP-Bir1p-expressing strain grown to stationary growth phase at 36° in minimal medium lacking glucose was induced to undergo synchronous meiosis by shift to medium lacking nitrogen. Samples were taken at 6, 7, 8, 9, and 10 hr after shift to nitrogen-free medium. Cells were fixed, stained with DAPI to visualize chromosomes, with α-GFP to visualize Bir1p-GFP, and with α-Tat1p to visualize microtubules. M1 and M2 refer to meiosis I and II, respectively. The panels represent cells in meiosis I prophase (i), metaphase I (ii), anaphase I (iii and iv), metaphase II (v), and anaphase II (vi and vii). Merge i represents an overlap of GFP (red) and DAPI (blue) staining. Merge ii–vii represent an overlap of GFP (red) and microtubule (green) staining. The arrows indicate spotty localization of GFP-Bir1p in metaphase I and II, possibly reflecting kinetochore staining.

kinetochores to the spindle was longer (~7 min) as compared to ~5 min in wild-type cells, consistent with the idea of extended mitosis in cells released from *cdc25*-22 arrest (Hagan *et al.* 1990). Together, these analyses suggested that Bir1p at the spindle midzone might originate from the kinetochores, after the kinetochores reach the spindle poles at the completion of anaphase A.

Bir1p localization in meiotic cells: Given the localization of Bir1p to kinetochores and the spindle midzone in mitotic cells, we tested the possibility of Bir1p detection at similar structures in cells proceeding through meiosis I and II. A homothallic strain that expressed GFP-Bir1p was constructed and induced to undergo synchronous meiosis, and localization of GFP-Bir1p was monitored through various stages of meiosis I and II. Growth in nitrogen-free medium for 5–6 hr led to the formation of conjugated cells. These cells displayed the presence of nuclei that assumed an elongated morphology (Figure 3, row i). This appearance was characteristic of the previously described "horsetail" nuclear morphology in meiotic cells during premeiotic S-phase and meiosis I prophase (Robinow 1977). Interestingly, cells with horsetail nuclei showed the localization of GFP-Bir1p to a spot that appeared to be located at the edge of the nuclear material (Figure 3, row i). This staining was reminiscent of the pattern of telomere clustering at the SPB that is thought to lead the horsetail movement of the meiotic nucleus (Chikashige *et al.* 1994). Presently it is unknown if Bir1p localizes to the telomeres, which are at the SPB during horsetail movement, or if Bir1p associates with the SPB itself during this stage of meiosis I. At the onset of metaphase in meiosis I, when sister chromatids are segregated to the same pole, cells showed the presence of condensed chromosomes and a short spindle that was reminiscent of metaphase in mitosis. At this stage, GFP-Bir1p localized to "distinct" spots (Figure 3, row ii) that bore a striking similarity to the pattern of localization of kinetochores in meiotic cells (Bernard *et al.* 2001). Subsequently, upon spindle elongation in meiosis I, GFP-Bir1p localized to the spindle midzone (Figure 3, rows iii and iv). Furthermore, during meiosis II, an identical sequence of kinetochore and midspindle localization of GFP-Bir1p was observed (Figure 3, rows v, vi, and vii). It can thus be concluded that the pattern of GFP-Bir1p localization during chromosome segregation in meiosis I and II is strikingly similar to its localization during chromosome segregation in mitotic cells. In addition, Bir1p was detected at the leading front of the horsetail chromosomes, which might represent localization of Bir1p to the SPB or the telomeres or both.

Conditional mutants of *bir1*: We generated conditional mutants of *bir1*$^+$ to analyze its essential function(s) in chromosome segregation more thoroughly. Two strategies were employed to generate conditional mutants of *bir1*. In the first, we isolated a temperature-sensitive mutant of *bir1* that was unable to form colonies at 36°. A modified method of hydroxylamine mutagenesis (Busby and Dreyfus 1983) yielded five mutants that displayed a cut phenotype upon shift to 36°, which was very similar to the phenotype displayed by the *bir1* null strain (Rajagopalan and Balasubramanian 1999; Uren *et al.* 1999), indicative of defects in chromosome segregation. Only one of these five mutants, termed *bir1*-1, displayed a tightly lethal cut phenotype at the restrictive temperature but formed relatively normal colonies at 24° and was hence chosen for further characterization (Figure 4A). Sequencing the *bir1*-1 mutant revealed a single mutation that altered codon ACA → GCA resulting in the substitution of threonine at position 194 by an alanine residue. A heterozygous diploid strain containing the mutated copy of *bir1* in combination with *bir1*$^+$ did not display temperature sensitivity at 36°, indicating that the *bir1*-1 mutation was recessive.

The second approach involved the construction of a strain in which *bir1*$^+$ expression was under the regulation of a thiamine-dependent promoter, P*nmt1*-81 (Basi *et al.* 1993). This strain was constructed using homologous recombination techniques, as described in materials and methods. Screening on the basis of lethality on thiamine-

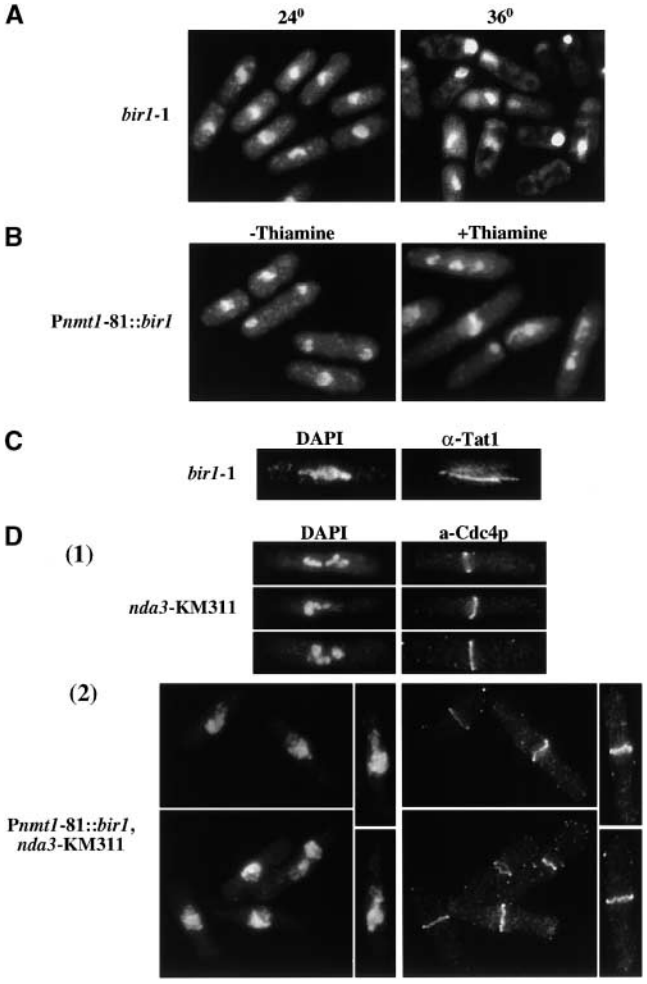

FIGURE 4.—Mitotic phenotype of *bir1* mutants. (A) *bir1*-1 cells were grown in minimal medium lacking uracil at 24° to exponential growth phase and shifted to 36° for 4 hr. The cells grown at both temperatures were fixed and stained with DAPI to visualize chromosomes. (B) P*nmt1*-81::*bir1* cells were grown to exponential growth phase at 32° in minimal medium lacking thiamine and shifted to medium containing 2 μM thiamine for 12 hr. The cells grown at both conditions were fixed and stained with DAPI to visualize chromosomes. (C) *bir1*-1 cells were grown to exponential growth phase at 24° and shifted to 36° for 4 hr. Cells were fixed and stained with DAPI to visualize chromosomes and with α-Tat1p to visualize microtubules. (D1) *nda3*-KM311 cells were grown to exponential growth phase at 32°, shifted to 18° for 6 hr, fixed, stained with DAPI to visualize chromosomes, and with α-Cdc4p to visualize the actomyosin ring. (D2) P*nmt1*-81::*bir1, nda3-KM311* cells were grown at 32° to exponential growth phase in minimal medium lacking thiamine and transferred to medium containing 2 μM thiamine for 5 hr. Cells were then shifted to 18° for 6 hr, fixed, stained with DAPI to visualize chromosomes, and with α-Cdc4p to visualize the actomyosin ring.

containing plates yielded a strain that showed severe chromosome segregation defects and was hence unable to grow and divide upon addition of thiamine as compared to normal nuclear and cell division in the absence of thiamine (Figure 4B). Both conditional mutants of *bir1*, *bir1*-1 and P*nmt1*-81::*bir1*$^+$, were rescued by plasmid-borne wild-type *bir1*$^+$, indicating that the observed phenotypes were attributable to loss of Bir1p function. These mutants were then analyzed in greater detail to characterize the mitotic role of Bir1p.

Bir1p is required for chromosome condensation: To investigate the mitotic phenotype of *bir1* conditional mutants in more detail, an asynchronous population of *bir1*-1 mutant cells was shifted to the restrictive temperature of 36°. The cells showed defects in chromosome segregation leading to the cutting of unsegregated chromosomes by the division septum. In addition, many mitotic cells displayed chromosomes that appeared to be defective in condensation, possibly leading to their "stretched-out" morphology along the length of the anaphase spindle (Figure 4C). To further analyze the chromosome condensation defect, the effects of Bir1p depletion were studied in the β-tubulin mutant strain, *nda3*-KM311. Inactivation of tubulin in the *nda3*-KM311 mutant by cold-arrest blocks cells at metaphase with highly condensed chromosomes (HIRAOKA *et al.* 1984). The P*nmt1*-81::*bir1*$^+$, *nda3*-KM311 strain was repressed for *bir1*$^+$ transcription by the addition of thiamine for 5 hr. Subsequently, cold arrest was used to enrich the population for cells blocked at metaphase. As a control, the *nda3*-KM311 strain was subjected to the same growth conditions. The actomyosin ring, stained with α-Cdc4p antibodies (MCCOLLUM *et al.* 1995), was used as a marker to ensure that only mitotic cells were scored for the phenotypic effects on chromosome morphology. It was observed that all *nda3*-KM311 cells with an actomyosin ring displayed highly condensed chromosomes, to the extent that individual chromosomes could be observed in some cells (Figure 4D1). In contrast, upon growth in thiamine-containing medium, ~45% of the Bir1p-depleted cells that stained for an actomyosin ring contained chromosomes that were uncondensed. The metaphase chromosomes appeared as loosely packed structures as opposed to the highly compact condensed chromosomes in the *nda3*-KM311 mutant (Figure 4D2). These data indicate that the cells depleted for Bir1p are unable to initiate/maintain condensation of chromosomes at the onset of mitosis.

Cells depleted of Bir1p display chromosomes that lag on the anaphase spindle: In addition to defects in chromosome condensation, a significant proportion of P*nmt1*-81::*bir1*$^+$ cells, depleted of Bir1p in thiamine-containing medium, showed the presence of discrete chromosome masses along the length of the spindle (Figure 5A). To test whether kinetochores were detected at several points along the length of the elongating anaphase B spindle, as would be expected if the chromosomes were lagging, *mis6*$^+$-13*myc* was introduced into the P*nmt1*-81::*bir1*$^+$ strain. This strain was shifted to thiamine-containing medium and the localization of Mis6p was monitored by staining with α-Myc antibodies. Approximately 69% of mitotic cells (138/200 mitotic cells

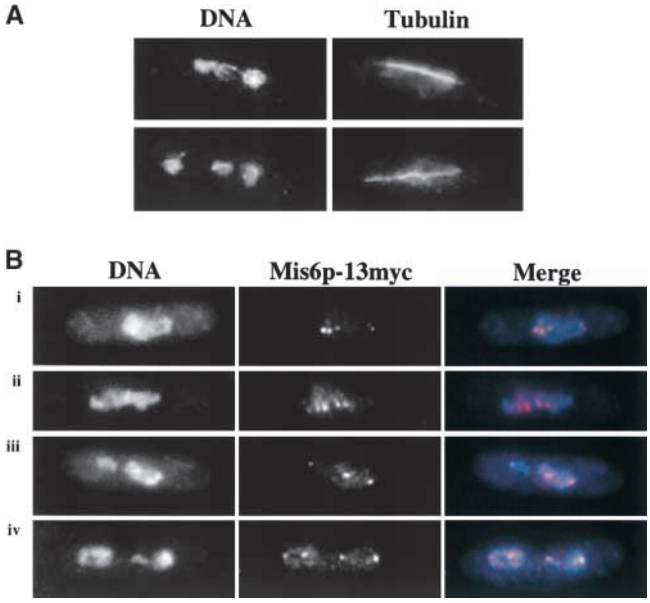

FIGURE 5.—Cells depleted of Bir1p display chromosomes that lag on the anaphase spindle. (A) P*nmt1*-81::*bir1* cells were grown at 32° to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 2 μM thiamine for 12 hr, fixed, stained with DAPI to visualize chromosomes, and with α-Tat1p to visualize microtubules. (B) A P*nmt1*-81::*bir1* strain expressing Mis6p-13myc was grown at 32° to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 2 μM thiamine for 12 hr, fixed, stained with DAPI to visualize chromosomes, and with α-myc to visualize Mis6p-stained kinetochores. Merge indicates an overlap of DAPI (blue)- and α-myc (red)-stained images. i demonstrates unequal segregation of the kinetochores.

scored) depleted of Bir1p displayed "lagging chromosomes" and did not contain all kinetochores at the ends of the spindle, as would be expected if mitosis had proceeded normally (Figure 5, A and B). The trailing kinetochores colocalized with the nuclear material, indicating that these lagging pieces of DNA were indeed entire chromosomes. Often, more than three kinetochores were observed, indicating that it was individual chromatids rather than nondisjoined chromosomes that lagged in anaphase B. It was also evident in certain cells that the localization of the kinetochores to the two poles was asymmetric (Figure 5B, row i), indicative of random segregation of the individual chromatids to the ends of the spindle. It is thus clear that in the absence of Bir1p, cells are defective in synchronous and/or symmetrical segregation of sister chromatids in anaphase.

Bir1p-depleted cells show a defect in complete elongation of the mitotic spindle in anaphase B: Spore germination studies had previously indicated that the *bir1* null strain was defective in spindle elongation in anaphase (RAJAGOPALAN and BALASUBRAMANIAN 1999; UREN *et al.* 1999). To investigate the effect of Bir1p on anaphase spindle elongation in more detail, cells were depleted for Bir1p and stained with tubulin antibodies to study the anaphase spindle morphology. As a control, P*nmt1*-81::*bir1*$^+$ cells were grown in the absence of thiamine. It was observed that cells grown in the absence of thiamine, hence unaffected for Bir1p expression, displayed normal segregation of chromosomes to the opposite ends of the cell with the aid of the anaphase B spindle that spanned the entire length of the cell (Figure 6A, i–v). In contrast, cells depleted for Bir1p displayed extensive structural and segregational defects of the mitotic chromosomes (as described in the earlier sections of this article). The cells, though capable of forming a short spindle at the onset of mitosis that elongated to a considerable length in anaphase, were unable to fully elongate the spindle to effectively segregate the chromosomes (Figure 6A, vi–x). The graph in Figure 6B depicts the percentage count comparison of mitotic cells with and without Bir1p. An ~80% reduction in the number of "end-to-end" elongated spindles in cells depleted of Bir1p was apparent. This reduction was compensated by an increase in the number of intermediate-length spindles. The drastic reduction in the formation of end-to-end spindles in cells depleted of Bir1p indicates that Bir1p is required for complete spindle elongation.

Bir1p is essential for localization of the aurora kinase homolog Aim1p to kinetochores and the spindle midzone: Studies in mammalian cells and in nematodes have suggested a functional link between Bir1p-related proteins and protein kinases of the Aurora family (SPELIOTES *et al.* 2000; WHEATLEY *et al.* 2001). An *S. pombe* protein kinase related to Aurora kinases has been identified by the *S. pombe* genome-sequencing project (Sanger Center, Hinxton, UK). We have designated this protein as Aim1p (*A*urora and *I*pl1p related *m*itotic kinase). To test the physiological role of *S. pombe* Aim1p, we created a diploid strain in which one copy of *aim1* was deleted and replaced with the marker gene *his3*$^+$. Analysis of the meiotic products from this strain established that Aim1p was essential for cell viability and appeared to be important for chromosome segregation and possibly chromosome condensation, leading to the prominent cut phenotype displayed by cells devoid of Aim1p (Figure 7A). To study the intracellular distribution of Aim1p, a strain that expressed Myc-epitope-tagged Aim1p under control of the native promoter sequences was constructed. Aim1p was detected at kinetochore-like structures and the spindle midzone in these cells (Figure 7B). Interestingly, unlike Bir1p, a diffuse nuclear localization pattern was not detected for Aim1p. To check if Aim1p colocalized with Bir1p, a strain that expressed GFP-Bir1p and Aim1p-13Myc was constructed. In this strain, Bir1p colocalized with Aim1p at kinetochores and the spindle midzone (Figure 7B, Merge). Interestingly, in a small proportion of cells that displayed prominent kinetochore staining of Bir1p, Aim1p was not detected at the kinetochores (Figure 7B, top). To test whether Bir1p was required for the mitotic localization of Aim1p, a P*nmt1*-81::*bir1*$^+$ strain expressing Aim1p-GFP was ana-

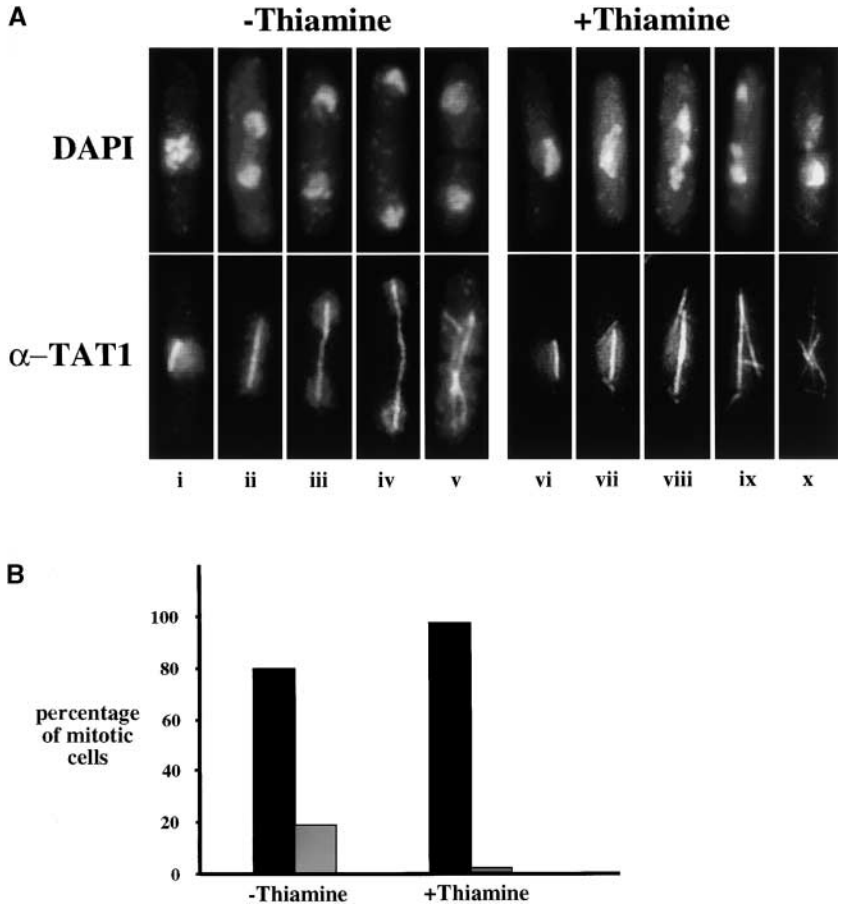

FIGURE 6.—Bir1p function is required for complete spindle elongation in mitosis. (A) P*nmt1*-81::*bir1*$^+$ cells were grown at 32° to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 2 μM thiamine for 12 hr, fixed, stained with DAPI to visualize chromosomes, and with α-Tat1p to visualize microtubules. i–v and vi–x represent different stages of mitosis in cells grown in the absence and presence of thiamine, respectively. i and vi represent cells with short spindles. ii, iii, vii, viii, and ix are examples of cells with intermediate-length spindles. iv represents an example of an end-to-end elongated spindle. A comparison of iv and ix points out the defect in spindle elongation in cells depleted of Bir1p. (B) P*nmt1*-81::*bir1*$^+$ cells grown in the absence and presence of thiamine (as described in A) were counted for the number of cells containing short, intermediate, and end-to-end elongated spindles. The graph represents a percentage comparison of cells with short and intermediate-length spindles (solid bar) and end-to-end elongated spindles (shaded bar).

lyzed. Cells depleted for Bir1p failed to localize Aim1p-GFP to the kinetochores and the spindle midzone (Figure 7C, i–iv), as opposed to Aim1p staining at the same structures in cells containing Bir1p (v–viii), suggesting a clear requirement for functional Bir1p for appropriate mitotic localization of Aim1p.

DISCUSSION

In this article, we have analyzed the role of the *S. pombe* BIR-domain-containing protein, Bir1p, in mitosis. We show that the nuclear protein Bir1p localizes to the kinetochores and the spindle midzone during chromosome segregation in both vegetative and meiotic cells. Time-lapse studies indicate that Bir1p may move from the kinetochores to the spindle at the onset of anaphase B. Bir1p colocalizes with the aurora kinase homolog, Aim1p, a protein essential for chromosome segregation, at kinetochores and the spindle midzone and is essential for this localization pattern of Aim1p during mitosis. Through analyses of *bir1* mutants, we also show evidence for the role of Bir1p in chromosome condensation, anaphase spindle elongation, and synchronous segregation of chromosomes in mitosis.

Localization of Bir1p: Using a fully functional GFP-Bir1p fusion molecule, we have shown that Bir1p localizes to the kinetochores at metaphase and the spindle midzone upon completion of anaphase A. These localization patterns were observed in both mitotic and meiotic cells, suggesting a fundamental role for Bir1p in events during mitosis and meiosis. That Bir1p localizes to kinetochores was confirmed by costaining with a *bona fide* marker of the kinetochore, Mis6p (SAITOH *et al.* 1997). It has been shown that *Saccharomyces cerevisiae* Bir1p interacts with kinetochore proteins such as Ndc10p and Skp1p (YOON and CARBON 1999). It is possible that the *S. pombe* Bir1p is a component of the kinetochore and is essential to effect chromosome segregation by interacting with other kinetochore proteins. That Bir1p is a component of the spindle midzone was established by costaining with antibodies against tubulin and from the fact that spindle midzone staining was lost under conditions of microtubule disassembly, such as incubation of cells on ice (data not shown). The mitotic localization pattern of *S. pombe* Bir1p to the kinetochores and the spindle midzone is similar to the localization patterns of Bir1p-related proteins that have been characterized in other eukaryotic organisms (SPELIOTES *et al.* 2000; WHEATLEY *et al.* 2001). This pattern of localization has also been observed for a variety of kinetochore proteins such as CENP-F (LIAO *et al.* 1995) and the INCENPs in animal cells (EARNSHAW and COOKE 1991; ECKLEY *et*

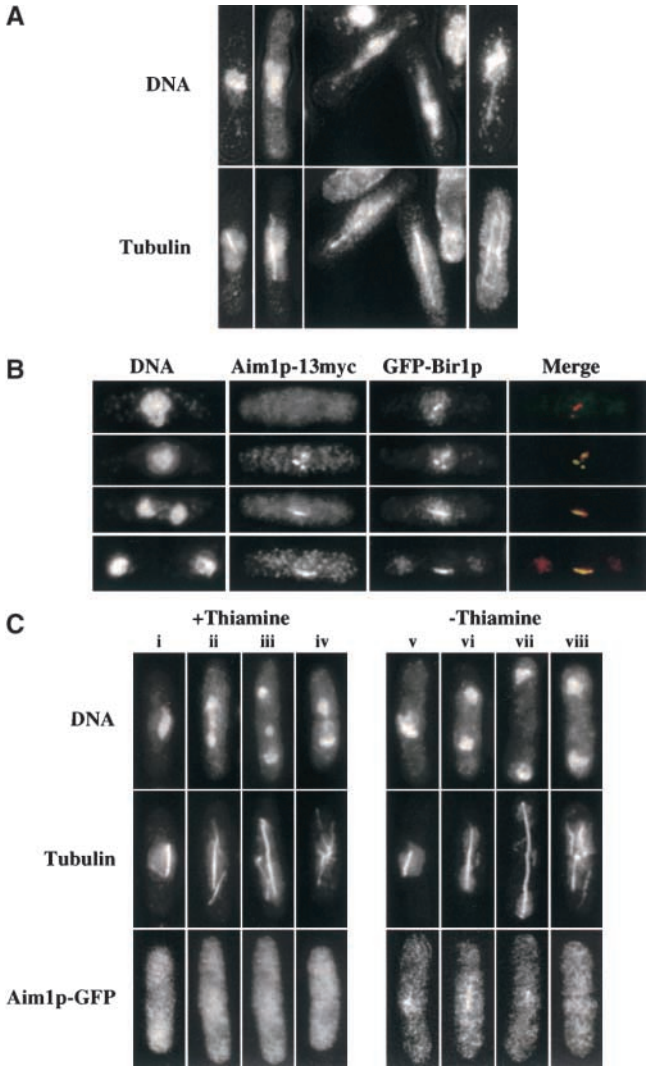

Figure 7.—Bir1p function is required for the localization of Aim1p, a gene essential for chromosome segregation, to kinetochores and the spindle midzone during mitosis. (A) *aim1* null mutant spores were germinated in medium lacking histidine for 20 hr, fixed, stained with DAPI to visualize chromosomes, and with α-Tat1p to visualize microtubules. (B) Cells expressing GFP-Bir1p and Aim1p-13myc were grown to exponential growth phase, fixed, stained with DAPI to visualize chromosomes, with α-GFP to visualize GFP-Bir1p, and with α-myc to visualize Aim1p-13myc. Merge represents an overlap of Aim1p (green) and Bir1p (red) staining. (C) P*nmt1*-81::*bir1*$^{+}$ cells were grown at 32° to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 2 μM thiamine for 12 hr, fixed, and stained with DAPI to visualize chromosomes, with α-Tat1p to visualize microtubules, and with α-GFP to visualize Aim1p-GFP. (i–iv) Examples of mitotic cells that were depleted of Bir1p, indicating the loss of Aim1-GFP staining to kinetochores and the spindle midzone as opposed to the cells that still retained Bir1p function (v–viii).

al. 1997). It has been proposed that a "chromosomal passenger" complex of proteins, including INCENP and aurora B kinase, moves from the inner centromeres in metaphase to the spindle midzone in anaphase and eventually to the midbody in telophase (Adams *et al.* 2000, 2001; Kaitna *et al.* 2000; Wheatley *et al.* 2001). Whether Bir1p is required at all different cellular locations where it can be seen should be a topic of future research. In addition, it will also be important to study a possible interaction of fission yeast Bir1p with chromosomal passenger proteins and the function of such complexes in the successful execution of mitosis and cytokinesis.

Bir1p is essential for chromosome condensation: *bir1* mutants show defects in chromosome condensation during mitosis. A close relationship has been established between Bir1p-like proteins and protein kinases of the Aurora family in other organisms. It has been shown that *Caenorhabditis elegans* AIR1 localization to chromosomes is dependent on Bir1p function (Speliotes *et al.* 2000). Furthermore, Aurora-like kinases have been shown to be important for chromosome condensation in a number of organisms, via phosphorylation of histone H3 on serine residues (Bischoff and Plowman 1999; Hsu *et al.* 2000; Giet and Glover 2001). We have shown that the *S. pombe*, Aurora kinase-related protein Aim1p is essential for mitosis and colocalizes with Bir1p at the kinetochores and the spindle midzone throughout mitosis and that this mitotic localization of Aim1p is dependent on Bir1p function, consistent with evidence from *C. elegans* (Speliotes *et al.* 2000). One possibility is that Bir1p may physically recruit Aim1p and/or other components of the condensation machinery such as the condensins (Sutani *et al.* 1999). Given that Bir1p localizes to the kinetochores, it is possible that kinetochores represent points from which the condensation machinery is loaded onto the chromosomes, although mutant alleles of Bir1p that specifically do not localize to the kinetochores will be necessary to firmly establish if this is the case.

Lagging chromosomes and spindle elongation defects: Cells depleted of Bir1p display chromosomes that lag on the anaphase spindle. Lagging chromosomes have been observed in a number of fission yeast mutants, such as *clr4*, *csp1*, and *swi6*, which affect kinetochore function (Ekwall *et al.* 1995, 1996, 1999; Sutani *et al.* 1999). One possible reason for this defect is that Bir1p-depleted cells may be impaired in the microtubule attachment aspect of kinetochore function. Localization of Bir1p to the kinetochore might ensure maintenance of kinetochore-microtubule attachment following onset of anaphase A. Alternatively, Bir1p at the kinetochore may be involved in establishment of sister-chromatid biorientation, the absence of which results in chromatids "held" in the middle by a balance of forces due to attachment of microtubules from both spindle ends. Our data also indicate that the lagging chromosomes seen in *bir1* mutants are individual chromatids rather than nondisjoined chromosomes, suggesting that *bir1* mutants are not defective in loss of cohesion between

sister chromatids that occurs at the onset of anaphase A (Uhlmann *et al.* 1999; Nasmyth *et al.* 2000; Tomonaga *et al.* 2000). Additionally, normal proteolysis of Cut2p (Funabiki *et al.* 1996) was observed in *bir1*-1 mutant during mitosis (our unpublished observations), reiterating that Bir1p may not be involved in the regulation of cohesion loss between sister chromatids in mitosis.

We have also shown that Bir1p function is required for complete elongation of the anaphase spindle. Our results (Rajagopalan and Balasubramanian 1999, and this study) are somewhat different from those reported by Uren *et al.* (1999) in that we have been able to detect spindles that are longer than metaphase-looking spindles. It is possible that the expression of a cut phenotype and septation might render it difficult to detect longer spindles and might explain the observed differences. However, given that intermediate-length spindles are detected in septation-defective cells that do not cut (S. Rajagopalan and M. K. Balasubramanian, unpublished observations), we conclude that elongation of the spindle is only partially compromised in cells lacking functional Bir1p. It has been previously reported that in cells with lagging chromosomes, the rate of spindle elongation is reduced to approximately half the wild-type elongation rates (Pidoux *et al.* 2000). It is possible that lagging chromosomes in Bir1p-depleted cells cause a slow down of anaphase spindle elongation. Perhaps, accumulation of all kinetochores at the SPBs is required to accelerate spindle elongation at the end of anaphase A. In the absence of Bir1p function, not all kinetochores may cluster at the SPBs, leading to a slowdown in the rate of spindle elongation. Time-lapse imaging supports the idea that Bir1p does indeed spread from the kinetochores at the pole end(s) to the spindle at the onset of anaphase B. Hence, Bir1p possibly relocates from the kinetochores to the spindle to coordinate anaphase B onset with completion of anaphase A. Alternatively, localization of Bir1p to the spindle midzone may physically stabilize the elongating anaphase B spindle.

Outstanding issues: This study has provided evidence for the role of Bir1p in chromosome condensation, in synchronous and symmetric segregation of chromosomes, and for proper elongation of the mitotic spindle during mitosis. In the future it will be important to determine if Bir1p plays a similar role in the processes of meiosis I and II. This does seem likely since Bir1p is detected at the kinetochore-like structures and the spindle midzone in meiosis I and II. Since Bir1p is related to caspase inhibitors (Crook *et al.* 1993; Duckett *et al.* 1996; Rajagopalan and Balasubramanian 1999; Uren *et al.* 1999), and proteins of the separin/separase class (that includes budding yeast Esp1p and fission yeast Cut1p) are related to caspases (Uzawa *et al.* 1990; Uhlmann *et al.* 2000; Yanagida 2000), it will be important to establish whether the function of separins is influenced by Bir1p-like proteins. The interactions of *S. pombe* Bir1p with potential chromosomal passenger proteins (such as Aurora kinases and the INCENP proteins) and their roles in regulation of the events of mitosis will also be of interest.

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