

Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1

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Fission yeast Cut5/Rad4 plays a unique role in the genome maintenance as it is required for replication, replication checkpoint, and normal UV sensitivity. It is unknown, however, how Cut5 protein is linked to other checkpoint proteins, and what part it plays in replication and UV sensitivity. Here we report that Cut5 interacts with a novel checkpoint protein Crb2 and that this interaction is needed for normal genome maintenance. The carboxyl terminus of Crb2 resembles yeast Rad9 and human 53BP1 and BRCA1. Crb2 is required for checkpoint arrests induced by irradiation and polymerase mutations, but not for those induced by inhibited nucleotide supply. Upon UV damage, Crb2 is transiently modified, probably phosphorylated, with a similar timing of phosphorylation in Chk1 kinase, which is reported to restrain Cdc2 activation. Crb2 modification requires other damage-sensing checkpoint proteins but not Chk1, suggesting that Crb2 acts at the upstream of Chk1. The modified Crb2 exists as a slowly sedimenting form, whereas Crb2 in undamaged cells is in a rapidly sedimenting structure. Cut5 and Crb2 interact with Chk1 in a two-hybrid system. Moreover, moderate overexpression of Chk1 suppresses the phenotypes of *cut5* and *crb2* mutants. Cut5, Crb2, and Chk1 thus may form a checkpoint sensor-transmitter pathway to arrest the cell cycle.

[Key Words: UV damage; hydroxyurea; DNA polymerase; phosphorylation; two-hybrid screen]

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The eukaryotic genome is maintained by a number of controlling elements. Weinert and Hartwell (1988) identified a checkpoint gene *RAD9* in the budding yeast *Saccharomyces cerevisiae*, which monitors the damage of DNA on UV irradiation, and delays the progression of cell cycle. In the absence of the *RAD9* function, cells containing the damaged DNAs enter fatal cell division without a delay. A number of checkpoint genes involved in DNA damage, replication, and spindle defects have been identified (e.g., Elledge 1996; Kitazono and Matsumoto 1997). The word checkpoint in the present paper is used as synonymous to a surveillance mechanism that blocks or delays cell-cycle transitions (Nasmyth 1996). Cell-cycle regulators are also important elements in maintenance of the genome. When the mitotic cyclin gene, *cdc13⁺*, is lost in the fission yeast *Schizosaccharomyces pombe*, cells are unable to enter mitosis but perform repeated DNA replications, leading to the formation of polyploidy giant nucleus (Hayles et al. 1994).

Similar phenotypes can be produced by overproducing Rum1, the inhibitor for CDK/cyclin B (Moreno and Nurse 1994) or constitutively activating Cdc18, an essential replication initiation factor (Kelly et al. 1993; Nishitani and Nurse 1995). In mammalian cells, the genome is maintained by tumor-suppressing proteins such as p53 and RB. p53 is a transcription factor, activating transcription of certain inhibitors against CDK kinase on DNA damage, leading to the arrest of cells in the G₁ phase (e.g., El-Deiry et al. 1993). If p53 is lost, the damage checkpoint in the G₁ phase no longer seems to be functional. These various gene functions contribute to the quality control of genome.

The fission yeast *cut5⁺* gene is uniquely implicated in the genome maintenance as it is essential for DNA replication, replication checkpoint control, and normal UV sensitivity (Saka et al. 1994a;b). Temperature-sensitive *cut5* mutants at the restrictive temperature (36°C) block DNA replication but enter mitosis, producing the cut phenotype (Saka and Yanagida 1993), which strikingly differs from the arrest phenotype of temperature sensitive *cdc* mutations in DNA polymerase or ligase, or ribonucleotide reductase (RNR) (Nurse et al. 1976; Gordon and Fantes 1986). The cell-cycle arrest of these replica-

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tion-defective *cdc* mutants is caused by the replication checkpoint control that also operates in the presence of hydroxyurea (HU), an inhibitor of RNR. The double-mutants between *cut5* and polymerase or ligase mutants also displayed no retardation in cell division at 36°C (Saka et al. 1994a;b). These results showed that Cut5 was required for the replication checkpoint arrest induced by HU or mutations in the replication enzymes. In *S. pombe*, the HU-induced replication checkpoint arrest is also abolished in *hus* and certain *rad* mutants, *rad1*, *rad3*, *rad9*, *rad17*, and *rad26* (Al-Khodairy and Carr 1992; Enoch et al. 1992; Rowley et al. 1992; Sheldrick and Carr 1993; Ford et al. 1994; Griffiths et al. 1995; Bentley et al. 1996; Lieberman et al. 1996; Kostrub et al. 1997).

In addition to the phenotypes described above, *cut5* mutants are sensitive to UV irradiation at the permissive temperature. Consistent with the phenotype, isolation of the *cut5⁺* gene (Saka and Yanagida 1993) indicated that it was identical to *rad4⁺* (Duck et al. 1976; Fenech et al. 1991). All *cut5/rad4* mutants were found to be UV sensitive (Duck et al. 1976; Saka and Yanagida 1993; Saka et al. 1994a). Intriguingly, however, the UV- or 4NQO-induced checkpoint control was retained in *cut5* mutants even at 36°C (Saka et al. 1994a,b; F. Esashi, Y. Saka, and M. Yanagida, unpubl.).

Cut5 may sense the replication defect and generate a signal to delay the cell cycle. Multiple phenotypes of *cut5* in replication and damage, however, are intriguing, and the actual molecular role of Cut5 in maintaining the genome is unknown. In this study, we addressed the question of what kind of proteins interact with Cut5. Identification of the interacting proteins should shed light on how Cut5 protein functions within cells. Cut5 is a 74-kD nuclear protein consisting of several regions (Saka et al. 1994a). The amino-terminal 100 amino acids long motif repeats twice, and is similar to the regions found in the human repair protein XRCC1 (Thompson et al. 1990), the oncoprotein Ect1 (Miki et al. 1993) and the yeast protein Rev1 (Larimer et al. 1989). This region may serve as the site for protein-protein interaction. The *S. cerevisiae* Dpb11 (Araki et al. 1995), a suppressor for mutations in an essential subunit of DNA Pol II (epsilon) has a weak resemblance to Cut5.

An initial investigation for searching Cut5-interacting proteins by the two-hybrid screen method (Fields and Song 1989) indicated that the GAL4 DNA-binding domain (GAL4D) fused to full-length Cut5 activated transcription without the activation domain. Therefore, Cut5 was divided into several regions and each of the regions was used as bait. We identified a novel gene product Crb2, which interacts specifically with the amino-terminal region of Cut5. The deletion of the *crb2⁺* gene profoundly affects checkpoint control induced by UV as well as DNA polymerase mutations. Crb2 is modified on DNA damage, and appears to mediate the signal for arrest of the cell cycle through Chk1, a protein kinase which restrains the activation of Cdc2 kinase by regulating phosphorylation of Tyr15 (Walworth et al. 1993; Walworth and Bernards 1996; O'Connell et al. 1997; Rhind et al. 1997).

Results

Mutations of cut5/rad4 in the conserved amino-terminus

To locate an essential region in Cut5, we determined mutation sites of *cut5*. The mutant gene was amplified from three *cut5/rad4* alleles [*rad4-116*; Duck et al. (1976); *cut5-580*, Hirano et al. (1986); *cut5-T401*, Samejima et al. (1993)] by PCR and sequenced. Surprisingly, all three mutations contained the same substitution at the 45th codon (from ACG to ATG), leading to the amino acid change T45M (Fig. 1A). An additional alteration (resulting in K62Q) was present in *cut5-T401*. The T45M mutation should create a new *EcoT22I* site (ATGCAT, Fig. 1B). This was verified by Southern hybridization (Fig. 1B, right), which produced expected sizes of the *EcoT22I* fragments for the mutant genomic DNAs. The T45M mutation resided in the middle of the first amino-terminal repeat R1 (consensus is indicated in the bottom of panel A; Saka et al. 1994b). T45 is present within the conserved stretch (consensus, VTHLIA), hereafter designated as the TH domain.

Isolation of Crb2

For the two-hybrid screening method (Fields and Song 1989), five constructs (Fig. 1C) were made as bait, each of which contained a region of Cut5 fused to the GAL4DB. GAL4DB-R1R2 thus contained the R1 and R2 region, and so on. More than 1×10^6 *S. pombe* cDNA clones were screened for each plasmid. Two identical clones (designated *crb2⁺*, *cut5-repeat binding*) were obtained by the bait of R1R2, whereas eight identical clones (*crb3⁺*) were isolated by the bait of R3. Combinations of R1R2 and Crb2, and of R3 and Crb3, produced β -galactosidase activities (Fig. 2A), respectively, as strong as the control interaction (p53 and T-antigen). Other control combinations showed low activities. The interaction of Cut5 with Crb2 or Crb3 was specific as seen by pairwise combinations (Fig. 2B; β -galactosidase positives are blue). No other positive clone was obtained by the screen with the other baits.

Interaction of Crb2 with Cut5

An in vitro-binding assay described below supported a direct interaction between Crb2 and Cut5. The cDNAs of Crb2 and Crb3 were expressed under the T7 promoter by the in vitro reticulocyte lysate system by use of [³⁵S]methionine for labeling Crb2 and Crb3 (Fig. 2C, left; luciferase was also labeled as control). To examine binding, GST-R1R2 and GST-R3 fusion proteins produced in *Escherichia coli* and purified by glutathione-agarose beads were incubated with ³⁵S-labeled proteins. The beads were washed, and bound labeled proteins were analyzed by SDS-PAGE and autoradiography (Fig. 2C; right). Radiolabeled Crb2 and Crb3 were detected to be bound to GST-R1R2 and R3, respectively, but luciferase was not. A Crb2 fragment cleaved during incubation was also efficiently bound to GST-R1R2. Another control

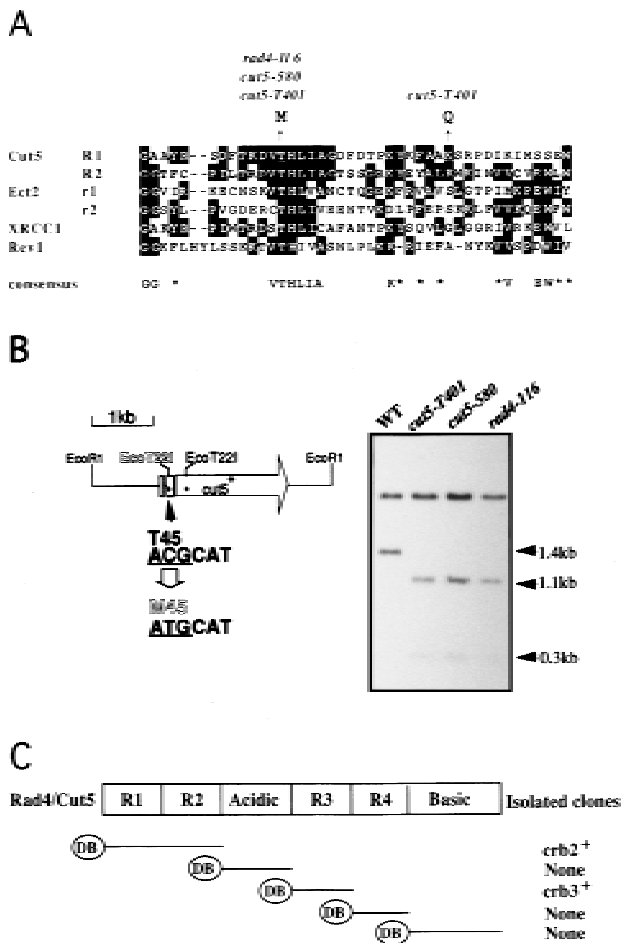


Figure 1. *cut5/rad4* mutations reside in the highly conserved T45. (A) Mutation sites for *rad4-116*, *cut5-580*, and *cut5-T401* were determined to be the identical T45M, residing in the R1 repeat. The amino-terminal regions R1 and R2 are similar to oncoprotein Ect2 (Miki et al. 1993), DNA repair proteins XRCC1 (Thompson et al. 1990), and Rev1 (Larimer et al. 1989). The consensus is indicated below. (B) The *cut5/rad4* mutation created a new site for *EcoT221* (left). This was verified by hybridization probed with *EcoRI* fragment containing the *cut5⁺* gene (right). Genomic DNAs from wild-type and mutant DNAs digested with *EcoT221* and *EcoRI* gave rise to the fragments with the expected sizes. (C) Construction of bait for two-hybrid screening. Each of five DNA fragments was placed under GAL4DB. The *S. pombe* cDNA library associated with the GAL4 activation domain was employed for screening. Crb2 and Crb3 cDNAs were obtained when the amino-terminal R1R2 and R3 were used as bait, respectively.

with the beads containing only GST showed that neither bound Crb2 nor Crb3.

To examine whether mutant Cut5^{T45M} retains the ability to interact with Crb2, the mutant R1R2 was isolated and fused to the GAL4DB. Two-hybrid interaction between Crb2 and mutant R1R2 was completely abolished at both 26°C and 36°C (Fig. 2D). Interaction of Cut5 with Crb2, at least at the level of the two-hybrid system, thus requires the TH domain.

Crb2 resembles *Rad9* and *p53*-binding protein

An *S. pombe* cosmid that contained the genomic *crb2⁺* gene was isolated with cDNA as the probe. Hybridization to an ordered cosmid bank indicated that *crb2⁺* was located near *nda2⁺* in the left arm of chromosome II (Mizukami et al. 1993). The 9-kb *PstI* fragment containing *crb2⁺* was subcloned from the cosmid (the map shown in Fig. 3A). Plasmid carrying *crb2⁺* (pFE15) fully suppressed the UV sensitivity of *cut5-T401* at 26°C, and this suppression was employed for subcloning of *crb2⁺* (+ indicates suppression). The same plasmid, however, did not suppress the temperature sensitive phenotype of *cut5-T401* at 36°C (data not shown).

Sequencing showed that *crb2⁺* encodes a 778 amino acid protein (predicted molecular mass of 87.5 kD; database accession no. D86478). The near amino-terminal region is required for interacting with Cut5 (data not shown). Database search revealed the highest scores for Rad9 of *S. cerevisiae* (Weinert and Hartwell 1988) and human 53BP1, which interacts with p53 (Iwabuchi et al. 1994). The carboxy-terminal region of Crb2 is ~30% and 25% identical to that of Rad9 and 53BP1, respectively. Crb2 is also similar to the carboxyl terminus of BRCA1, a mammary cancer gene, and other repair and cancer-related genes (Koonin et al. 1996; Callebaut and Morion 1997). A common domain termed BRCT was proposed from detailed sequence analyses (Bork et al. 1997); a number of damage-responsive proteins including Cut5 also contained the BRCT motif (Fig. 3D). The BRCT-containing domain in BRCA1 is implicated in transcriptional activation.

Crb3 is a WD repeat containing protein

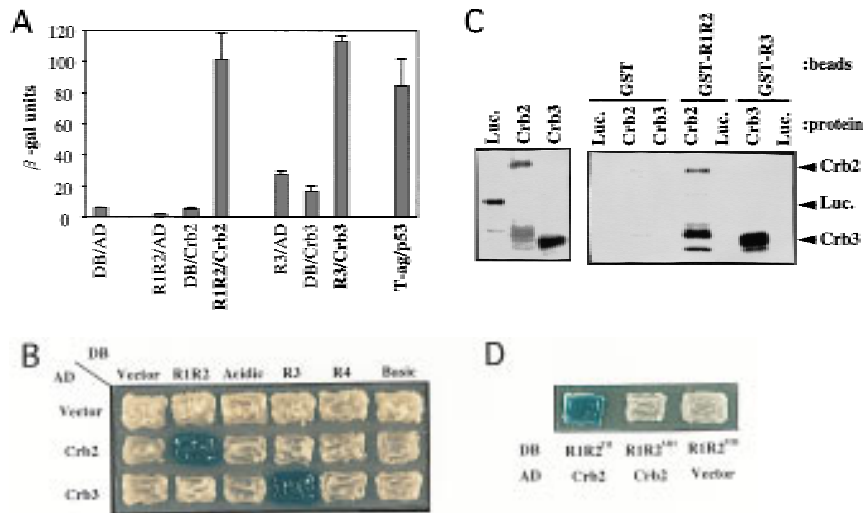
The nucleotide sequence of Crb3 cDNA has been determined (database accession no. D45883): *crb3⁺* encodes a protein with the WD repeats (Neer et al. 1994) partly resembling β -transducin (Fig. 4). The predicted sequence contains 446 amino acids (calculated molecular mass of, 49.5 kD) with 6 WD repeats. It is an essential protein for viability and may be implicated in the G₁/S progression (T. Matsusaka and M. Yanagida, unpubl.). Structure and functional analyses of the *crb3⁺* gene will be described elsewhere.

Identification and localization of *Crb2*

Rabbit antibodies against Crb2 were affinity-purified for detection of Crb2 in *S. pombe* extracts. Diffused immunoblot bands were obtained at the position of ~100–110 kD (Fig. 5A, lane 2), the intensity of which increased in cells carrying plasmid pCRB2 (lane 3), but which were absent in Δ *crb2* strain (lane 1). These diffuse bands may be caused by post-translational modification (see below).

Jellyfish green fluorescence protein (GFP) was tagged to the amino-terminus of Crb2 under a moderate promoter REP41 and used to determine intracellular localization of Crb2 (Figure 5B). The nuclear chromatin region (verified by double stain with DAPI; data not

Figure 2. Crb2 and Crb3 interact with Cut5. (A) β -Galactosidase activities were measured for different combinations. (DB) GAL4 DNA-binding domain; (AD) GAL4 activation domain; (R1R2) the R1R2 region of Cut5; (R3) the central R3 region of Cut5; (T-ag) large T-antigen; (p53) human tumor suppressor. (B) Pairwise combinations were examined. The interactions between Crb2 and R1R2, and between Crb3 and R3, are positive (blue). (C) Binding of Crb2 and Crb3 with Cut5 in vitro. The reticulolysate translation system was employed to synthesize luciferase, Crb2, and Crb3 by use of [³⁵S]methionine (left). GST-R1R2 and GST-R3 made in bacterial cells were purified by association with glutathion-beads. Beads were then incubated with labeled luciferase, Crb2 or Crb3, followed by washing of the beads. ³⁵S-labeled proteins bound to beads were analyzed by SDS-PAGE and autoradiography (right). (D) The mutant R1R2 DNA containing M⁴⁵H instead of T⁴⁵H failed to interact with Crb2 in the two-hybrid method at 26°C and 36°C.



shown) showed green fluorescence when the promoter was repressed in the presence of thiamine (+Thi). When Crb2 was mildly overproduced in the absence of thiamine (-Thi), the same nuclear region was intensely fluorescent. Hence, Crb2 appears to be a nuclear chromatin protein like Cut5 (Saka et al. 1994). We have been unsuccessful in detecting the stable complex formation between Cut5 and Crb2 in *S. pombe* extracts, however. Immunoprecipitation by anti-cut5 antibodies was performed under different buffer conditions. Cut5 was clearly detected in the precipitates, whereas Crb2 was not (data not shown). Conversely, Crb2 was immunoprecipitated by anti-crb2 antibodies, but Cut5 was not detected in the precipitates.

Hypermodification of Crb2 upon irradiation

We examined whether Crb2 was further modified after cells were UV irradiated (100 J/m²) at 26°C. Additional upper bands (apparent molecular mass of 120–150 kD) became visible, greatly intensified after 1–2 hr, and diminished after 4–5 hr (Fig. 5C, top). This transient band-shift clearly indicated the occurrence of strong post-translational modification in Crb2 after irradiation. The intensity of 74-kD Cut5 increased (approximately two-fold) after irradiation (Fig. 5C; second panel).

Chk1 kinase has been reported to be transiently phosphorylated in UV irradiated cells (Walworth and Bernards 1996). We compared the timing of Crb2 band-shift with that of Chk1 phosphorylation by immunoblot with anti-HA antibodies and a strain integrated with the HA-tagged *chk1*⁺ gene (a kind gift of Dr. N. Walworth, Robert Wood Johnson Medical School, Piscataway, NJ; this strain was also used to obtain data for Crb2 modification described above). The upper phosphorylated band of

Chk1 was seen at approximately the same timing (Fig. 5C, third panel) as Crb2 modification. Cdc2 detected by anti-PSTAIRE antibodies was shown as control (Fig. 5C, bottom panel). UV irradiation thus induced a change in Crb2 during the arrest of cell cycle (see below).

We examined whether this 120- to 150-kD super-band shift required the presence of other damage checkpoint genes. Extracts of wild type, *cut5-T401*, Δ *chk1*, *rad1-1*, Δ *rad3*, Δ *rad9*, Δ *rad17*, and Δ *rad26* were prepared before (-) and 1 hr after (+) UV irradiation at 26°C and immunoblotted with anti-crb2 antibodies (Fig. 5D). The 120- to 150-kD band shift of Crb2 after irradiation was observed in Δ *chk1* deleted cells and also in *cut5-T401* (the level in *cut5* somewhat reduced) but not in other checkpoint mutants, suggesting that the damage-sensing checkpoint genes, Rad1, Rad3, Rad9, Rad17, and Rad26, were needed to modify Crb2 upon irradiation.

We wanted to know whether the band shift of Crb2 was produced by phosphorylation, and performed phosphatase treatment of UV irradiated cell extracts as described in Walworth and Bernards (1996) at 30°C and 0°C. The intensity of the unmodified sharp 100-kD band clearly increased after the phosphatase treatment at 30°C (Fig. 5E, second lane). The control Chk1 was also dephosphorylated after phosphatase treatment. The upper 110-kD and 120- to 150-kD bands of Crb2 thus formed at least partly because of phosphorylation. Crb2 in nonirradiating cells thus appeared to be already phosphorylated and this phosphorylation was greatly enhanced further following UV irradiation. This hypermodification of Crb2 is not caused by the cell-cycle arrest, because it did not occur in G₂-arrested *cdc25* mutant cells (data not shown). UV-induced DNA damage is probably the direct cause of this hypermodification.

To know whether Crb2 and Cut5 were present in mo-

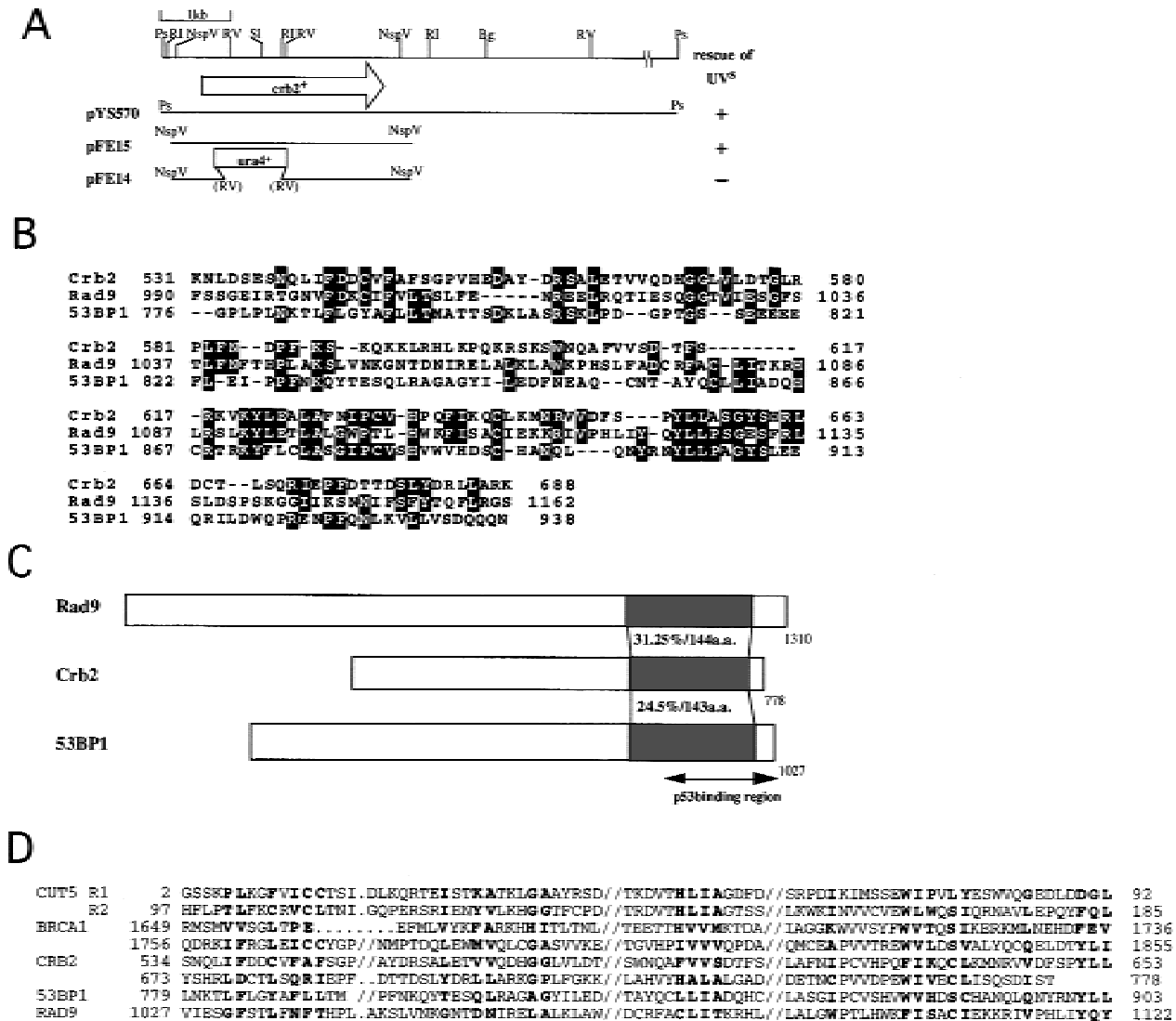


Figure 3. Crb2 suppressing the UV sensitivity of *cut5* resembles yeast Rad9 and human 53BP1 and BRCA1. (A) A cosmid containing *crb2+* was obtained by the probe of Crb2 cDNA. Plasmid carrying *crb2+* was subcloned by its ability to suppress the UV phenotype of *cut5* mutant (indicated by +). pFE15 contained the full-length *crb2+* gene. pFE14 was used for gene disruption. The accession no. for Crb2 is D86478. (B, C) The carboxyl terminus of Crb2 resembles that of yeast Rad9 and human 53BP1. Identical amino acids are boxed. (D) The BRCT motifs present in Cut5, Crb2, BRCA1, 53BP1, and Rad9 are shown (based on Bork et al. 1997). Thick characters represent identical or similar amino acids.

meric or oligomeric complexes, sucrose gradient centrifugation of *S. pombe* extracts was run to determine the sedimentation profiles of Crb2 and Cut5 (Fig. 5F). Cell extracts were first centrifuged at 14,000 rpm for 20 min, and the supernatants were overlaid on the top of a 15%–40% linear gradient, followed by centrifugation at 40,000 rpm for 12 hr. A broad peak ranging from 20S to the near bottom of the centrifugal tube (>50S) was obtained by immunoblot with anti-*crb2* antibodies (Fig. 5F, top panel, α -Crb2), suggesting that Crb2 was associated with relatively large heterogeneously sized particles in extracts. For Cut5, the native 74-kD band was also present in the heavy fractions, whereas the cleaved 61-kD band (Saka et al. 1974) sedimented much more slowly (α -Cut5). The cleavage of 74-kD Cut5 occurs during ex-

tract preparations and centrifugations, and is difficult to control; the cleaved 61-kD Cut5 band is enriched in slowly sedimenting fractions. Chk1 kinase sedimented very broadly from the top to the bottom of sucrose gradient (α -HA). Fractions of Crb2, Cut5, and Chk1 thus sedimented as large structures though they did not form any cosedimenting peak.

Sedimentation profiles in cell extracts prepared 1 hr after UV irradiation (100 J/m^2) differed strikingly from those in nonirradiated cells. The modified forms of Crb2 sedimented very slowly at $\sim 3\text{--}5\text{S}$ (Fig. 5F, bottom panel, α -Crb2), and the upper band of Chk1 was also present in the slowly sedimenting fractions. Cut5 showed a broad profile, with a small fraction also present in the slowly sedimenting fractions. These results strongly suggested

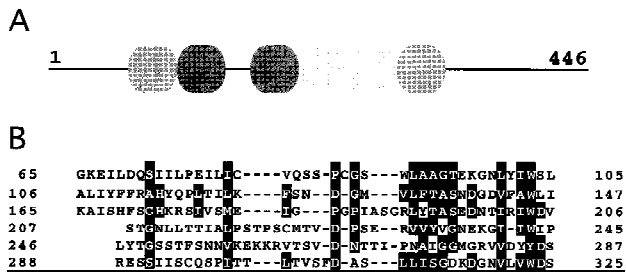


Figure 4. The amino acid sequence of Crb3 protein. Six WD repeats are present in Crb3 protein, which contains 446 amino acids. (A) Location of the WD repeats is schematized; (B) the repeats are aligned. Identical amino acids are boxed. The accession no. for the database is D45883.

that the modified populations of Cut5, Crb2, and Chk1 were dissociated from the larger complexes after UV irradiation. We did immunoprecipitation of the upper slowly-sedimenting fractions after UV irradiation using antibodies against Crb2, Cut5, and Chk1, and could detect respective immunoprecipitated proteins, but were not able to detect coimmunoprecipitation with others under the conditions employed (data not shown).

crb2 null is sensitive to UV and HU

The genomic copy of *crb2*⁺ was disrupted (Fig. 6A, Materials and Methods). Resulting heterozygous diploids produced all four spores that were viable in each tetrad, while Ura⁺ and Ura⁻ segregated 2 : 2. The *crb2*⁺ gene is thus not essential for viability. The gene disruption was confirmed by genomic Southern hybridization of haploid segregants (Fig. 6B) and the lack of protein confirmed by immunoblotting (Fig. 5A, lane 1). Haploid *crb2* null cells (designated Δ *crb2*) divide normally as do wild type at both 26°C and 36°C in minimal or rich culture medium, with a slightly longer generation time (3.4 hr) than wild type (2.9 hr at 26°C in YPD). Δ *crb2* cells showed no anomaly in DNA content as determined by FACScan analysis (data not shown).

We found that Δ *crb2* deletion was both UV and HU sensitive (Fig. 6B,C,D). It was more UV sensitive than *cut5* (Saka et al. 1993). This UV hypersensitivity of Δ *crb2* was suppressed by plasmid pCRB2 but not by pCUT5 (Fig. 7C). Δ *crb2* was also HU sensitive and its sensitivity was similar to that of *cdc2-3w* (Fig 6D).

UV damage checkpoint is abolished in Δ crb2

We examined whether UV-induced damage checkpoint was retained in Δ *crb2* cells. When wild-type cells were irradiated with UV (100 J/m² at 26°C), their mitotic entry was greatly delayed, and cell number increase was arrested (Fig. 7A, WT). The septation index (SI) decreased for ~2 hr because of the activation of damage checkpoint control (Al-Khodairy and Carr 1992; Rowley et al. 1992). The damage checkpoint mutant *rad1-1*, however, did not show such delay nor a decrease in SI. We found that

Δ *crb2* also lost the delay, basically showing the identical damage phenotype to that of *rad1-1* (Fig. 7A).

UV-irradiated wild-type cells are known to be elongated with the single nucleus (Fig. 6B, 2 hr after irradiation). In sharp contrast, after irradiation Δ *crb2* and *rad1-1* cells were divided and the septated cells, with occasional cut phenotype, were frequently seen. The deletion of *crb2*⁺ thus led to loss of the cell-cycle arrest in response to UV irradiation. The UV sensitivity of the double mutant *cut5* Δ *crb2* was identical to Δ *crb2*, and its UV-induced checkpoint control was also abolished (data not shown).

Polymerase mutant-induced arrest abolished in Δ crb2

We wanted to know whether the cell-cycle arrest phenotypes of polymerase mutants at 36°C were maintained in the absence of *crb2*⁺. Double-mutants were constructed by crossing of Δ *crb2* with *cdc6-121*, *swi7-H4*, or *cdc20-M10* (Singh and Klar 1993; Francesconi et al. 1995; Murakami and Okayama 1995). These single polymerase mutants (α , δ , and ϵ) were arrested at 36°C and the SI decreased (Fig. 7C). The double-mutants, however, were not arrested at 36°C, and the level of SI remained the same after the temperature-shift. The double-mutant cells frequently displayed the cut phenotype at 36°C for 4 hr (Fig. 7D, right), whereas single mutant cells were elongated with the single nucleus (left). Checkpoint control was thus lost if the *crb2*⁺ gene was disrupted in the polymerase mutants at 36°C. The phenotype of the other double-mutant with *cdc17-K42* (defective in DNA ligase) was also examined. The arrest phenotype was abolished in the double-mutant (data not shown). We concluded from these results that Crb2 was required for the cell-cycle arrest induced by defects in the replication machinery enzymes.

Nucleotide pool-dependent replication checkpoint retained in Δ crb2

We then determined whether the HU-induced arrest was maintained in Δ *crb2*. In sharp contrast to the results with polymerase mutations, Δ *crb2* cells were arrested in the presence of HU (+HU) at 36°C (Fig. 8A). Δ *crb2* cells in the presence of HU were elongated with the single nucleus as wild-type cells, and did not show the *cut* phenotype (right panel). The SI index was strikingly lowered (data not shown). Similar results were obtained at 26°C (data not shown). The HU-induced cell-cycle arrest was thus completely retained in Δ *crb2*.

Basically the same result was obtained for the double mutant Δ *crb2* temperature sensitive *cdc22*. The *cdc22*⁺ gene encodes the large subunit of ribonucleotide reductase (Gordon and Fantes 1986; Fernandez-Sarabia et al. 1993), and is known to be directly inhibited by HU. The double mutant Δ *crb2* *cdc22* grew normally at 26°C, and was arrested at 36°C, displaying the identical arrest phenotypes of HU-added Δ *crb2* or single *cdc22* mutant cells (Fig. 8B). These results established that the cell-cycle ar-

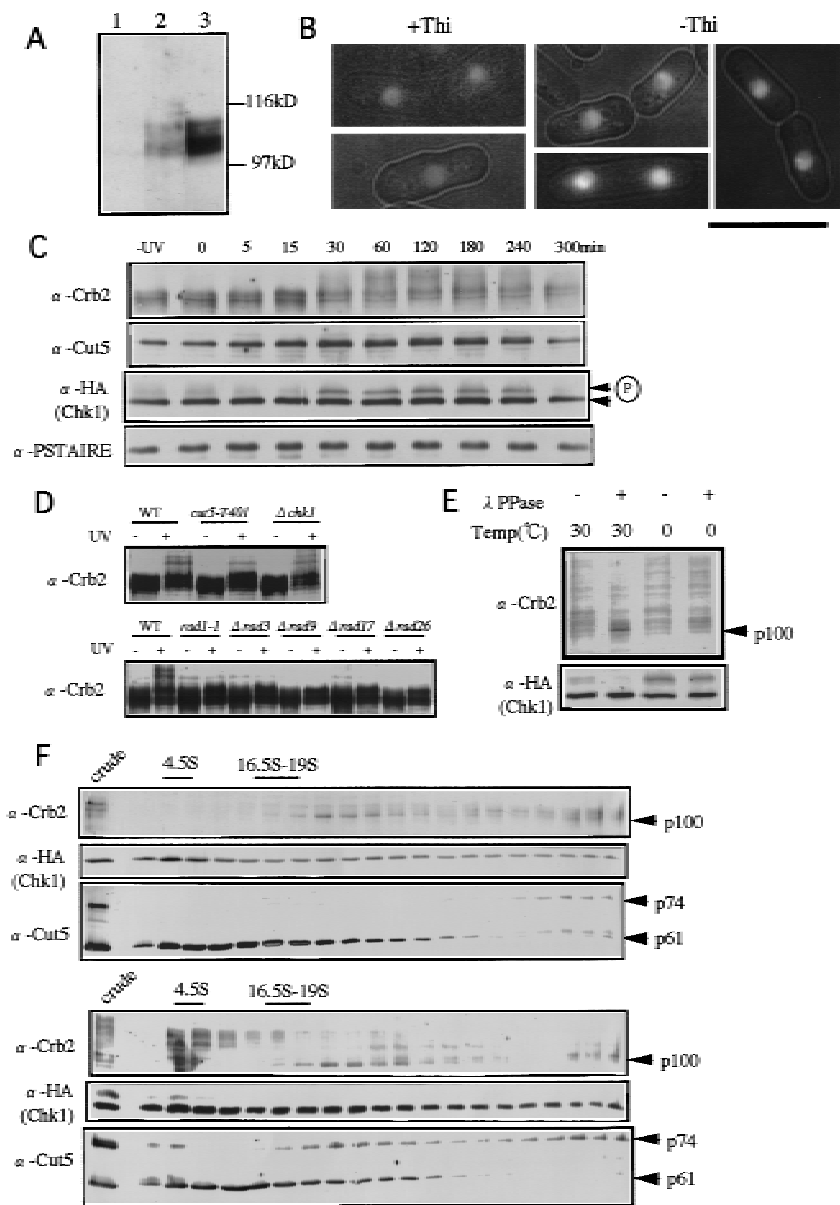


Figure 5. Crb2, a putative nuclear phosphoprotein, alters sedimentation profile upon UV damage. (A) Polyclonal antibodies made against the fusion protein of Crb2 were used for detecting Crb2 in *S. pombe* extracts. The diffused 100–110-kD band was detected (lane 2), the intensity of which increased in cells carrying pCRB2 (lane 3). No band detected in $\Delta crb2$ (lane 1). (B) GFP-tagged Crb2 was placed under a mild inducible promoter REP41. Cells cultured in the presence of thiamine (+Thi, the promoter repressed) or the absence (–Thi, derepressed) were observed. Nuclear fluorescence of cells in +Thi was weak, and photographically enhanced. The contour of cell shape was produced with the merged exposures of fluorescence and transmission light source. Bar, 10 μ m. (C) *S. pombe* cells integrated with HA-tagged *chk1*⁺ (Walworth and Bernards 1996) were irradiated with UV (100 J/m²). Extracts were prepared and immunoblotted by use of antibodies against Crb2, Cut5, HA, and Cdc2 (PSTAIRE). Modification of the Crb2 band occurred ~30 min and was clearly seen 60 min after irradiation. The upper band of Chk1, intensified from 30 to 240 min, represents phosphorylation (Walworth and Bernards 1996). (D) Immunoblot patterns of wild type (WT), *cut5-T401*, $\Delta chk1$, *rad1-1*, $\Delta rad3$, $\Delta rad9$, $\Delta rad17$, and *rad26* extracts before (–) and after (+) UV irradiation with anti-crb2 antibodies are shown. Cells irradiated by UV (100 J/m²) were cultured at 26°C for 1 hr. The upper band was seen in wild-type and $\Delta chk1$ but not in other mutant extracts. (E) Extracts of the UV-irradiated cells were treated with (+) or without (–) λ protein phosphatase at 0°C or 30°C. Upper modified bands of Crb2 and Chk1 were diminished, and the lower bands at 100 and 56 kD, respectively, were intensified after treatment with phosphatase at 30°C. (F) Centrifugation patterns of nonirradiated (top) and UV irradiated (bottom) cell ex-

tracts run in a 15–40% sucrose gradient at 40,000 rpm for 12 hr. Fractions collected were immunoblotted with antibodies against Crb2, HA (Chk1), and Cut5. After irradiation, the upper bands of Crb2 and Chk1 were clearly seen in the top, slowly sedimenting fractions. A small population of Cut5 was also shifted to the slowly sedimenting fractions. The positions of the control markers (4.5S and 16.5–19S) are indicated by the bars. The immunoblot patterns of crude extracts are shown in left lanes.

rest induced by HU or *cdc22* mutation did not require Crb2.

Interaction of *crb2*⁺ with other checkpoint genes

We examined whether the UV sensitivity of $\Delta crb2$ can be suppressed by overexpression of other checkpoint genes. For this purpose, plasmids carrying *crb2*⁺, *cut5*⁺, *rad1*⁺, *rad3*⁺, *rad9*⁺, *rad17*⁺, *rad26*⁺, or *chk1*⁺/*rad27*⁺ (Al-Khodairy and Carr 1992; Rowley et al. 1992; Sheldrick and Carr 1993; Al-Khodairy et al. 1994) placed at the downstream of the moderate promoter REP41 (a gener-

ous gift of Dr. A.M. Carr, Medical Research Council Cell Mutation Unit, University of Sussex, Brighton, UK, except for the first two strains constructed in the present study) were introduced into $\Delta crb2$, *cut5-T401* and $\Delta chk1$ strains, and the UV-sensitive phenotype of transformants was investigated. The results are summarized in Table 1. None of the damage-sensing checkpoint genes (*rad1*⁺, *rad3*⁺, *rad9*⁺, *rad17*⁺, *rad26*⁺) could suppress the UV sensitivity of $\Delta crb2$, *cut5-T401*, and $\Delta chk1$. In contrast, moderate overexpression of Chk1 kinase, a checkpoint signal transmitter, suppressed the UV phenotype of $\Delta crb2$ (see Fig. 6C) and *cut5-T401*. Moreover, pCRB2

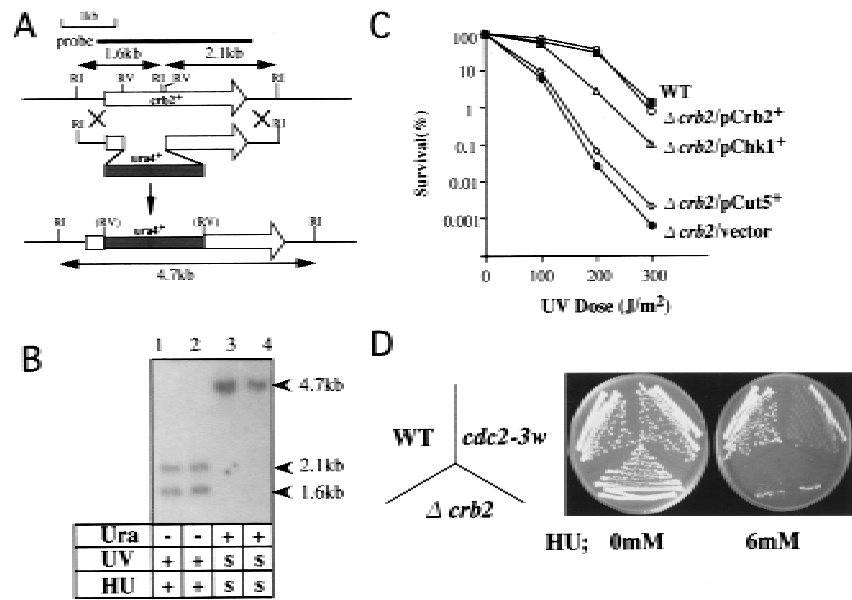


Figure 6. Deletion of *crb2⁺* is sensitive to UV and HU. (A) Gene disruption of *crb2⁺*. Resulting *crb2::ura4⁺* was used for transformation after linearization with *EcoRI*. (B) Disrupted *Ura⁺* segregants were viable. 4.7-kb *EcoRI* fragment was detected in the disrupted cells when probed with the *crb2⁺* DNA. S, hypersensitive to UV or HU. (C) The deletion strain of *crb2⁺* ($\Delta crb2$) is hypersensitive to UV. This UV sensitivity was suppressed by pCRB2 and pCHK1, but not by pCUT5. (D) Colony of $\Delta crb2$ was scarcely formed on YPD containing 6 mM HU at 26°C. (WT) wild type; (*cdc2-3w*) a HU sensitive *cdc2* allele (Enoch et al. 1992).

suppressed the UV sensitivity of *cut5*, but pCUT5 suppressed neither $\Delta crb2$ nor $\Delta chk1$. These results suggested that Crb2 played an upstream role for Chk1 and acted at the downstream of, or parallel to, other checkpoint gene products in the UV damage-signaling pathway (see Discussion).

Interactions of Chk1 with Crb2 and Cut5

More evidence for genetic interactions among Crb2, Cut5, and Chk1 was obtained in following experiments. HU, as well as UV, sensitivities were examined for $\Delta crb2$ or $\Delta chk1$ carrying multicopy vector plasmid (1, 2 in Fig. 9A), pCHK1(3,4), pCRB2 (5,6), or pCUT5 (7,8). These plasmid-borne cells were spotted after dilution, and cultured at 26°C with the addition of HU to the medium (right, HU) or the pretreatment by UV irradiation (100 J/m², UV, middle, +UV) as control. pCHK1 suppressed the HU-phenotype of $\Delta crb2$, whereas pCRB2 did not suppress that of $\Delta chk1$.

We wanted to know whether the loss of UV-induced checkpoint arrest in $\Delta crb2$ could be restored by moderate overproduction of Chk1 (Fig. 9B). REP41, a moderate inducible promoter, was derepressed in the absence of thiamine (-Thi). The ability to arrest cells following UV damage was restored in $\Delta crb2$ cells that overexpressed Chk1 (-Thi; shown by a temporal decrease of the SI) but not in cells without overproduction (+Thi), showing that moderate overexpression of Chk1 also suppressed the loss of damage checkpoint. Hence, the UV-induced checkpoint is partly restored in $\Delta crb2$ mutant by overproducing Chk1, suggesting that the gene product other than Crb2 might be able to activate Chk1, but in a way much less efficiently.

We found that phosphorylation of Chk1 after UV irradiation required functional Crb2. The upper band of Chk1 formed 1 hr after UV irradiation at 26°C (+UV) in

wild type and *cut5* mutant cells but did not in $\Delta crb2$ (Fig. 9C, +UV); the intensity of Chk1 upper band in *cut5* mutant was significantly reduced, however. However, after UV irradiation, Crb2 may become essential for cells to survive by sending a signal for Chk1 phosphorylation, that leads to the accumulation of activated Chk1 (Walworth and Bernards 1996).

Moreover, strong two-hybrid interactions existed between Chk1 and Crb2 and between Chk1 and the central region (Acidic R3R4) of Cut5 (Fig. 9D). The degree of interactions were the highest for Chk1 and Crb2, whereas the interaction between Chk1 and the region of Cut5 spanning from the acidic to R3R4 region (acidicR3R4) was high. No two-hybrid interaction was found between Chk1 and R1R2 of Cut5, however. The interaction between Chk1 and R3R4 of Cut5 was relatively weak. Immunoprecipitation experiments, however, did not show the presence of a detectable stable complex. Neither Chk1 and Crb2, nor Chk1 and Cut5, existed as the stable complex in cell extracts.

Discussion

We report in this study (1) identification and characterization of *crb2⁺*, an *S. pombe* checkpoint gene, which is required for both DNA damage and replication machinery checkpoint; and (2) interactions among three checkpoint gene products Cut5, Crb2, and Chk1 for proper maintenance of the genome. Cut5 and Crb2 contain BRCT-motif present in a number of damage-responsive proteins, whereas Chk1 is a protein kinase. Although the stable complex has not been detected in *S. pombe* extracts, evidence for their putative direct interaction is presented. Functions of Crb2 and Cut5 are distinct, but overlapping. Crb2 is responsive to the DNA damage, whereas Cut5 responds to the impaired precursor nucleotide supply for replication. Both Crb2 and Cut5,

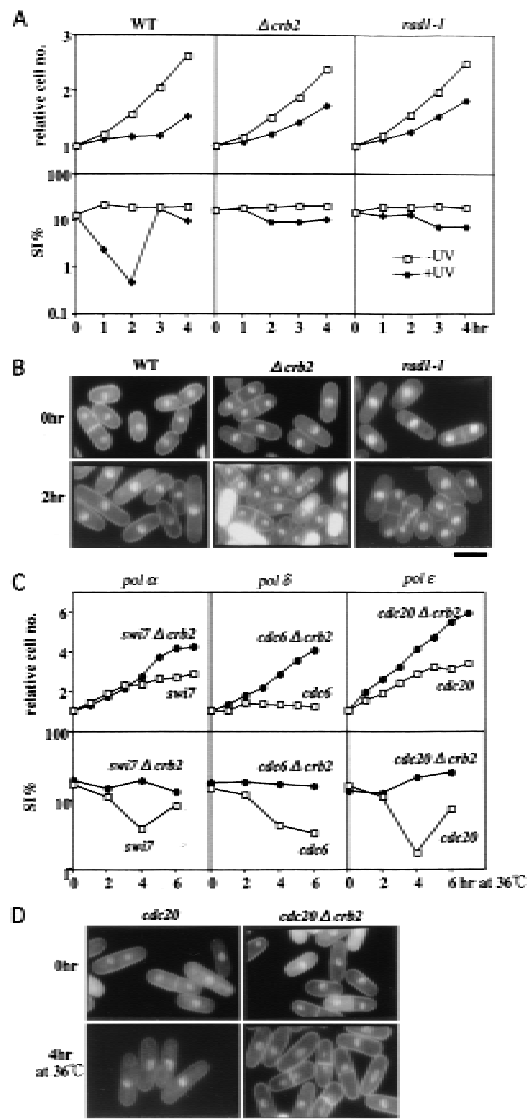


Figure 7. Damage- and RC mutation-induced checkpoint abolished in $\Delta crb2$. (A) Wild-type (WT), $\Delta crb2$, and *rad1-1* cells were irradiated by UV (+UV, 100 J/m²) at 26°C, and the cell number and the septation index (SI%) were measured. The control of no irradiation (-UV) is also shown. (B) Cells 0 and 2 hr after irradiation were stained by DAPI. Bar, 10 μ m. (C) temperature sensitive strains *swi7*, *cdc6*, and *cdc20*, and double mutants *swi7- $\Delta crb2$* , *cdc6- $\Delta crb2$* , and *cdc20- $\Delta crb2$* were grown at 26°C and then transferred to 36°C. The cell number and the SI% were measured. (D) Single *cdc20* and double *cdc20 $\Delta crb2$* cultured at 36°C for 0 and 4 hr were stained by DAPI. Bar, 10 μ m.

however, are required for the checkpoint response to defects in DNA polymerases and ligase. Cut5 and Crb2 may act as sensors that sense damage and/or replication defect, and generate signals to activate Chk1 for regulating Cdc2. Thus, Cut5, Crb2, and Chk1 may form a checkpoint sensor-transmitter pathway to arrest the cell cycle.

The present paper also shows that replication check-

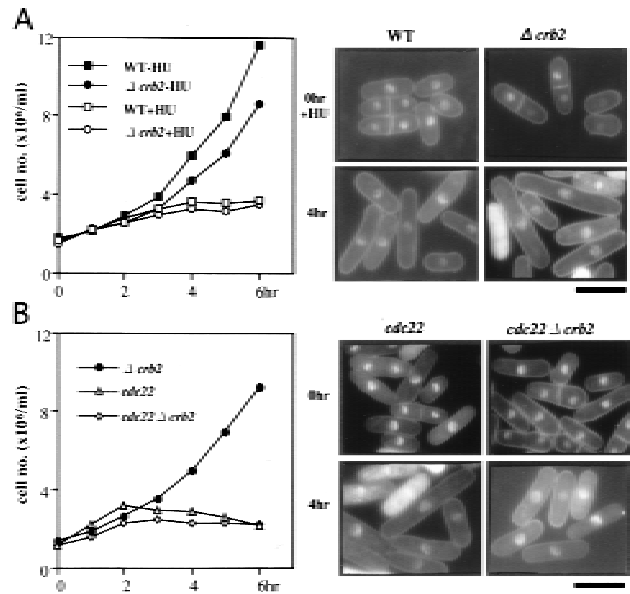


Figure 8. HU- and *cdc22*-induced checkpoint occurs in $\Delta crb2$. (A) Wild type (WT) and $\Delta crb2$ were cultured at 36°C in the presence (+) or the absence (-) of HU, and their cell numbers were counted (left). Cells were stained by DAPI (right). The cell number of wild type and $\Delta crb2$ did not increase in the presence of HU. Cell elongation and single nuclear phenotype was observed for both wild-type and $\Delta crb2$ strains in the presence of HU. Bar, 10 μ m. (B) Single mutants $\Delta crb2$ and temperature sensitive *cdc22*, and the double mutant *cdc22 $\Delta crb2$* were cultured at 36°C. The double mutant showed the *cdc* phenotype and no cut phenotype. Cells were stained by DAPI after 4 hr at 36°C. Both *cdc22* and *cdc22 $\Delta crb2$* showed the checkpoint arrest at 36°C. Bar, 10 μ m.

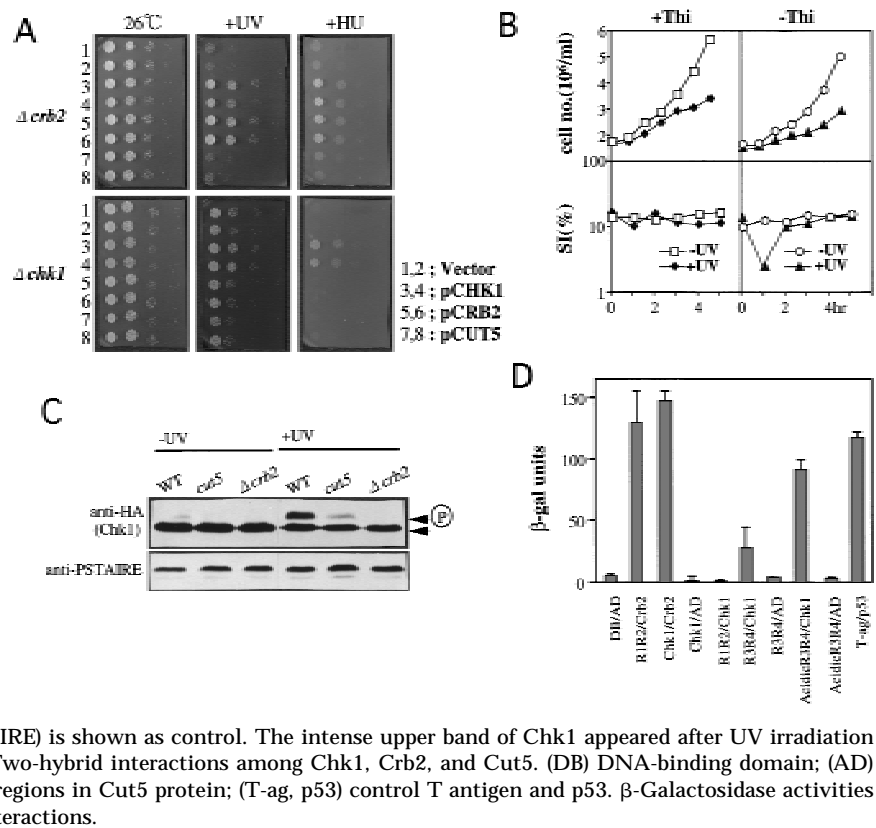
points present in *S. pombe* may be categorized into two classes: one caused by the defect in replication machinery (mutations in polymerases and ligase), and the other caused by the inhibition of normal nucleotide supply (HU and *cdc22* mutation). Crb2 is required only for the former, but Cut5 is necessary for both (Saka et al. 1994a,b). A possible explanation for this difference is that the mutant polymerase and ligase enzymes may

Table 1. UV sensitivity of $\Delta crb2$ suppressed by plasmid *pCHK1* carrying the *chk1⁺/rad27⁺* gene

Mutant	Plasmids			
	pCRB2	pCUT5	pCHK1	pRAD1 pRAD3 pRAD9 pRAD17 pRAD26
$\Delta crb2$	+	-	+	-
<i>cut5-T401</i>	+	+	+	-
$\Delta chk1$	-	-	+	-

(+) Normal sensitivity; (-) hypersensitivity; ($\Delta crb2$, $\Delta chk1$) deletion mutants of *crb2⁺* and *chk1⁺*, respectively.

Figure 9. Chk1 interacts with Crb2 and Cut5. (A) Elevated gene dosage of *chk1⁺* suppresses the UV- and HU-sensitive phenotypes of Δ *crb2*. Each of the four transformant strains of Δ *crb2* (top) and Δ *chk1* (bottom) strains carrying plasmids (1 and 2, vector; 3 and 4, pCHK1; 5 and 6, pCRB2; 7 and 8, pCUT5) were spotted after dilution, and incubated at 26°C (left). Identical cells were also incubated after UV irradiation (middle) or in the presence of HU (right). The plates did not contain thiamine. (B) Δ *crb2* null cells carrying plasmid with the *chk1⁺* gene under a moderate inducible promoter REP41 were precultured at 26°C for 18 hr in the presence of thiamine (+Thi; repressed condition) or the absence (-Thi; derepressed condition), and then irradiated by UV and cultured for 4 hr at 26°C. The cell number and the septation index were measured. (C) Phosphorylation of Chk1 does not take place in Δ *crb2* on UV irradiation. Three strains (wild type, *cut5-T401* and Δ *crb2*) integrated with *chk1⁺* tagged with the HA antigen (Walworth and Bernards 1996) were irradiated with UV (+UV) and cultured at 26°C for 1 hr. The level of Cdc2 (anti-PSTAIRE) is shown as control. The intense upper band of Chk1 appeared after UV irradiation in the wild type was absent in Δ *crb2*. (D) Two-hybrid interactions among Chk1, Crb2, and Cut5. (DB) DNA-binding domain; (AD) activation domain; (R1, R2, acidic, R3, R4) regions in Cut5 protein; (T-ag, p53) control T antigen and p53. β -Galactosidase activities were assayed for each pair of two-hybrid interactions.



synthesize bad or damaged DNAs that are sensed by Crb2, whereas such bad DNAs are not made in HU-arrested cells. This implies that Crb2 may monitor the damaged DNAs accumulated during the S phase, consistent with the concept that DNA damage causes replication to be blocked (Paulovich and Hartwell 1995).

Chk1 appeared to act at the downstream of Crb2 and Cut5 as a checkpoint signal transmitter. This hypothesis explains a number of results presented in this paper (Table 1; Figs. 5, 6, and 9). Consistent with this hypothesis, Chk1 is required not only for the damage checkpoint (Walworth et al. 1993; Al-Khodairy et al. 1994) but also for the replication checkpoint (Francesconi et al. 1997). Cdc2 kinase is affected by Chk1, which was recently shown to regulate Tyr15 phosphorylation of Cdc2 through Cdc25 and/or Wee1 (O'Connell et al. 1997; Rhind et al. 1997).

We showed that a fraction of Crb2 was transiently modified, probably phosphorylated, after UV irradiation. In sucrose gradient centrifugation, the modified Crb2 was slowly sedimenting, possibly released from the large complex structures. Similarly, the phosphorylated form of Chk1 kinase, which was reported to be activated by autophosphorylation when damaged (Walworth and Bernards 1996), was also present in the slowly sedimenting fractions. We speculate that damage leads to Crb2 modification, and the modified Crb2 can produce a signal to activate Chk1, possibly by direct interaction. This hypothesis is consistent with two-hybrid interaction, the

suppression of Δ *crb2* by the elevated gene dosage of *chk1⁺* and the absence of Chk1 phosphorylation in Δ *crb2* cells. The occurrence of Crb2 modification induced by damage in Δ *chk1* cells supports the upstream nature of Crb2 to Chk1.

Following UV irradiation, the products of the damage-sensing checkpoint genes (*rad1⁺*, *rad3⁺*, *rad9⁺*, *rad17⁺*, and *rad26⁺*; Al-Khodairy et al. 1994) may act in conjunction with Crb2 to generate the signal for the cell-cycle block. Functional relationship between Crb2 and these five checkpoint genes is unclear. The fact that Crb2 is not modified upon UV irradiation in these *rad* mutants and that plasmids carrying any one of these *rad⁺* genes fail to suppress the UV phenotype of Δ *crb2* cells suggests that Crb2 functions in the downstream of these Rad gene products. Crb2 may be activated by the Rad gene products. It is also possible, however, that Crb2 independently senses damaged DNAs and sends an essential signal for promoting cell-cycle arrest, separately from the Rad genes. A more complex relationship is likely, as pCRB2 can suppress the UV sensitivity of *rad1-1*, but not that of *rad3*, *rad9*, or *rad17* mutants (F. Esashi and M. Yanagida, unpubl.). Crb2 may thus have a close functional link to Rad1. The *S. cerevisiae* Rad17, an *S. pombe* Rad1 homolog, has an exonuclease activity to produce a gap in DNA and plays an important role in the damage checkpoint (Lyddall and Weinert 1995; Nugent et al. 1996).

The predicted amino acid sequence of Crb2 is signifi-

cantly similar to the budding yeast Rad9 (Weinert and Hartwell 1988; Hartwell and Weinert 1989) that acts in the processing of DNA damage for repair (Lydall and Weinert 1995), as a component in the sensor/transducer pathway of UV damage outside of S phase (Navas et al. 1996). However, the similarity between budding yeast Rad9 and fission yeast Crb2 is restricted to the carboxyl termini. Hence, *RAD9* and *crb2⁺* are unlikely to be true homologs. Elevated dosage of the budding yeast *RAD9* or the fission yeast *crb2⁺* gene introduced into the fission yeast Δ *crb2* or the budding yeast *rad9* mutant, respectively, did not suppress the UV sensitivity (S. Mochida and M. Yanagida, unpubl.).

Mammalian proteins involved in oncogenesis, 53BP1 (Iwabuchi et al. 1994) and BRCA1 (Koonin et al. 1996), contain regions similar to the carboxyl termini of Crb2. 53BP1 binds to a tumor suppressor protein p53 by the two-hybrid method, and the carboxyl terminus of 53BP1 is the site for binding to p53 (Iwabuchi et al. 1994). Mutations in BRCA1 lead to familial breast and ovarian cancers. The common carboxyl terminal motifs present in Crb2, Rad9, 53BP1, and BRCA1 (and other related proteins, Koonin et al. 1996) may be the sites for interaction with proteins implicated in the maintenance of the genome in response to damages. It is of considerable interest to determine whether any protein bound to the carboxyl terminus of Crb2 exists in fission yeast, and whether such protein(s) is a transcription factor sharing a property with p53.

During the preparation of this paper, Wilson et al. (1997) reported that one of the fission yeast methylmethane sulfonate (MMS)-sensitive alleles, *rhp9⁺*, was required for the DNA damage checkpoint but not the replication checkpoint. Comparison of the sequences indicated that Rhp9 was identical to Crb2. Though their conclusion apparently differed from ours, the experimental results on *rhp9* null were not inconsistent with ours, because only the replication checkpoint induced by HU was investigated for *rhp9* null. Crb2/Rhp9 may respond to a variety of DNA toxins.

XRCC1, a human repair protein, the amino acid sequence of which partly resembles Cut5, plays a scaffold role in interactions with polymerase β and ligase III (Kubota et al. 1996). Cut5 might also have a scaffold structure, which makes complex regulations possible through protein-protein interactions. The amino-terminal T45, which appears to be necessary for interacting with Crb2, is the temperature sensitive mutation site. Because the mutants were also defective in replication, the amino terminus might also interact with an unidentified protein(s) essential for replication. The central-to-carboxy-terminal region is probably the site for interaction with Chk1 and Crb3. The amino-terminal and the central-to-carboxy-terminal regions contain multiple BRCT motifs (Saka et al. 1994; Bork et al. 1997). As both Cut5 and Crb2 contain BRCT motif, pursuing their protein functions will shed light on the actual role of BRCT motif in maintaining the genome.

It is not a simple matter to explain the reason *cut5* mutants are sensitive to UV because the UV- and 4NQO-

induced checkpoint is maintained in *cut5* mutant at both 26°C and 36°C (Saka et al. 1994a,b; Saka et al., unpubl.). We speculate that the interaction between Cut5 and Crb2 is needed for promoting efficient repair of the damaged DNA. In *cut5/rad4* mutants, where the interaction of Cut5 with Crb2 might be lost even at 26°C, repair replication may become inefficient, leading to UV hypersensitivity. However, Crb2, which is responsible for generating a damage signal for cell-cycle arrest is present. The reason for the HU sensitivity in Δ *crb2* is also unclear because the HU-induced checkpoint is retained in Δ *crb2*. We propose that only Cut5 is required for HU-induced checkpoint and that the Cut5-Crb2 interaction is needed for an unidentified function to facilitate replication under an inhibited nucleotide supply. Under the HU-induced checkpoint arrest, Cut5 may send an arrest signal through a protein other than Crb2 or directly to Chk1. Two-hybrid interaction favors the latter case. To explain the result that both Crb2 and Cut5 are necessary for the cell-cycle arrest by defects in DNA polymerases or ligase, we propose that neither Crb2 nor Cut5 alone can detect the replication defect caused by defects of polymerases or ligase.

It remains to be determined whether the stable complex between Crb2 and Cut5 is actually present in the nucleus in vivo but difficult to detect in *S. pombe* extracts, or if the stable complex never exists in vivo and the interaction is only transient such as is true of a substrate-enzyme relationship. In sucrose gradient centrifugation, fractions of Crb2 and Cut5 were sedimented as large heterogeneous materials that might contain both Crb2 and Cut5. A small fraction of Chk1 was also in the large complex form. The properties of these large complexes are unknown, and whether Cut5, Crb2, and Chk1 exist in the same large structure remains to be determined. Alternatively, slowly sedimenting forms of Crb2, Cut5, and Chk1 were obtained after UV irradiation, and these smaller forms possibly phosphorylated may interact each other in a transient style.

Materials and Methods

Strains and media

Haploid wild-type *S. pombe* *h⁻ 972* and *h⁺ 975* (Gutz et al. 1974) and their derivative mutant strains were used. *cut5* mutants (*cut5-T401*, *cut5-580*, *rad4-116*; Duck et al. 1976; Saka and Yanagida 1993; Samejima et al. 1993), *rad* mutants (*rad1-1*, *rad3::ura4⁺*, *rad9::ura4⁺*, *rad17::ura4⁺*, *rad26::ura4⁺*, and *rad27::ura4⁺* mutants; Al-Khodairy et al. 1994; gifts of Dr. A.M. Carr), *cdc* mutants (*cdc6-121*, *cdc17-K42*, *cdc20-M10*, *cdc22-C1*, Nurse et al. 1976; Gordon and Fantes 1986) and *swi7-H4* (Singh and Klar 1993) mutants were described previously. Rich YPD (2% glucose, 2% polypeptone, and 1% yeast extract) and minimal EMM2 (Mitchison 1970) were employed.

Determination of UV sensitivity

The procedures for examining UV sensitivity were described previously (Saka and Yanagida 1993; Saka et al. 1994). Briefly, *S. pombe* cells diluted were plated and incubated for 30 min, followed by UV irradiation (0–300 J/m²; Stratalinker, Stratagene)

and culturing for several days at 26°C (the permissive temperature for temperature sensitive mutants). The percent septation index was obtained by Calicoflour staining after glutaraldehyde fixation. To study the checkpoint response, exponentially growing *S. pombe* cells were irradiated by 100 J/m².

FACScan analysis, Southern transfer, and sequencing

The procedures for FACScan were described previously (Costello et al. 1986; Saka and Yanagida 1993). Southern hybridization was performed by the procedures described (Saka et al. 1994a). The dye-terminator method was employed for nucleotide sequencing by use of the ABI 373A Sequencing System.

Cell extracts and immunoblotting

Cells were collected and suspended in ice chilled phosphate buffer-saline at pH 7.5 containing 10 mM Na₃N and 50 mM NaF, rapidly frozen by liquid nitrogen and kept at -20°C. Cells were then thawed and suspended at a concentration of 1 × 10⁹/ml in HB buffer (25 mM Tris-HCl at pH 7.5 containing 15 mM MgCl₂, 15 mM EGTA, 0.1% NP-40, 1 mM DTT, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.5 mM Na₃V₀₄, 0.1 mM NaF, 1 mM PMSF). Cells were disrupted by glass beads and centrifuged at 4°C at 14,000 rpm for 20 min. Supernatants were used for immunoblotting. As secondary antibodies, HRP-labeled protein A (200-fold dilution, Bio-Rad) or HRP-labeled sheep anti-mouse antibody (Amersham, 500-fold dilution) was employed.

Two-hybrid screening

The *S. pombe* cDNA library adapted to the two-hybrid system in yeast was purchased from Clontech, Inc. (XL4000AA), and the instruction procedures for screening were followed by use of the HF7c strain. Yeast cells were grown in the SD synthetic medium supplemented with the dropout solution at 30°C. DNAs of different domains of the *cut5*⁺ gene were obtained by amplification by the PCR method (the R1R2 region) or by isolating the restriction fragments (the central acidic domain, R3, R4, and the carboxy-terminal region). These DNAs were ligated in frame with pGBT9 containing the GAL4 DNA-binding domain. All of these plasmids were verified by nucleotide sequencing. The filter assay for β-galactosidase activity was done in accordance with the company's instructions. Strong signals were obtained from colonies by incubating them on the filter for 30 min or up to 2 hr. Liquid assay for the β-galactosidase activity was also done following the company's instructions by use of the SFY526 strain.

Preparation of GST fusion proteins and in vitro binding assay

*Nde*I-*Bam*HI fragment of *cut5*⁺ containing the R1R2 region was blunt-end ligated at the *Xho*I site of plasmid pGEX-KG (Guan and Dixon 1991), whereas *Bam*HI-*Sal*I fragment containing the R3 region was inserted at the same site of pGEX-KG. Resulting plasmids pYS583 and pR308, respectively, were used for producing fusion proteins that were dissolved in the presence of 8 M urea, followed by successive dialysis in PBS containing reduced concentrations of urea and 10 mM DTT. Resulting GST proteins in the absence of urea were incubated with glutathione beads. In vitro translation of Crb2 and Crb3 proteins was performed with the Promega TNT Coupled reticulocyte lysate system and [³⁵S]methionine (ICN). Luciferase, Crb2, and Crb3 proteins were in vitro translated and mixed with the beads bound to GST-R1R2 or GST-R3, and incubated at 4°C for 1 hr. Beads

were washed three times by 10 volumes of 20 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml of leupeptin, 0.5 μg/ml of aprotinin, followed by SDS-PAGE and autoradiography by use of the Amersham Amplify system.

Sucrose gradient centrifugation and phosphatase treatment

The procedure of sucrose gradient centrifugation was followed to that reported previously (Yamashita et al. 1996; Yamada et al. 1997). Extracts prepared from each 4 × 10⁸ cells nonirradiated or irradiated with UV (100 J/m²) and cultured 1 hr at 26°C were run at 40,000 rpm for 12 hr at 4°C with a SW50.1 rotor. BSA (4.5S) and thyroglobulin (16.5-19S) were used as the markers for the S values. The procedure of Walworth and Bernards (1996) was followed for treatment of Crb2 and Chk1 in cell extracts by λ protein phosphatase (New England Biolab).

GFP tagging and immunofluorescence microscopy

The jellyfish GFP gene was introduced to the amino-terminal site of the *crb2*⁺ gene by creating a new *Bam*HI site at the amino-terminus. A mild inducible promoter REP41 (Maundrell 1990) was placed upstream of the fused GFP-Crb2 gene by use of pGFT41 (Heim et al. 1995). Resulting plasmid was introduced into Δ*crb2* strain and fluorescence was observed in the presence (repressed) or the absence (induced) of thiamine (Nabeshima et al. 1995). Cells cultured in the presence or the absence of thiamine carrying pGFT-Crb2 were observed by blue light without fixation.

Gene disruption

The procedure of gene disruption was as reported previously (Rothstein 1983).

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