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The Drosophila *mus101* Gene, Which Links DNA Repair, Replication and Condensation of Heterochromatin in Mitosis, Encodes a Protein With Seven BRCA1 C-Terminus Domains

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ABSTRACT

The *mutagen-sensitive-101* (*mus101*) gene of *Drosophila melanogaster* was first identified 25 years ago through mutations conferring larval hypersensitivity to DNA-damaging agents. Other alleles of *mus101* causing different phenotypes were later isolated: a female sterile allele results in a defect in a tissue-specific form of DNA synthesis (chorion gene amplification) and lethal alleles cause mitotic chromosome instability that can be observed genetically and cytologically. The latter phenotype presents as a striking failure of mitotic chromosomes of larval neuroblasts to undergo condensation of pericentric heterochromatic regions, as we show for a newly described mutant carrying lethal allele $mus101^{lcd}$. To gain further insight into the function of the Mus101 protein we have molecularly cloned the gene using a positional cloning strategy. We report here that *mus101* encodes a member of the BRCT (BRCA1 C terminus) domain superfamily of proteins implicated in DNA repair and cell cycle checkpoint control. Mus101, which contains seven BRCT domains distributed throughout its length, is most similar to human TopBP1, a protein identified through its *in vitro* association with DNA topoisomerase IIβ. Mus101 also shares sequence similarity with the fission yeast Rad4/Cut5 protein required for repair, replication, and checkpoint control, suggesting that the two proteins may be functional homologs.

MORE than 30 genes of *Drosophila melanogaster* implicated in DNA repair, replication, recombination, and/or cell cycle checkpoint control have been identified through mutations conferring larval hypersensitivity to DNA-damaging agents (reviewed by Dusenbery and Smith 1996; Sekelsky *et al.* 1998; Henderson 1999a). The X-linked locus *mus101* was among the very first such *mutagen-sensitive* (*mus*) genes to be identified. The two original alleles, $mus101^{D1}$ and $mus101^{D2}$, confer hypersensitivity to methyl methanesulfonate (MMS), nitrogen mustard, and γ-rays, but not to UV radiation or *N*-acetyl-2-aminofluorene, a UV mimetic (Boyd *et al.* 1976). Other alleles of *mus101* causing different phenotypes were later isolated: the $mus101^{K451}$ (*fs(1)K451*) mutant is both female sterile (Komitopoulou *et al.* 1983; Orr *et al.* 1984) and MMS sensitive (this report); $mus101^{lcd}$ and $mus101^{SM}$ are late larval lethals (this report; A. Schalet, personal communication); and $mus101^{ts1}$ is a temperature-sensitive lethal (Gatti *et al.* 1983; Smith *et al.* 1985).

The $mus101^{D1}$ mutant is partially defective in postreplication repair (PRR; Boyd and Setlow 1976; Brown and Boyd 1981), as shown by a reduction in the average size of the newly synthesized DNA molecules compared to wild type after UV treatment. Nonirradiated cells from this mutant accumulate high molecular weight DNA molecules more slowly than control cells, suggesting a defect in S phase (Boyd and Setlow 1976). In common with other PRR mutants, $mus101^{D1}$ disrupts "magnification" of ribosomal DNA genes in the male germline, a little-understood process that restores the copy number of 18S and 28S rDNA tandem repeats lost through deletion (Hawley *et al.* 1985).

The female sterile $mus101^{K451}$ mutant was isolated in a screen for mutants affecting eggshell formation (Komitopoulou *et al.* 1983; Orr *et al.* 1984). The eggshell abnormalities observed in this mutant result from defective amplification of clusters of chorion protein genes, which is a form of DNA replication specific to follicle cells (for review see Calvi and Spradling 1999). The recent finding that the Drosophila homolog of the yeast origin recognition complex 2 (ORC2) subunit is also required for chorion gene amplification (Landis *et al.* 1997) raises the intriguing possibility that Mus101 protein may physically interact with DmORC2 at replication orgins of chorion gene clusters.

Animals hemizygous for $mus101^{ts1}$ and heterozygous

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for the autosomal recessive cell marker *mwh* exhibit high levels of chromosomal instability, evidenced by the frequent occurrence of *mwh* clones (Smith *et al.* 1985). This chromosome instability observed genetically is also observed cytologically: neuroblast cells from hemi- or homozygous $mus101^{ts1}$ show a striking failure to condense heterochromatic regions of chromosomes (Gatti *et al.* 1983; Smith *et al.* 1985). Similar genetic and cytological phenotypes have been observed for other *mus101* alleles (Baker and Smith 1979; Smith *et al.* 1985; this report).

The phenotypes associated with the various *mus101* alleles summarized above suggest roles for the $mus101^{+}$ gene product in different aspects of chromosome metabolism. It is evident that the molecular cloning of this gene should bring important new insights into the function of its encoded protein. Here we describe the cloning of *mus101*, achieved through a strategy of chromosome walking and mutation mapping in region 12B. We report that *mus101* encodes a protein with seven BRCT (BRCA1 C terminus) domains, a motif originally noted as a repeated region in the carboxy-terminus of the human ovarian and breast cancer protein BRCA1, in the budding yeast checkpoint protein Rad9, and in a human protein that binds to p53, named 53BP1 (Koonin *et al.* 1996). More recent additions to the BRCT domain superfamily include the human proteins XRCC1, poly (ADP-ribose) polymerase (PARP), DNA ligases III and IV (Altschul *et al.* 1997; Bork *et al.* 1997; Callebaut and Mornon 1997), and TopBP1 (Yamane *et al.* 1997) among others. Mus101 is most similar to human TopBP1, a protein identified in a two-hybrid screen through its association with DNA topoisomerase IIβ (Yamane *et al.* 1997). Mus101 also shares sequence similarity with the fission yeast Rad4/Cut5 protein required for repair, replication, and checkpoint control (Fenech *et al.* 1991; Saka and Yanagida 1993), suggesting that the two proteins may be functional homologs.

MATERIALS AND METHODS

Drosophila stocks and culture conditions: The Drosophila stocks, balancer chromosomes, and phenotypic markers described in this work are catalogued in Lindsley and Zimm (1992) or FlyBase (1999), except as noted below. Flies were maintained on standard yeast-cornmeal-agar medium at 25° unless mentioned otherwise.

***mus101* lethal alleles:** $mus101^{lcd}$ was recovered from a collection of X-linked lethal mutations induced by hybrid dysgenesis (Simmons *et al.* 1984) that we screened for mitotic mutants. Of 101 lethal lines, 37 produced mutant males dying as late larvae or pupae. These were examined for mitotic defects in squash preparations of brain ganglia from third-instar larvae, essentially as described in Gonzalez and Glover (1993). Two stocks, including $mus101^{lcd}$, showed obvious differences from wild type in these preparations.

A second late larval lethal allele, $mus101^{SM}$, was generously provided to us by A. Schalet (Yale University). This mutation arose spontaneously from a cross of wild-type (Amherst) males to females carrying a *mei-9* mutation [$In(1)dl\text{-}49,\ y^{31d}\ w^{a}\ mei\text{-}9^{L1}\ rb\ v^{Of}\ f$] (A. Schalet, personal communication). $mus101^{SM}$ fails to complement the larval lethality of $mus101^{lcd}$, and both $mus101^{lcd}$ and $mus101^{SM}$ fail to complement the mutagen sensitivity of $mus101^{D1}$.

Molecular biology: Standard molecular biology techniques were employed following Ausubel *et al.* (1989) and Sambrook *et al.* (1989). The plasmid vectors pBluescript I and II (Stratagene, La Jolla, CA) were used for all subcloning. The $mus101^{lcd}$ genomic DNA library was constructed in the λ vector EMBL4 (Frischauf *et al.* 1983) according to Axton (1990). Microdissection of polytene chromosome bands 12B1,2 was performed according to Saunders *et al.* (1989). Cosmids comprising the chromosome walk were recovered from the European Drosophila Genome Project (EDGP) gridded library (Sidén-Kiamos *et al.* 1990) by filter hybridization. Initial localization of the gene *garnet* (*g*) to a 6.5-kb *Eco*RI fragment of the chromosome walk was made through the identification of restriction fragment length polymorphisms (RFLPs) on Southern blots containing genomic DNAs of three *garnet* mutants [g^{S2}, g^{S3}, and $In(1)g^{w}$] probed with this fragment. This location was later confirmed by the hybridization of this *Eco*RI fragment to a *garnet* cDNA (gift of V. Lloyd, Dalhousie University, Halifax, Nova Scotia, Canada).

Creation of $Df(1)w^{LCD}$ by γ-irradiation: $In(1)z^{+}\,64b9$ is a rearranged X chromosome carrying an inversion between 3C1 and 12B9, with w^{+} at 3C1 not included within the inverted segment (Sorsa *et al.* 1973). $In(1)z^{+}\,64b9$ males were irradiated with 45 Gy from a ^{60}Co source and mated to a fourfold excess of balancer-bearing virgin females [either *FM7*, $y\ w^{a}\ sn^{x2}\ v\ g/DFB$, $y\ w^{a}$ or *FM7*, $y\ w^{a}\ sn^{x2}\ v\ g/FM6$, or $y\ ml(1)$]. Balanced lines were established by allowing the F_1 progeny to mate *inter se*, since the irradiated chromosomes were balanced in the F_1 females and all the F_1 males were *FM7* (the *DFB* and *FM6* balancer chromosomes carry lethal mutations). Irradiated chromosomes balanced over *FM7* and *DFB* were scored for garnet and/or white phenotypes in the F_1 generation. For irradiated chromosomes balanced over *FM6* (~25% of the F_1), the F_2 generation was examined for visible mutations. The lethality of $Df(1)w^{LCD}$ is not complemented by the duplications $Dp(1;f)LJ9$ and $y^{+}\ g^{+}\ na^{+}\ Y$. It also fails to complement the lethality of the *LS* allele of *crm*, a gene adjacent to *w*, indicating that the deficiency extends into one or more lethal loci in 3C.

Creation of new deficiencies in region 12B by transposase-mediated *P*-element excision: Deficiencies in region 12B were created by "imprecise excision" of a single *P*-element transposon in the strain P[w^{+}, ry^{+}E]2 (Levis *et al.* 1985) that we had localized to the distal part of the walk (12B1,2). This *P* element resides within a 12-kb *Bam*HI fragment found in wild-type DNA (Oregon-R and Canton-S), detected using a 4.7-kb *Bam*HI fragment (B4.7) from the end of the insert in the cosmid 29E9.

To mobilize this element, females homozygous for the *P* insert were crossed *en masse* to $+/Y$; $\Delta 2\text{-}3(99B)$, $Sb/+$ males to generate P/Y; $\Delta 2\text{-}3(99B)$, $Sb/+$ transposase-expressing males in the F_1 generation. Such F_1 males were crossed to *crm/FM7* virgin females and the F_2 progeny examined for the presence of phenotypically white, Bar, non-Stubble females (*i.e.*, $p^{*}/FM7$; $+/+$, where the asterisk indicates deletion or mutation of the w^{+} gene of the *P* insert). Such mutants were crossed individually to *FM7* males to establish stocks. Only those chromosomes bearing lethal mutations (*i.e.*, lines in which white-eyed F_3 males were not recovered) were kept for further study. A total of 23 lethal mutants were isolated from 1698 F_2 crosses. These were analyzed in complementation tests with the lethal mutant $mus101^{SM}$. Virgin $p^{*}/FM7$ females were crossed with $mus101^{SM}/y^{+}g^{+}na^{+}\,Y$ males, and their heterozygous mutant female progeny were examined for viability. These mutants can be divided in two classes: 11 mutants that complement $mus101^{SM}$ and 12 mutants that fail to complement

mus101^{SM}. All 12 of the *P*-element excision mutants that delete *mus101* were found to delete *garnet* as well. This result indicated that the deletions in these mutants extend from the distal part of the walk to the *garnet* locus or beyond and therefore were not informative with respect to mapping the *mus101* locus, since they remove too large a portion of the walk. Five of the 11 strains that complement *mus101*^{SM} were also tested for their ability to complement *garnet*. These strains (including *p116D*, *p205A*, *p281A*, and *p490D*), when heterozygous with *g*^{4}, have wild-type eye color. The 11 lethal mutants that complement *mus101*^{SM} were analyzed at molecular level in Southern-blotting experiments to be able to exclude those regions of the walk not containing *mus101*. In 6 of these mutants, no deletion had occurred proximal to the site of the *P* insertion. In a 7th mutant, a chromosomal rearrangement appeared to have occurred. None of these mutants were characterized further. The 4 remaining mutants (*p116D*, *p205A*, *p281A*, and *p490D*) are discussed in RESULTS.

Isolations of cDNA clones: cDNAs prefixed with an "x" were recovered from a 0–3-hr embryonic library constructed in the vector λgt10 (POOLE *et al.* 1985). A λZAP 2–14-hr embryonic cDNA library (Stratagene) probed with the *mus101* cDNA x99 yielded a single partial-length cDNA. An ovary library constructed in λgt22a (STROUMBAKIS *et al.* 1994) was found to be a rich source of *mus101* cDNAs.

We have isolated two further cDNAs corresponding to distinct genes, in addition to those reported in RESULTS (Figure 2). Both cDNAs were obtained from a 0–4-hr embryonic cDNA library (BROWN and KAFATOS 1988). cDNA F3 was isolated using the 4.1-kb *Bam*HI fragment (B4.1) from cosmid 29E9. F3 is ~1.5 kb and has no significant homology to any sequence in the database. This cDNA also hybridized to genomic fragment B6.2, immediately distal to B4.1. cDNA F5 hybridized to three independent genomic probes: an 8-kb *Pst*I fragment (P8) from cosmid 110D5; a 15-kb *Bam*HI fragment (B15) from cosmid 165E3; and a 5-kb *Xba*I fragment (X5) from phage λA26-7. cDNA F5 is ~3.5 kb and hybridized to genomic fragments spanning an ~30-kb region of the genomic walk, suggesting that the gene corresponding to this cDNA has several introns. cDNA F5 has similarity to a hypothetical 80-kD protein of *Saccharomyces cerevisiae* (accession no. 731675) and a *Caenorhabditis elegans* expressed sequence tag (accession no. 3874484).

DNA sequencing: Sequencing reactions were performed using the ABI PRISM big dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). *mus101* accession no. is AF257463.

***P*-element-mediated germline transformation:** Embryos of the genotype w^{1118}/w^{1118}; +/+; Δ*2-3(68C)*/+ were used for injection. A 9-kb *Xho*I fragment from cosmid 44F7 was subcloned into the transformation vector pW8 (KLEMENZ *et al.* 1987) to give the rescue construct P[w^{+}, X9]. The 10-kb *Bam*HI fragment from cosmid 22F12 was subcloned into the transformation vector pCaSpeR (PIRROTTA 1988) to form the negative control construct P[w^{+}, B10].

Mutagen sensitivity tests: These tests were carried out as described in HENDERSON (1999b). The mutagen sensitivity of the female sterile mutant *mus101*^{K451} has not been documented previously. We have found this mutant to be more sensitive to MMS than either *mus101*^{D1} or *mus101*^{D2}. A dose as low as 0.02% v/v MMS is sufficient to kill all *mus101*^{K451} males, compared to doses of 0.04 and 0.07% required to kill *mus101*^{D1} and *mus101*^{D2} animals, respectively.

RESULTS

Isolation of a new lethal allele of *mus101* showing defective condensation of pericentric heterochromatin: The isolation and cytological characterization of the temperature-sensitive (ts) lethal mutant *mus101*^{tsl} revealed that *mus101* is an essential gene whose wild-type product is necessary for condensation of heterochromatin in mitosis (GATTI *et al.* 1983). We isolated a further (non-ts) lethal allele, *mus101*^{lcd}, from a collection of X-linked lethal mutations induced by hybrid dysgenesis (SIMMONS *et al.* 1984), in a screen for *P*-induced alleles that would facilitate the molecular cloning of the gene through the recovery of sequences flanking the *P* element. *mus101*^{lcd} mutants die as late third-instar larvae or shortly after pupariation and have very small imaginal discs (hence the superscript *lcd* for *lethal-compact-disc*). Examination of neuroblasts of *mus101*^{lcd}/*Y* third-instar larvae revealed numerous chromosomes with striking undercondensation of pericentric heterochromatic regions (Figure 1B), reminiscent of the mitotic phenotype observed in brains of *mus101*^{tsl} mutants shortly after they are shifted to the nonpermissive temperature (GATTI *et al.* 1983). This phenotype may be the earliest to appear in *mus101*^{lcd} neuroblasts, since most of the mitoses in *mus101*^{lcd} brains show more extreme defects, characteristically grossly undercondensed chromatin or chromosome breakage (it is difficult to distinguish between these two phenotypes in orcein-stained preparations). A typical example is shown in Figure 1C. These mitotic phenotypes are also observed in neuroblasts of *mus101*^{lcd}/

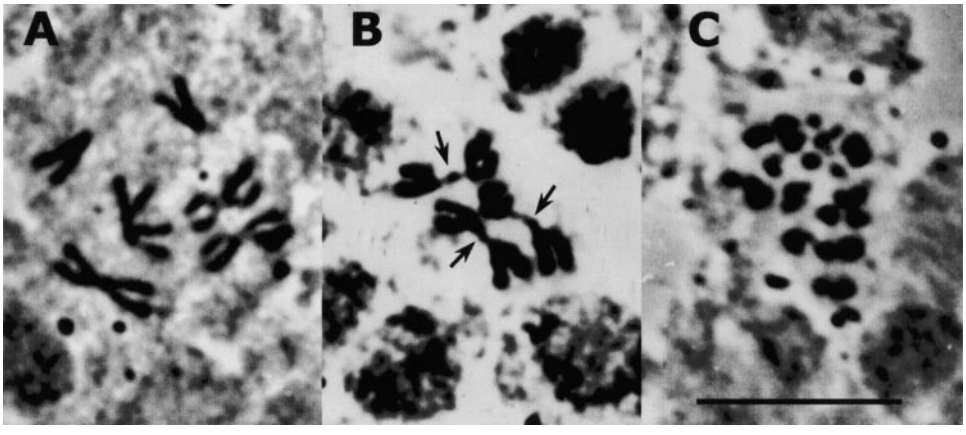

FIGURE 1.—Mitotic chromosome defects in *mus101*^{lcd} neuroblasts. (A) Wild-type *XX* metaphase. (B) Chromosomes from a *mus101*^{lcd}/*Y* larva showing undercondensed pericentric heterochromatin (arrows). (C) Extreme phenotype more typical of *mus101*^{lcd}/*Y*, with undercondensed or broken chromosomes. Blocks of chromatin are probably chromatid arms. Brains in A and B were incubated in 10 μM colchicine for 1 hr followed by hypotonic treatment. Bar, ~10 μm.

TABLE 1

Mitotic chromosome condensation defects in *mus101^{lcd}* neuroblasts

Genotype	No. of brains examined[a]	% aberrant mitoses[b] (total no. of mitoses examined)
mus101^{lcd}/mus101^{+}	12	0 (383)
mus101^{lcd}/mus101^{lcd}	13	95 (168)
mus101^{lcd}/Df(1)HA92	3	100 (50)
mus101^{lcd}/Y	12	80 (103)

[a] Brains were incubated in 10 μM colchicine for 1 hr followed by treatment in hypotonic saline. The appearance of abnormal mitotic figures in mutant brains was similar with and without colchicine treatment (not shown).

[b] Aberrant cells are those exhibiting abnormal condensation of at least one chromosome.

Df(1)HA92 hemizygous females (not shown). Table 1 summarizes the frequency of observed chromosome condensation defects in *mus101^{lcd}* larval brains.

Refinement of the cytological map position of *mus101*: Although *mus101^{lcd}* had been induced in a dysgenic cross, in which the strain π2 provided both *P*-element "ammunition" and a source of transposase, an observed *P* element in this mutant at region 12C1 was found to be separable by recombination from the *mus101^{lcd}* mutation. Furthermore, introduction of a transposase source [*Δ2-3(99B)*] into this strain failed to revert the *mus101^{lcd}* mutation to wild type. Therefore, as an alternative means to isolate the gene we adopted a positional cloning strategy.

The *mus101* gene was originally localized to between bands 12A6,7 and 12D3 on the basis of the failure of *mus101* mutants to complement *Df(1)HA92* (Mason *et al.* 1981). We obtained a further refinement of the *mus101* location by using available *T(1;Y)* stocks to synthesize interstitial duplications and deficiencies (Axton 1990) and by creating a small deficiency, *Df(1)w^{LCD}*, that is missing the cytological interval 12B2-9 and uncovers *mus101*. These mapping experiments, taken together with the recombination studies of A. Schalet, which had positioned *mus101* ~0.1 cM distal to *garnet* (*g*; A. Schalet, personal communication), established the cytological location of *mus101* as 12B2-6.

Molecular cloning of region 12B by chromosome walking: Molecular entry into the 12B region was gained using several approaches. Although the *P* element at 12C1 in the *mus101^{lcd}* line was not responsible for the *mus101* mutation (see above), its molecular cloning nonetheless provided the first step toward the isolation of the *mus101* gene. Characterization of a recovered clone revealed that the *P* element at 12C1 had inserted adjacent to the *Yolk protein 3* (*Yp3*) gene. This flanking genomic DNA was used in turn as a probe to identify cloned wild-type genomic segments (as phages and cosmids) from 12C1 and, more distally, into region 12B6,7. Recovered EDGP cosmids belonging to this contig (12.2) include 7C5, 22F12, and 189B8 (Figure 2). Microdissection of polytene chromosome bands 12B1,2 and PCR amplification of the recovered DNA provided probes corresponding to the distal part of 12B. EDGP cosmids belonging to this contig (12.6) include 29E9, 36G4, 94D8, and 110D5 (Figure 2). Contig 12.2 was extended distally with the isolation of the cosmid 44F7, and the two contigs were joined and the relative orientation of contig 12.6 established by the isolation of the bridging cosmid, 165E3.

The locations of two important landmarks on the chromsome walk were identified: the distal breakpoint of *Df(1)w^{LCD}* near the distal end of the walk and the *garnet* locus in its proximal part (Figure 2). These two landmarks define the boundaries of the genomic region containing *mus101* (see above), an interval of ~90 kb.

Creation of new deficiencies in region 12B by excision of a marked *P* element: We sought to create new deficiencies in region 12B through transposase-mediated excision of a single *P* element located in the distal part of the walk in the strain P[*w*$^{+}$, *ry*$^{+}$E]2 (Levis *et al.* 1985; Figure 2). To enrich for such deletion mutants, we screened for loss of the *w*$^{+}$ marker of P[*w*$^{+}$, *ry*$^{+}$E]2 associated with organismal lethality. Four of 23 mutants generated in this way (*p116D*, *p205A*, *p281A*, and *p490D*) were informative with respect to narrowing the location of *mus101*; each had a deletion extending proximally from the site of insertion of the *P* element into the region covered by the chromosome walk (Figure 2). The most proximal breakpoint, that of deficiency *p490D* (*Df(1)p490D*), is near the distal end of the *Bam*HI fragment B15 (Figure 2). This result positioned *mus101* to within the interval defined by this breakpoint distally and the *garnet* locus proximally, ~30 kb of cloned DNA.

Pinpointing the location of *mus101*: To further narrow the location of *mus101* we sought to determine whether any of the *mus101* alleles showed a RFLP in the ~30-kb interval between the breakpoint of *Df(1)p490D* and *garnet*. To achieve this we used subcloned *Bam*HI and *Eco*RI fragments to probe Southern blots of genomic DNAs of *mus101* mutants. Of the various probes used, only the fragment B10 detected RFLPs in the lethal mutant *mus101^{lcd}* and in the mutagen-sensitive strains *mus101^{D1}* and *mus101^{D2}* (not shown). The presence of RFLPs in three independent *mus101* mutant alleles detected by the B10 probe thus identified this genomic fragment as a putative *mus101* locus. This possibility was further strengthened because B10 partially overlaps a 6.5-kb *Eco*RI fragment that we had shown to contain *garnet*, which mapping experiments had placed proximal but very near to *mus101* (see above).

Identification of transcription units in the *mus101* region: We used two methods to identify transcription units in the *mus101* region: Northern blotting and isolation of cDNAs. Northern blots of total RNA extracted from 0- to 4-hr wild-type embryos were probed separately

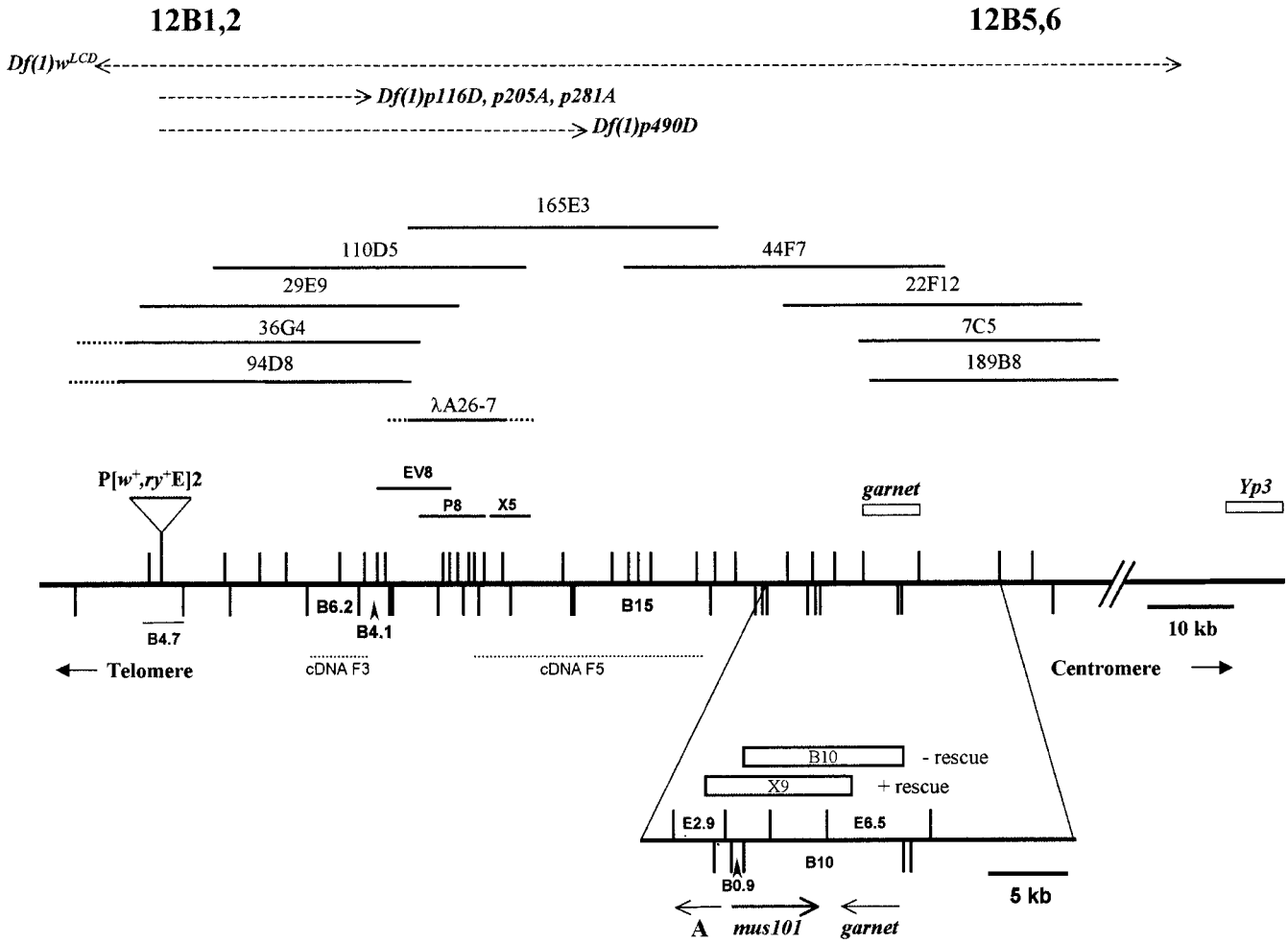

FIGURE 2.—Molecular map of region 12B. Genomic region 12B is represented by ~150 kb of cloned DNA. The main map (center) shows the restriction enzyme recognition sites for *Eco*RI and *Bam*HI, indicated by vertical lines above and below the horizontal line, respectively. Subcloned restriction fragments mentioned in the text are labeled (*e.g.*, B15). EDGP cosmids that comprise the chromosome walk are indicated above the map as overlapping horizontal solid lines. λA26-7 represents a phage clone. EV8, P8, and X5 represent subcloned restriction fragments (see MATERIALS AND METHODS). The dashed lines above the cosmids represent genomic regions deleted in the indicated deficiency strains; the arrowheads mark the approximate positions of the breakpoints defined molecularly. The triangle in the distal part of the walk (region 12B1,2) represents the site of integration of the *P* element in the strain P[w^+, ry^+E]2. The positions of the genes *garnet* and *Yp3* are indicated. Not all of the ~30-kb interval between these two genes is represented on the map; this discontinuity is indicated by the double slash. cDNAs F3 and F5 are referred to in MATERIALS AND METHODS. An expanded view of the region of the walk containing *mus101* is shown at the bottom right. The fragments used to search for transcription units and RFLPs are labeled (*e.g.*, B10). The arrows below this map show the identified transcription units and direction of transcription (see text for details). The fragments X9 and B10 were used in germline transformation rescue experiments.

with the contiguous genomic fragments E2.9, B0.9, and B10 (Figure 2). Probe E2.9 detected an ~3-kb transcript (corresponding to transcription unit "A" in Figure 2). Probe B10 detected two transcripts, of ~5 kb and ~3.5 kb (corresponding to *mus101* and *garnet*, respectively). Probe B0.9, distal to B10, also recognized the ~5-kb transcript, indicating that this transcription unit spans the B0.9 and B10 restriction fragments.

We used a 9-kb *Xho*I fragment (X9) that partially overlaps fragments E2.9, B10, and E6.5 and contains the entire B0.9 fragment (Figure 2) to recover cDNAs corresponding to the three transcripts detected by Northern analysis. Six partial cDNAs belonging to three different classes were isolated: *garnet* (x91, x94, x95, and x96) (OOI *et al.* 1997), transcription unit "A" (x93), and *mus101* (x99). A homology search using the BLASTX program (ALTSCHUL *et al.* 1997) revealed that the partial sequence of the cDNA x93 is similar to the human clone KIAA0544 (NAGASE *et al.* 1998; accession no. 3043612) and to the *C. elegans* locus CEC11H1 (accession no. Z70205), identified in the genome project. Although no function has been assigned to these loci, KIAA0544 has significant similarity to human cell growth regulators and apoptosis inhibitors (NAGASE *et al.* 1998).

A BLASTX search using the x99 sequence revealed similarity to a human cDNA (NAGASE *et al.* 1996), which

TABLE 2

Rescue of *mus101* mutant phenotypes by *P*-element-mediated germline transformation

mus101 allele	Phenotype tested	P[w^+, X9]	P[w^+, B10]
D1	MMS sensitivity	+	−
D2	MMS sensitivity	+	−
K451	Female sterility	+	NT
K451	MMS sensitivity	+	−
lcd	Lethality	+	NT
SM	Lethality	+	−
tsl	Temperature-sensitive lethality	+	−

Transformant males in which the *P*-element construct (either rescue construct P[w^+, X9] or negative control construct P[w^+, B10]) had inserted into one of the autosomes were crossed to $mus101^{D1}$ and $mus101^{D2}$ homozygotes and to $mus101^{lcd}/FM7c$, $mus101^{SM}/FM7c$, $mus101^{tsl}/FM7c$, and $mus101^{K451}/FM3$ heterozygotes. Progeny from these crosses were treated with MMS ($mus101^{D1}$, $mus101^{D2}$, and $mus101^{K451}$), left untreated ($mus101^{lcd}$ and $mus101^{SM}$), or grown at restrictive temperature ($mus101^{tsl}$). Rescue of the *mus101* mutant phenotypes was monitored by the recovery of non-Bar-eyed (*i.e.*, nonbalancer) male progeny carrying the *mus101* mutant chromosome. Rescue of $mus101^{K451}$ female sterility was monitored in $mus101^{K451}$ homozygous females carrying a $mus101^+$ *P*-element construct on chromosome 2. +, rescue of the phenotype; −, nonrescue; NT, not tested.

in turn is similar to the *Schizosaccharomyces pombe* $rad4^+/cut5^+$ gene (Fenech *et al.* 1991; Saka and Yanagida 1993). *rad4/cut5* mutants show sensitivity to a variety of DNA-damaging agents, and the Rad4/Cut5 protein is essential for S phase (Saka *et al.* 1994) and important in a DNA replication checkpoint. The similar phenotypes presented by *mus101* and *rad4/cut5* mutants suggested that a gene corresponding to cDNA x99 was a very strong candidate for *mus101*. Therefore we sought and recovered additional cDNAs using x99 as a probe (see materials and methods).

Identification of the *mus101* gene by *P*-element-mediated germline transformation: To confirm the cloning of *mus101*, we designed two genomic constructs for use in *P*-element-mediated germline transformation experiments. The X9 fragment referred to above was selected as a rescue fragment (Figure 2). This fragment was predicted to contain a complete *mus101* gene together with truncated versions of transcription unit "A" and *garnet*. The B10 fragment, which is missing ~2 kb of promoter elements and coding region from the 5′ end of *mus101*, was selected as a negative control. The construct P[w^+, X9] fully rescued the phenotypes of all *mus101* alleles tested (Table 2). In constrast, the construct P[w^+, B10] rescued none of the *mus101* mutant phenotypes. These experiments prove that *mus101* corresponds to the identified gene between transcription unit "A" and *garnet* depicted in Figure 2.

Genomic organization of *mus101*: We determined the complete genomic sequence of *mus101* (~5.3 kb) by sequencing both the distal 6 kb and very proximal end of the X9 restriction fragment (~700 bp). The distal end of X9 overlaps transcription unit "A" (620 bp), and the proximal end overlaps *garnet*. Conceptual translation of the *mus101* sequence revealed that it encodes a polypeptide with a predicted molecular weight of 158 kD and a calculated pI of 6.4. The gene contains no introns. There are three in-frame methionines at the N terminus of the predicted protein at positions 1, 5, and 7. The region upstream of the first ATG has the best match to the Drosophila consensus sequence for translation initiation (Cavener and Ray 1991). However, it is also possible that the ATGs corresponding to the methionines at positions +5 and +7 are used to initiate translation, since the region upstream of these ATGs also has considerable similarity to the Cavener and Ray (1991) consensus sequence.

Further upstream, at positions −377 to −370 and −352 to −345 with respect to the first ATG, are two DRE (DNA replication-related element) motifs. DRE is a *cis*-acting positive regulatory element (TATCGATA) present in the promoters of a variety of genes, including those encoding proteins with functions in DNA replication, transcription, translation, signal transduction, and cell cycle control (Matsukage *et al.* 1995). We have not carried out extensive searches for other regulatory motifs in this region.

***mus101* encodes a protein with seven BRCT domains and is similar to the human protein TopBP1:** Searches of the databases using the PSI-BLAST program (Altschul *et al.* 1997) for proteins similar to Mus101 revealed the highest scores for a human protein first described as having similarity to the fission yeast Rad4/Cut5 gene product (Nagase *et al.* 1996). This human protein is identical to TopBP1, a protein identified through its *in vitro* binding to DNA topoisomerase IIβ (Yamane *et al.* 1997). In addition to TopBP1 and Rad4/Cut5, proteins showing significant similarity to Mus101 are *C. elegans* clone F37D6.1, *Arabidopsis thaliana* clone T10M13.12, the Drosophila protein Pebble (Pbl) required for cytokinesis (Prokopenko *et al.* 1999), the mouse transforming protein Ect2, and the human DNA repair protein XRCC1.

All these proteins have BRCT domains, a module

composed of ~100 amino acids with a characteristic hydrophobic profile, easily identified in a hydrophobic cluster analysis plot (Callebaut and Mornon 1997). In fact, the regions of highest similarity that these proteins show with Mus101 are in the BRCT domains, with the exception of TopBP1 and the *C. elegans* clone F37D6.1, where the regions of similarity extend beyond the BRCT domains. As an example, Figure 3A shows the similarities between the highly conserved BRCT domain I in Mus101, TopBP1, *C. elegans* clone F37D6.1, Rad4/Cut5, Pbl, and Ect2. The number and distribution of BRCT domains in these proteins are compared in Figure 3B.

The pairwise alignment of Mus101 and TopBP1 generated using the BLAST2 program (Tatusova and Madden 1999) is shown in Figure 4. Both proteins have similar size: Mus101 is 1425 and TopBP1 is 1522 amino acids long. The human TopBP1 protein has eight BRCT domains, the greatest number of domains so far observed in a single protein (Yamane *et al.* 1997). Mus101 has seven BRCT domains, and their distribution is similar to those of TopBP1, with only the central BRCT domain (VI) of TopBP1 not represented in Mus101 (Figure 3B). The N-terminal and central part of Mus101 has 24% identity and 42% similarity to the N terminus of TopBP1. This region contains the Mus101 and TopBP1 BRCT domains I, II, III, IV, and V. The C terminus of Mus101 has 33% identity and 51% similarity with the C terminus of TopBP1. This region contains the Mus101 BRCT domains VI and VII and the TopBP1 BRCT domains VII and VIII.

The region between BRCT domains V and VI of the Mus101 protein that does not have similarity to TopBP1 contains two distinct subregions with similarity to other proteins. Residues 825–909 of Mus101 share 27% identity and 46% similarity with the C terminus of human treacle protein (TCOF1), a putative nucleolar trafficking phosphoprotein that is defective in patients with Treacher Collins syndrome, a craniofacial developmental disease (Wise *et al.* 1997). Residues 1046 to 1129 of Mus101 are 26% identical (38% similar) to the C terminus of Drosophila posterior sex combs (Psc) protein. Psc is a product of one of the *Polycomb* group genes (*Pc-G*). *Pc-G* genes are needed to maintain patterns of expression of homeotic genes during Drosophila development by repression of target genes (for review see Pirrotta 1995).

DISCUSSION

We have molecularly cloned the *mus101* gene and found it to encode a predicted protein of 1425 amino acids containing seven BRCT modules distributed throughout

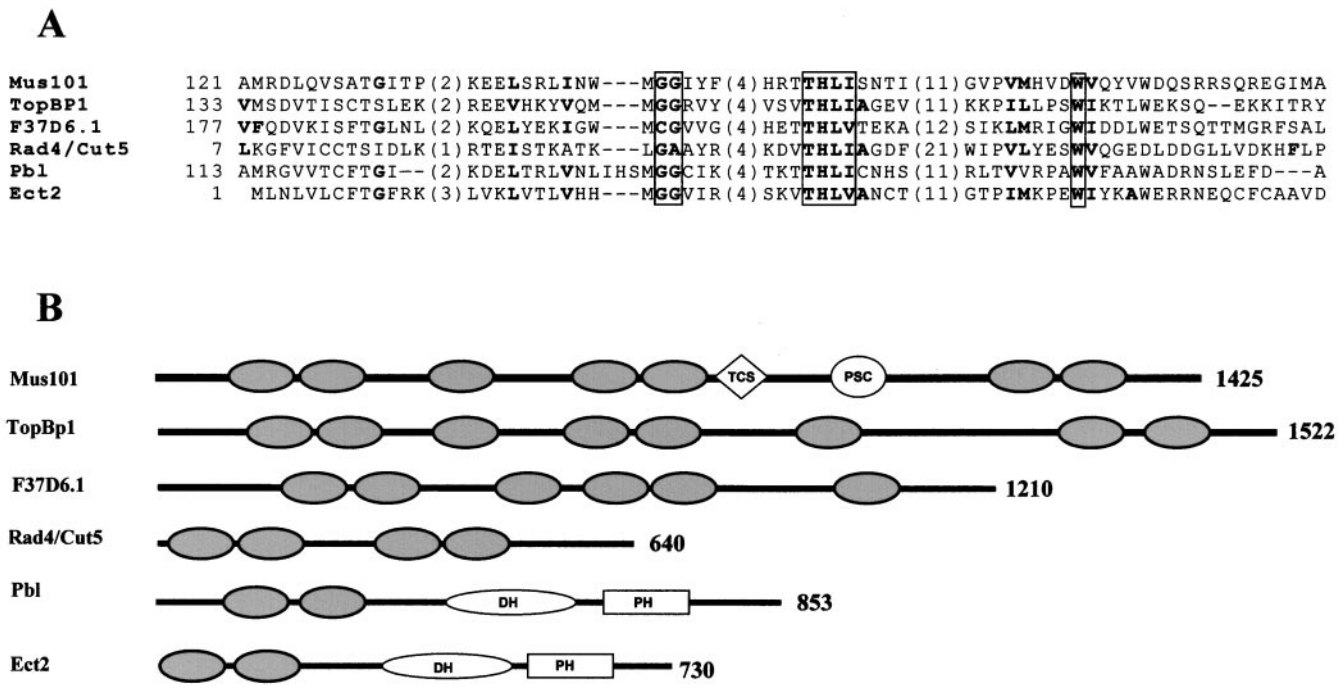

Figure 3.—A comparison of proteins structurally related to Mus101. (A) Alignment of the primary structures of BRCT domain I from Mus101, TopBP1, *C. elegans* clone F37D6.1, fission yeast Rad4/Cut5, Drosophila Pebble (Pbl), and mouse Ect2. The boxed residues are highly conserved and the boldface residues conserved among BRCT domains, according to Callebaut and Mornon (1997). (B) Comparison of the number and distribution of BRCT domains in Mus101, TopBP1, F37D6.1, Rad4/Cut5, Pbl, and Ect2. BRCT domains are represented by solid ovals. The diamond labeled TCS and the circle labeled PSC represent regions of homology shared by Mus101 and human TCOF1 and Drosophila posterior sex comb proteins, respectively. The open ovals labeled DH and the boxes labeled PH of Pbl and Ect2 represent Dbl and pleckstrin homology domains, respectively (Prokopenko *et al.* 1999).

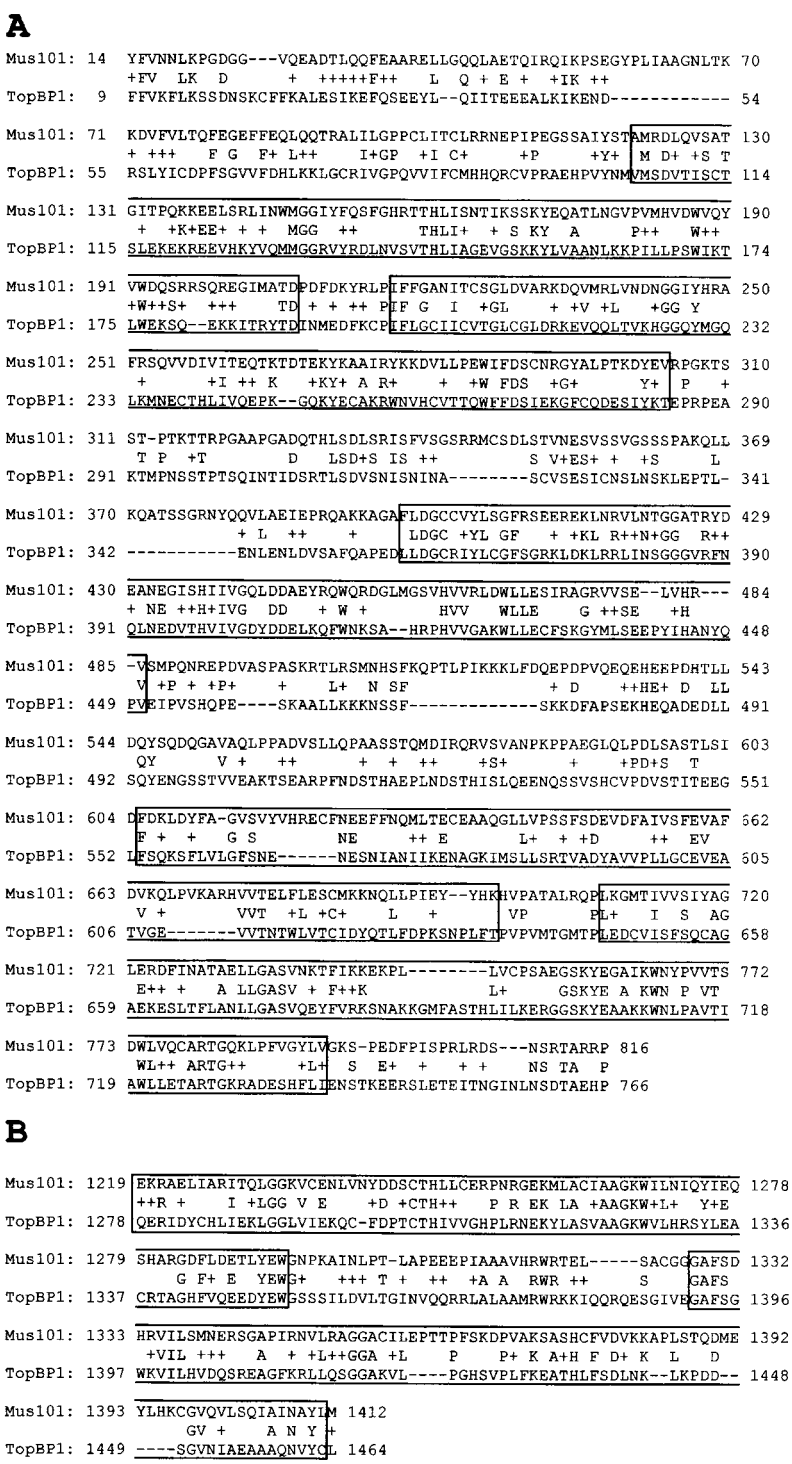

FIGURE 4.—BLAST2 alignment of Mus101 and TopBP1. (A) Alignment of the N-terminal-to-central regions of Mus101 and TopB1. Identity, 206/828 (24%); positives, 361/828 (42%); gaps, 95/828 (11%). Residues comprising each BRCT domain are boxed. Domains were predicted on the basis of CALLEBAUT and MORNON (1997) and through comparisons of multiple sequence alignments of BRCT domain-containing proteins. Actual domains may include up to five or six additional residues at each end. (B) Alignment of the C-terminal regions of Mus101 and TopBP1. The BRCT domain names VI and VII refer to the Mus101 protein; the homologous domains in TopBP1 are VII and VIII, respectively. Identity, 67/200 (33%); positives, 104/200 (51%); gaps, 19/200 (9%).

its length. The number and position of BRCT domains vary in different proteins. For example, BRCA1, Rad9, and XRCC1 each have two domains in their C termini, Ect2 has two domains in its N terminus, and Rad4/Cut5 has two domains in its N terminus and two domains in the central part of the protein (BORK *et al.* 1997; CALLEBAUT and MORNON 1997). The majority of proteins described so far have on average two to four BRCT domains, although some have a greater number, like the *C. elegans* clone F37D6.1, of unknown function, which has six domains (BORK *et al.* 1997; CALLEBAUT and MORNON 1997) and TopBP1, which has eight (YAMANE *et al.* 1997). It has been suggested by CALLEBAUT and MORNON (1997) and BORK *et al.* (1997) that BRCT domains may be sites for interaction between proteins implicated in the maintenance of the genome in response to damage. The knowledge of such interactions is beginning to shed light upon poorly understood re-

pair mechanisms. Examples are accumulating showing that BRCT domain-containing proteins can participate not only in different aspects of DNA repair and cell cycle checkpoint control, but also in other essential cellular processes, like transcription (Anderson *et al.* 1998; Gowen *et al.* 1998; Kleiman and Manley 1999; Xu *et al.* 1999; Zhong *et al.* 1999).

The protein most closely resembling Mus101 in searches of the current databases is TopBP1, which interacts with DNA topoisomerase IIβ (Topo IIβ). This interaction is mediated through the C terminus of TopBP1, where two consecutive BRCT domains are located (Yamane *et al.* 1997). Topo IIβ is a member of the type II subfamily of topoisomerases that generates transient double-strand breaks to catalyze topological changes in DNA (Austin and Marsh 1998). Yamane *et al.* (1997) have speculated that TopBP1 may function in the repair of strand breaks resulting from a failure of the DNA rejoining reaction of Topo IIβ. This hypothesis is supported by their more recent finding that regions of TopBP1 containing BRCT domains are able to bind nicked circular and linear DNA molecules, but not intact circular DNA (Yamane and Tsuruo 1999).

D. melanogaster has only a single Topo II isoform, present in at least three separate functional pools: one for chromosome condensation, one for chromosome segregation, and one pool that remains associated with the chromosome throughout the cell cycle (Swedlow *et al.* 1993). Drosophila Topo II associates with Barren, a member of the condensin/cohesin family of proteins necessary for segregation of sister chromatids at mitosis (Bhat *et al.* 1996). Drosophila Topo II was also recently identified as a component of the chromatin-remodeling factor CHRAC (chromatin-accessibility complex; Varga-Weiz *et al.* 1997), suggesting a structural role for this protein in chromatin organization. The C-terminal region of TopBP1 that interacts with Topo IIβ has 33% identity and 51% similarity with Mus101. It is possible that Mus101 is a functional homolog of TopBP1 and that Mus101 also binds Topo II. Such a putative interaction between Mus101 and Topo II, if it were specific to heterochromatic regions, could explain the failure of heterochromatin to condense properly in the lethal mutants $mus101^{lcd}$ and $mus101^{ts1}$.

Mus101 BRCT domains I and II are very similar to BRCT domains I and II of the fission yeast replication checkpoint protein Rad4/Cut5 and the oncogene product Ect2, regions that are important to the roles of these proteins in cell cycle control. Truncation of the N-terminal region of Ect2 to remove these two BRCT domains increases its transforming activity, suggesting that this region has a negative effect on cell division (Miki *et al.* 1993). The N terminus of Rad4/Cut5 containing these BRCT domains is essential for complementation of the temperature-sensitive phenotype in *rad4/cut5* mutants. Moreover, its overexpression blocks cell division (Saka and Yanagida 1993). The localization of the mutations in three independent *rad4/cut5* alleles revealed the same amino acid substitution T45M in the conserved stretch VTHLIA in BRCT domain I (Saka *et al.* 1997; see Figure 3A). This mutation prevents the interaction of Rad4/Cut5 with the DNA replication and DNA damage checkpoint protein Crb2 (Saka *et al.* 1997).

There are many similarities between *mus101* and $rad4^{+}/cut5^{+}$. Both are essential genes and both are required for DNA repair. They also both encode members of the BRCT superfamily, with similar BRCT domains I and II. Rad4/Cut5 is a component of the replication checkpoint control system (Saka and Yanagida 1993) and together with Crb2 forms a complex with the checkpoint protein Chk1. It has been suggested by Saka *et al.* (1997) that these proteins may form a checkpoint sensor-transmitter pathway to arrest cell cycle progression. Is Mus101 also necessary for the DNA replication checkpoint? In *S. pombe*, the replication checkpoint has been categorized into two classes: one activated by a defect in the replication machinery (mutations in polymerases and ligases) and the other activated by limitation of normal nucleotide supply [effected by hydroxyurea (HU) treatment or mutation of *cdc22*]. Rad4/Cut5 is necessary for both checkpoints, whereas Crb2 is necessary only for the nucleotide supply checkpoint (Saka *et al.* 1997). Tests of $mus101^{D1}$, $mus101^{D2}$, and $mus101^{K451}$ larvae have revealed wild-type levels of sensitivity to HU (Banga *et al.* 1986; R. R. Yamamoto and D. S. Henderson, unpublished results), suggesting that the nucleotide supply checkpoint is still largely intact in these mutants and perhaps highlighting a functional distinction between Rad4/Cut5 and Mus101.

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