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Functional Analysis of the Putative *BUB2* Homologues of *C. elegans* in the Spindle Position Checkpoint

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Abstract: Spindle position checkpoint monitors the orientation of mitotic spindle for proper segregation of replicated chromosomes into mother cell and the daughter, and prohibits mitotic exit when mitotic spindle is misaligned. *BUB2* forms one of the key upstream element of spindle position checkpoint in budding yeast, but its functional homologues have not been identified in higher eukaryotes. Here, we analyzed the functions of two putative *BUB2* homologues of *C. elegans* in the spindle orientation checkpoint. From the *C. elegans* genome database, we found that two open reading frames (ORFs), F35H12_2 and C33F10_2, showed high sequence homology with *BUB2*. We obtained the expressed sequence tag (EST) clones for F35H12_2 (yk221d4) and C33F10_2 (yk14e10) and verified the full cDNA for each ORF by sequencing and 5' RACE with SL1 primer. The functional complementation assays of yk221d4 and yk14e10 in Δ*bub2* of *S. cerevisiae* revealed that these putative *BUB2* homologues of *C. elegans* could not replace the function of *BUB2* in spindle position checkpoint and mitotic exit. Our attempt to document the component of spindle position checkpoint in metazoans using sequence homology was not successful. This suggests that structural information about its components might be required to identify functional homologues of the spindle position checkpoint in higher eukaryotes.

Key words: *BUB2, S. cerevisiae, C. elegans*, F35H12_2, C33F10_2, spindle position checkpoint, mitotic exit network, human GAPCenA

During mitosis, genomic integrity is maintained by the proper coordination of anaphase entry and mitotic exit through mitotic checkpoints. Mitotic checkpoints arrest cells at metaphase or prevent the exit from mitosis through the regulation of cyclin-dependent kinase (CDK) activity by sensing defects in the microtubule cytoskeleton. Genetic studies in budding yeast *Saccharomyces cerevisiae* have identified several components of mitotic checkpoints by isolating mutants that could no longer sense spindle depolymerization and died rapidly in the presence of microtubule-depolymerizing drugs such as nocodazole or benomyl (Hoyt et al., 1991; Li and Murray, 1991). These components include *MAD1, MAD2, MAD3, BUB1, BUB2,* and *BUB3*. Mitotic checkpoints bifurcate into two separate signaling pathways, the *MAD/BUB* spindle assembly checkpoint for metaphase arrest and the *BUB2*-dependent spindle position checkpoint to block mitotic exit and cytokinesis (Li, 1999; Alexandru et al., 1999).

Bub1p, Bub3p, Mad1p, Mad2p, and Mad3p are present at kinetochores and form spindle assembly checkpoint that monitors the attachment of bipolar microtubules to the kinetochores of sister chromatids and delays the metaphase-to-anaphase transition in response to spindle assembly defects (Hwang et al., 1998; Fang et al., 1998). Homologues of the components of yeast spindle assembly checkpoint including Mad1p, Mad2p, Bub1p, and Bub3p have been identified in higher eukaryotes such as mammals and their conserved functions have been confirmed (Cleveland et al., 2003; Gillett et al., 2004; Ikui et al., 2002). In *C. elegans*, *mdf-1* and *mdf-2* are identified as yeast *MAD2* homologues (Kitagawa and Rose, 1999).

Bub2p is present in the spindle pole body (SPB), the microtubule-organizing center in budding yeast, and forms the spindle position checkpoint that controls mitotic exit and the timing of cytokinesis (Pereira et al., 2000). In the fission yeast *Schizosaccharomyces pombe*, the Bub2p homologue Cdc16p interacts with Byr4p to form a two-component GTPase activating protein (GAP) that stimulates the GTPase activity of Spg1p to regulate the onset of

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cytokinesis (Jwa and Song, 1998; Furge et al., 1998). The presented model for spindle position checkpoint in budding yeast would be that Bub2p along with Bfa1p forms a GAP activity that inhibits GTPase Tem1p to prevent the untimely activation of mitotic exit by monitoring defects in mitotic spindle orientation including the migration of the SPB into the bud and astral microtubule structures (Bloecher et al., 2000; Wang et al., 2000; Pereira et al., 2001). Therefore, Bub2p plays a critical role to avoid improper mitotic exit, which targets degradation of the mitotic cyclin in response to defects in spindle orientation (Schwab et al., 1997; Visintin et al., 1997; Zachariae et al., 1998). However, the regulation of late mitotic events by spindle position checkpoint and its components are poorly understood in metazoans. Only the sequence homologues of *MOB1* and *CDC14* of yeast mitotic exit network (MEN) in the downstream of spindle position checkpoint have been identified in mammalian cells (Kaiser et al., 2002). Previously, GAPCenA (for GAP and centrosome-associated) that shares 63% identity to F35H12_2 of *C. elegans* has been identified in human as a GAP for Rab6 GTPase, which plays a role in the Golgi dynamics and the organization of microtubule cytoskeleton (Cuif et al., 1999). However, the functional homology between GAPCenA and Bub2p has not been elucidated. In addition to F35H12_2, we also found that C33F10_2 of *C. elegans* shows high sequence homology to *S. cerevisiae BUB2* using wormbase blast (http://www.wormbase.org/).

Here, we analyzed the function of two putative *BUB2* homologues of *C. elegans* in the spindle orientation checkpoint, to document the presence of spindle position checkpoint and its components in metazoans.

MATERIALS AND METHODS

Yeast strains

W303 (*MATa ura3-1trp1-1 ade2-1 leu2-3, 112 his3-11, 15*) and YSK 11(*MATa BUB2:: TRP1 ura3-1 ade2-1 leu2-3, 112 his3-11,15*) *S. cerevisiae* strains are used in this study.

C. elegans EST clones

EST clones, yk221d4 (F35H12_2) and yk14e10 (C33F10_2), were identified in *C. elegans* EST database and were provided by Dr. Yuji Kohara (Center for Genetic Resource Information, National Institute of Genetics, Japan). These EST clones in pBSK were verified by sequencing with T7 and T3 primers.

Subcloning for expression in yeast

yk221d4 and yk14e10 were subcloned into pRS426-*GAL1* (2 μ galactose-inducible yeast expression vector) and pRS413 -promoter of *BUB2* (CEN yeast expression vector with endogenous promoter of *BUB2*). yk221d4 amplified by PCR with 5'-CCCGAGCTCATGGAGGATTTTAAAGATTTC -3' and 5'-CAAGAGCTCTCACGGCGTCACTGAGCTATTCGA -3' was subcloned into the *Sac*I site, and yk14e10 with 5'-CCCGGATCCATGGGTAGTTTTCCAAACTTC-3' and 5'-CCCGGATCCTTATGGCTTCACAACAACTGA-3' was subcloned into the *Bam*HI site of pRS426-*GAL1* and pRS413-promoter of *BUB2*. The full *S. cerevisiae BUB2* was also amplified by PCR with 5'-CCACTGCAGATG-ACCTCAATTGAAGAT-3' and 5'-GGGCTGCAGTTAC GGTATATATATGTC-3' from genomic DNA and subcloned into the *Eco*RI site of pRS426-*GAL1* and PstI site of pRS413-*BUB2* promoter. The *BUB2* homologous region of yk221d4 and yk14e10 was also amplified by PCR to be cloned into pTS903CL (a gift of Dr. Sasaki, University of Tokyo) to make pTS903CL-yk221d4*BUB2*HR and pTS903CL-yk14e10*BUB2*HR: 5'-ATGG ATCGGCCGCCAGA AAATA-3' and 5'-TCATCCCTGACTCAAAAATAA-3' for yk221d4 and 5'-ATGATATCTCGACTTACA-3' and 5'-TCAAGGGATGAGCTTCTC-3' for yk14e10 were used and cloned into *Xba*I and *Bam*HI sites.

Western blots

YSK11 cells transformed with *ScBUB2-GST*/pRS426, yk221d4-*GST*/pRS426, yk14e10-*GST*/pRS426, yk221d4HR -*HA*/pTS903CL, and yk14e10HR-*HA*/pTS903CL were grown to 5×10^6 cells/ml and proteins were extracted in SDS sample buffer (2x). Protein extracts were resolved using 10% SDS-PAGE, transferred onto nitrocellulose membrane, and then incubated with monoclonal HA antibody (Santa Cruz) or polyclonal rabbit GST antibody (Santa Cruz), respectively. Bound antibodies were detected with anti-rabbit or anti-mouse IgG-HRP (Jackson Immunochemicals) by enhanced chemiluminescence (ECL) reagents.

Total RNA isolation, RT-PCR and RACE

Total RNAs of yeast were prepared using easy-BLUE™ Total RNA Extraction kit by iNtRON. Total mRNAs of *C. elegans* embryo were a gift of Dr. J. Lee (Seoul National University). RT-PCR was respectively performed with total RNAs from yeast and mRNAs from *C. elegans* embryos by ONE-STEP RT-PCR Pre-Mix Kit of iNtRON using each primer set for 5' and 3' end of the ORF: 5'-CCCGAGCTCATGGAGGATT TTAAAGATTTC-3' and 5'-CAAGAGCTCTCACGG CGTCACTGAGCTATTCGA-3' for yk221d4, 5'-CCCGGATCCATGGGTAGTTTTCCAAACTTC -3' and 5'-CCCGGATCCTTATGGCTTCACAACAACTGA-3' for yk14e10, and 5'-CCACTGCA GATGACCTCAATTGAAGAT -3' and 5'-GGGCTGCAGTTACGGTATATATA TGTC-3' for *S. cerevisiae BUB2*. Primer extension of the 5' end of yk14e10 was performed with its cDNA synthesized from mRNAs of *C. elegans* embryo by RT- PCR using SL1 primer (5'-GGTTTAATTACCCAAGTT TGAG-3') and an internal primer (5'-GCTTCCAGTGAGAT TCCCAA-3') of yk14e10.

Benomyl sensitivity test, re-budding assay, and flow cytometry

Δ*bub2* (YSK11) cells were respectively transformed with pRS426-*GAL1-BUB2*, pRS426-*GAL1-GST*-yk221d4, pRS426 -*GAL1-GST*-yk14e10, pRS413-promoter of *BUB2*-yk221d4, pRS413-promoter of *BUB2*-yk14e10, pTS903CL-yk221d4*BUB2*HR and pTS903CL-yk14e10*BUB2*HR. For benomyl sensitivity test, each transformed cell in exponential growth was adjusted to 10^7 cells/ml and its tenfold serial dilutions were spotted on YPG (yeast rich media supplemented with 4% galactose) plates with or without 30 μg/ml benomyl. For re-budding assay, exponentially growing cells of each transformant were incubated with nocodazole (15 μg/ml) for 0, 3, and 6 h, and cells collected at each time point were briefly fixed in 70% ethanol (V/V). Cells with extra new buds were counted under bright-field microscopy after light sonication to disperse cell clumps. For flow cytometry, cells of each tranformant were arrested with 6 μM α-factor for 3 h, released in the presence of 15 μg/ml nocodazole, and collected every hour for 4 h. Their DNA contents were analyzed by fluorescence-activated cell sorter as described by Kim et al. (2004). For each sample, 20,000 cells were analyzed with a Becton Dickinson (San Jose, CA) fluorescence-activated cell sorter analyzer.

Abbreviations used: *S. cerevisiae, Saccharomyces cerevisiae*; *C. elegans, Caenorhabditis elegans*; ORF = open reading frame; EST = expressed sequence tag; FACS = fluorescence-activated cell sorter; CDK = cyclin-dependent kinase; SPB = spindle pole body; MEN = mitotic exit network; GAP = GTPase-activating protein; RACE = rapid amplification of cDNA ends.

RESULTS AND DISCUSSION

Search for Bub2p homologues in *C. elegans*

Cell cycle regulatory mechanisms are well conserved in all eukaryotic cells, but the regulation of late mitotic events by spindle position checkpoint and its components are poorly understood in metazoans. In an attempt to study spindle position checkpoint and mitotic exit network (MEN) in multi-cellular organisms, we looked for the homologues of the components of this checkpoint pathway in *C. elegans* because it provides a nice metazoan model system for studying the function of genes during cell division and development. When worm protein database (http://www.wormbase.org/db/searches/blast) was searched with *S. cerevisiae* Bub2p, C33F10_2 was identified by blast search. C33F10_2 contained a Bub2p homology region in the residues 453-688 that shared 25% identity and 43% similarity with *S. cerevisiae* Bub2p (Fig. 1A). In addition, the central domain (residues 502-709) of human Rab6-interacting protein called GAPCenA, which showed high

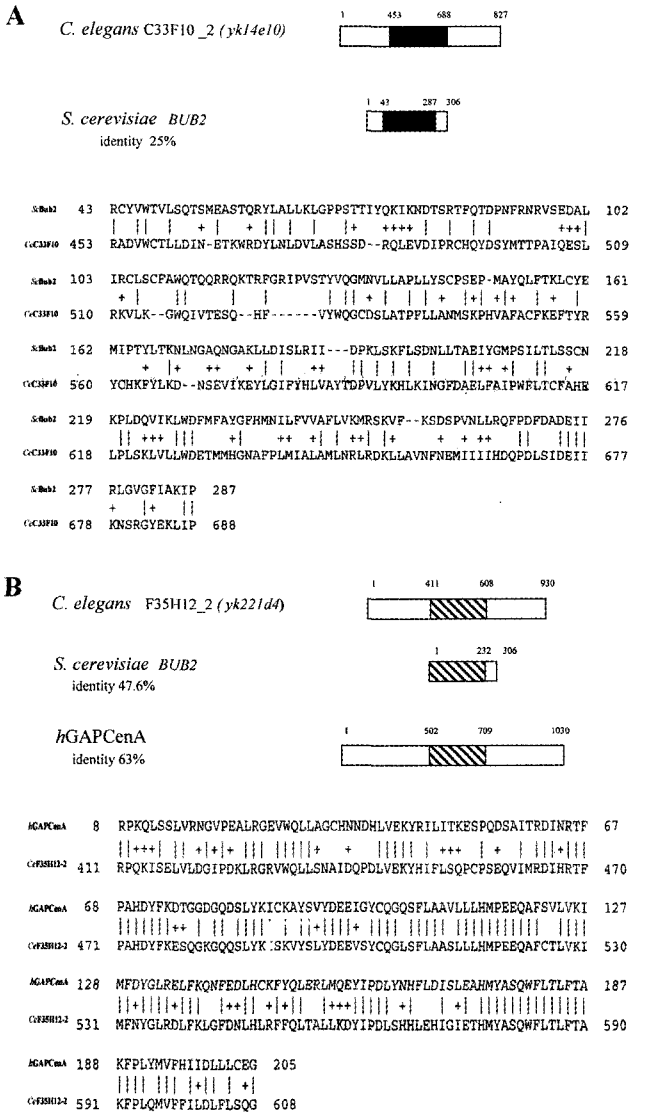

Fig. 1. Homology comparison between *S. cerevisiae BUB2* and its putative *C. elegans* homologues C33F10_2 and F35H12_2. Sequences of the predicted ORF of C33F10_2 and F35H12_2 in *C. elegans* were compared with *S. cerevisiae* Bub2p and the alignment of homology region was presented. A, The residues 453-688 of *C. elegans* C33F10_2 share 25% identity with *S. cerevisiae* Bub2p. B, *C. elegans* F35H12_2 shares high sequence identity with the residues 502-709 of *h*GAPCenA (a GTPase activationg protein for Rab6) (63%) and *S. cerevisiae* Bub2p (47.6%).

homology with *S. cerevisiae* Bub2p and its *S. pombe* homologue Cdc16p, also displayed significant similarity with F35H12_2 of *C. elegans* (Cuif et al. 1999). The Bub2p homology region of GAPCenA shares 63% identity with F35H12_2 (Fig. 1B).

Sequence analysis of both EST clones

EST clones for F35H12_2 (yk221d4) and C33F10_2 (yk14e10) in pBSK were obtained from Dr. Yuji Kohara (Center for Genetic Resource Information, National Institute of Genetics, Japan), and sequenced to confirm that these

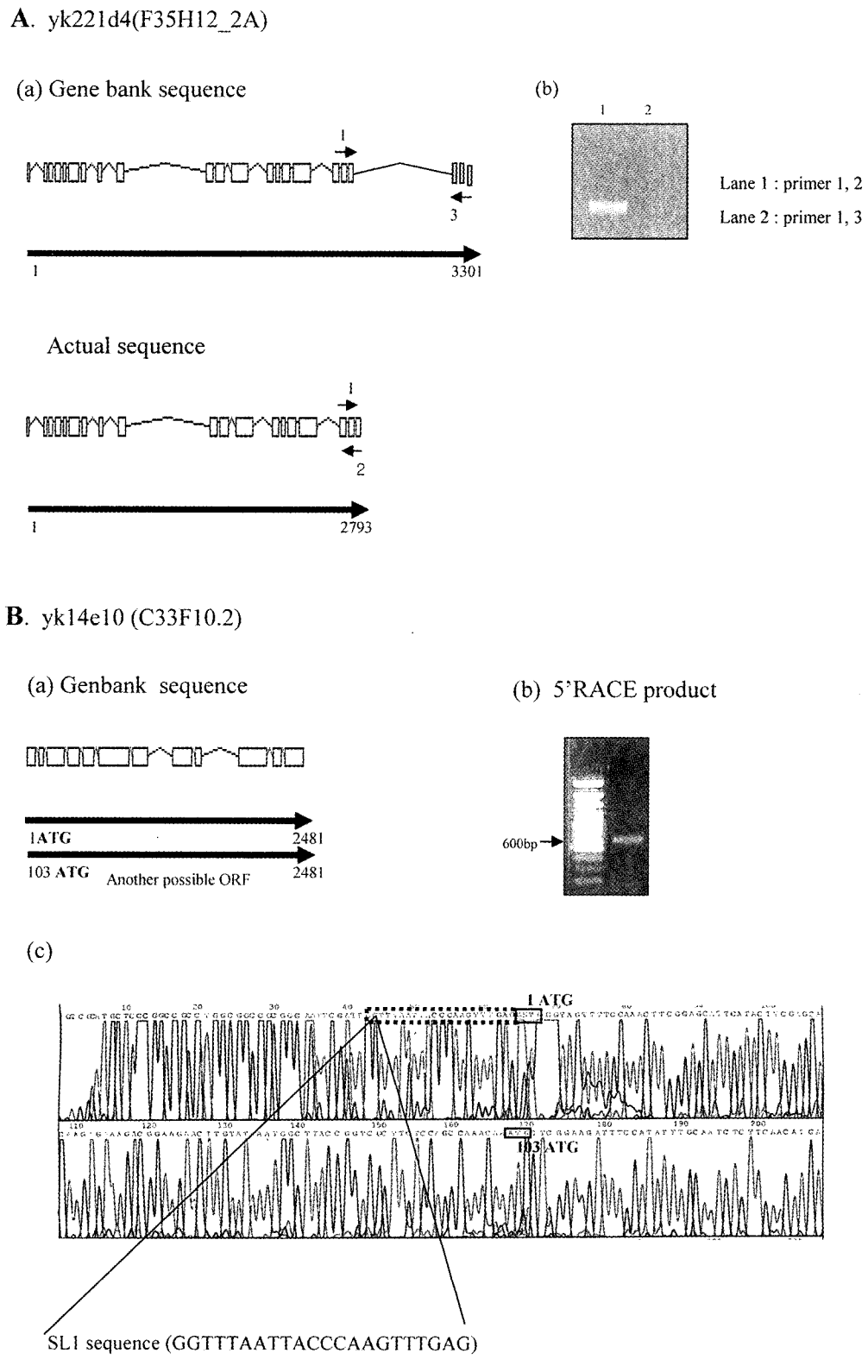

Fig. 2. Verification of the EST clones of F35H12_2 (yk221d4) and C33F10_2 (yk14e10). A, F35H12_2 (yk221d4) (a) Sequence analysis of yk221d4 revealed that 3' end of the EST of F35H12_2 is 508 bases shorter than the predicted sequence of Genbank. (b) The 3' end of F35H12_2 was verified by PCR of its cDNA prepared by RT-PCR of *C. elegans* embryonic mRNAs, using the same 5' primer (primer 1) and each of two different 3' primers (primer 2 and 3) derived from sequences of the actual (primer 2) or the predicted region (primer 3). B, C33F10_2 (yk14e10) (a) Sequence analysis of yk14e10 shows two start codons in frame that are 103 base apart. (b) The start codon was confirmed by 5' RACE of its cDNA generated by RT-PCR with embryonic mRNAs using SL1 primer and a 3' primer derived from yk14e10. (b) The size of 5' RACE product. C, Sequence analysis of the 5' RACE product revealed the position of SL1 sequence and two ATGs.

ESTs contained the full cDNA for each ORF. Sequence analysis of yk221d4 demonstrated that its 3' end was 508 base pair shorter than the sequence predicted from Genbank (Fig. 2A(a)). To confirm the 3' end of yk221d4, its cDNA was prepared by RT-PCR of total embryonic mRNAs of *C. elegans* (a gift from Dr. J. Lee), and PCR was performed using the same 5' primer (primer 1) and each of two different 3' primers (primer 2 and 3) derived from sequences of the actual (primer 2) or the predicted region (primer 3), as shown in Fig. 2A(a). Only primer 1 and 2 gave the expected 490 base pair PCR product (Fig. 2A(b)), indicating that the predicted ORF was not correct and the stop codon starting 508 base ahead of the stop predicted was used for the full ORF of F35H12_2.

Sequencing of yk14e10 showed two start codons in frame that are 103 base apart (Fig. 2B(a)). To verify the right start codon for C33F10_2, 5' RACE was performed with cDNA prepared by RT-PCR of the total embryonic mRNAs of *C. elegans*, using nematode trans-splicing leader sequence SL1 primer (5'-GGTTTAATTACCCAAGTTTGAG -3') and an internal primer derived from yk14e10 (5'-GCTTCCAGTGAGATTCCCAA-3'). In *C. elegans*, one gene always generates transcripts with different 5' ends by cis- and/or trans-splicing mechanisms, and the transcript with 5' SL1 trans-splicing leader sequence is considered as the full-length transcript (Liou and Blumenthal 1990; Van and Hirsh 1990). Since SL1 presents the 5' end of the full-length transcript, the 5' RACE product of yk14e10 using SL1 primer should show the correct ORF start. The 5' RACE product of yk14e10 was 660 base pairs as expected and was confirmed by nested PCR with internal primers of yk14e10 (data not shown). When this 5' RACE product was sequenced, SL1 trans-splicing leader sequences are positioned at just upstream of the first ATG, strongly suggesting that first ATG is the start codon (Fig. 2B(c)). Altogether, these data verified that yk221d4 (2793bp) and yk14e10 (2481bp) contained the full cDNA for F35H12_2 and C33F10_2.

F35H12_2 and C33F10_2 can not rescue the defects of *BUB2* in *S. cerevisiae*

Considering that most cell cycle regulatory components are conserved in eukaryotes by cross-species complementation tests (Kitagawa and Rose, 1999), the role of F35H12_2 (yk221d4) and C33F10_2 (yk14e10) in spindle orientation checkpoint and MEN was tested by functional complementation assays with Δ*bub2* of *S. cerevisiae*.

The full cDNAs of F35H12_2 (yk221d4) and C33F10_2 (yk14e10) respectively were subcloned in pRS426-*GAL1-GST* (2 μ galactose-inducible yeast expression vector) and pRS413-promoter of *BUB2* (CEN yeast expression vector with endogenous promoter of *BUB2*) for their expression in yeast. When the expression of F35H12_2 (yk221d4) and C33F10_2 (yk14e10) was driven in galactose media by *GAL1* promoter, the sensitivity of Δ*bub2* to benomyl, a microtubule-destabilizing drug, was not rescued (Fig. 3A). Similar to benomyl plate assay, neither F35H12_2 (yk221d4) nor C33F10_2 (yk14e10) recovered the lack of mitotic arrest of Δ*bub2* in the presence of nocodazole (15 μg/ml), and F35H12_2 (yk221d4) and C33F10_2 (yk14e10) transformed Δ*bub2* cells showed multiple budding phenotypes (Fig. 3B). The lack of complementation by these two Bub2p homologous EST clones of *C. elegans* was also verified by examining the DNA content of Δ*bub2* cells transformed with pRS413-*BUB2*, pRS413-yk221d4, or pRS413-yk14e10 after treated with nocodazole. When these cells were arrested with α-factor at G1 and released into medium containing nocodazole for 4 h, wild type (W303) and pRS413-*BUB2* transformed Δ*bub2* cells were arrested with 2N DNA content, but Δ*bub2* and Δ*bub2* cells transformed with yk221d4 or yk14e10 progressed to the next cell cycle and showed DNA content higher than 2N (Fig. 3C). These observations disappointingly suggested that F35H12_2 and C33F10_2 were not able to rescue the spindle checkpoint function of *BUB2* in Δ*bub2*. In addition to the complementation assays of two EST clones containing the full-length ORF, we also tested the function of Bub2p homology region of F35H12_2 and C33F10_2, 411-608 residues of F35H12_2 and 453-688 residues of C33F10_2. Bub2p homology region of F35H12_2 and C33F10_2 could not recover the multiple budding phenotypes of Δ*bub2* cells in the presence of nocodazole, demonstrating that they can not rescue Bub2p functions in Δ*bub2* (Fig. 3D). Expression and size of the full and Bub2p homology region of F35H12_2 and C33F10_2 ORF in transformed yeast cells were verified by western blots, as shown in Fig. 3E.

To examine whether yk221d4 and yk14e10 of *C. elegans* are real homologues of *S. cerevisiae BUB2* and vice versa, we also performed RT-PCRs with primers for 5' and 3' ends of *BUB2*, yk221d4, and yk14e10, using total RNAs of *S. cerevisiae* and total embryonic mRNAs of *C. elegans* respectively. When RT-PCR was performed with total RNAs of *S. cerevisiae*, only *BUB2* was produced and no product was detected with primers of yk221d4 and yk14e10 (Fig. 4A). Similarly, only yk221d4 and yk14e10 were produced and no *BUB2* was detected with embryonic mRNAs of *C. elegans* (Fig. 4B). These observations suggested that the similarities between *BUB2* and yk221d4, or yk14e10 might be limited and other ORFs with high sequence homology to *BUB2* of *S. cerevisiae* might not exist in *C. elegans*.

Consistent with our results, the RNAi of F35H12_2 and C33F10_2 reported that the knock-down of these *BUB2* homologues did not induce any maternal phenotypes or embryonic lethality in *C. elegans* (Kamath and Ahringer, 2003). We also tested *C. elegans* homologue of *TEM1* that

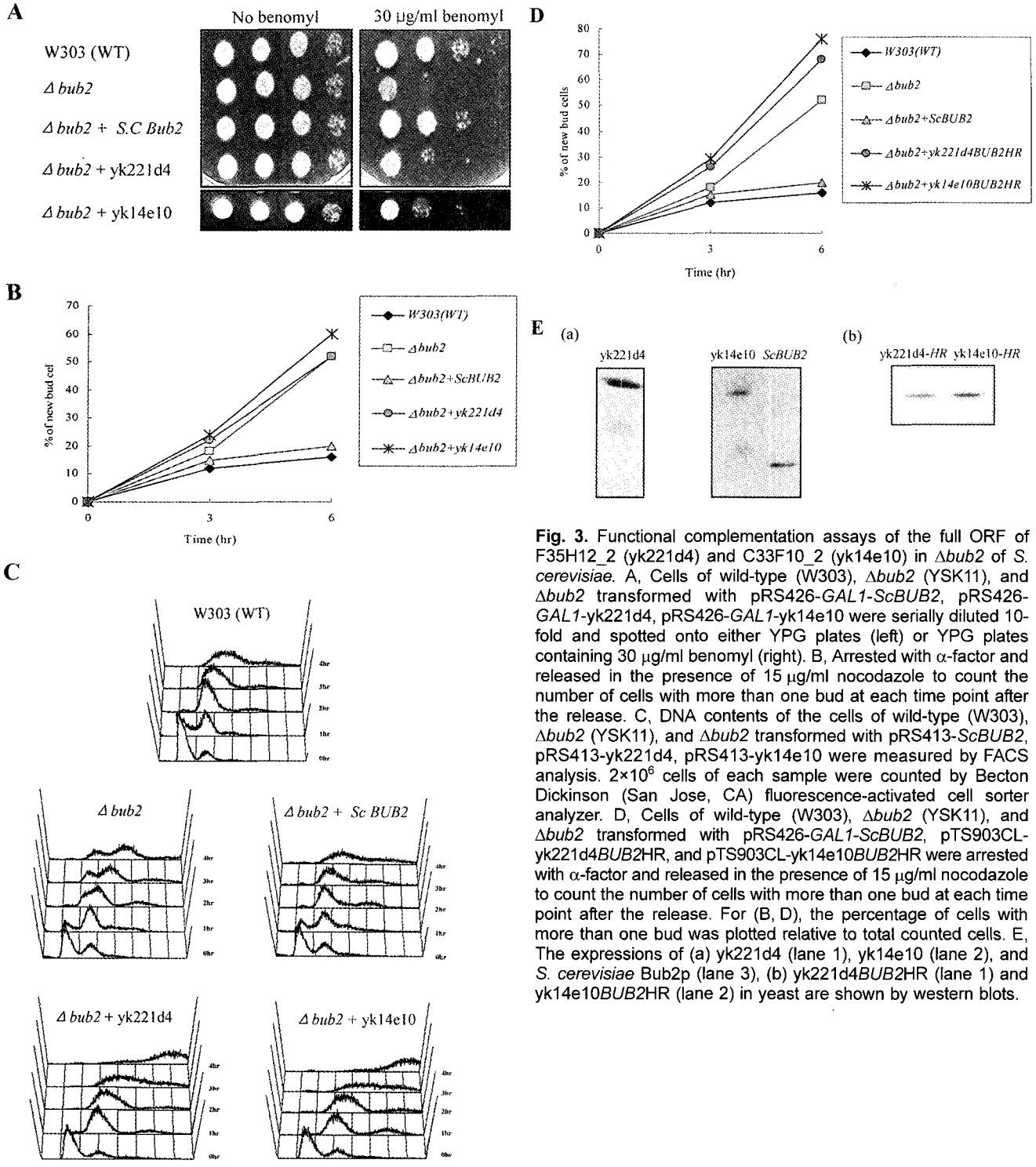

Fig. 3. Functional complementation assays of the full ORF of F35H12_2 (yk221d4) and C33F10_2 (yk14e10) in Δ*bub2* of *S. cerevisiae*. A, Cells of wild-type (W303), Δ*bub2* (YSK11), and Δ*bub2* transformed with pRS426-*GAL1-ScBUB2*, pRS426-*GAL1*-yk221d4, pRS426-*GAL1*-yk14e10 were serially diluted 10-fold and spotted onto either YPG plates (left) or YPG plates containing 30 μg/ml benomyl (right). B, Arrested with α-factor and released in the presence of 15 μg/ml nocodazole to count the number of cells with more than one bud at each time point after the release. C, DNA contents of the cells of wild-type (W303), Δ*bub2* (YSK11), and Δ*bub2* transformed with pRS413-*ScBUB2*, pRS413-yk221d4, pRS413-yk14e10 were measured by FACS analysis. 2×10^6 cells of each sample were counted by Becton Dickinson (San Jose, CA) fluorescence-activated cell sorter analyzer. D, Cells of wild-type (W303), Δ*bub2* (YSK11), and Δ*bub2* transformed with pRS426-*GAL1-ScBUB2*, pTS903CL-yk221d4*BUB2*HR, and pTS903CL-yk14e10*BUB2*HR were arrested with α-factor and released in the presence of 15 μg/ml nocodazole to count the number of cells with more than one bud at each time point after the release. For (B, D), the percentage of cells with more than one bud was plotted relative to total counted cells. E, The expressions of (a) yk221d4 (lane 1), yk14e10 (lane 2), and *S. cerevisiae* Bub2p (lane 3), (b) yk221d4*BUB2*HR (lane 1) and yk14e10*BUB2*HR (lane 2) in yeast are shown by western blots.

functions just downstream of *BUB2* in spindle position checkpoint, but this *C. elegans* homologue could not rescue the function of Tem1p in *S. cerevisiae* Δ*tem1* (data not shown). Functional analyses of the potential MEN homologues using RNAi in *C. elegans* reported that only the depletion of *CeCDC14* (C17G10_4), the *C. elegans* homologue of *S. cerevisiae CDC14* that is a key downstream regulator of mitotic exit, caused embryonic lethality by failing to establish a central spindle and cytokinesis (Gruneberg et al., 2002). These reports along with our results suggest that the ultimate functions of spindle position checkpoint and MEN are conserved in metazoans but upstream

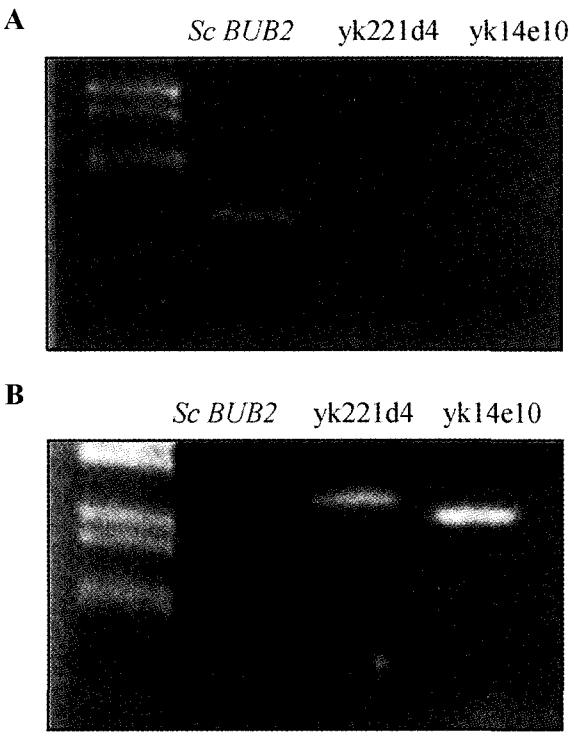

Fig. 4. RT-PCRs to confirm F35H12_2 (yk221d4) and C33F10_2 (yk14e10) of *C. elegans* are sequence homologues of *S. cervisiae BUB2*. RT-PCRs using 5' and 3' primers for the ORF of *S. cerevisiae BUB2*, yk221d4, and yk14e10 were respectively performed with total RNAs from *S. cerevisiae* (A) and mRNAs from *C. elegans* embryos (B), as described in Materials and Method.

components are not conserved at least at the sequence level.

In short, our effort to identify one of the components of spindle position checkpoint, *BUB2*, in *C. elegans* by sequence homology was not successful, and we suggest that structure-based homology screen might be necessary to predict the functional homologues of spindle position checkpoint and MEN in metazoans. Further studies on the structure-based homology screen to detect functional homologues with remote homology such as Bayesian fold recognition method (Bienkowska et al., 2000, 2003), protein domain structure models by DSM (Discrete State-space Models) (Bienkowska et al., 2000, 2003), ISS (Intermediate Sequence Search) method (John and Sali 2004) might be necessary.

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