To the Graduate Council:

I am submitting herewith a dissertation written by Siuk Yoo entitled "Functional analysis of *Drosophila* Rad51 protein in DNA repair, recombination, and apoptosis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

Bruce D. McKee, Major Professor

We have read this dissertation
and recommend its acceptance

Accepted for the Council

Interim Vice Provost and
Dean of The Graduate School

Functional analysis of Drosophila Rad51 protein in DNA repair, recombination, and apoptosis

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

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DEDICATION

This dissertation is dedicated to my mother

Mrs. Jeong-Ja Choi (최 정 자)

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ABSTRACT

Among proteins involved in homologous recombination, Rad51 is an essential enzyme possessing DNA binding, ATPase, and strand transfer activities. The functions of Rad51 in repair of double-strand breaks (DSB) and meiotic recombination have been extensively studied in yeast and mammals, but little is known about its roles in cell cycle regulation and apoptosis. In this study, we examine the roles of Rad51dm protein in *Drosophila melanogaster*.

Like other Rad51 proteins, Rad51dm is involved in DNA repair and recombination. The function of Rad51dm in DNA repair was demonstrated by examining the sensitivity of transgenic animals to methyl methanesulfonate (MMS) that induces double-strand breaks in DNA. When treated with MMS, transgenic larvae carrying extra copies of the Rad51dm gene showed higher survival rates. To down-regulate the function of native Rad51dm, hsp26-IRrad51 transgenic animals carrying Rad51dm gene in an inverted-repeat orientation (IR) driven by a heat shock inducible promoter, hsp26, were generated and the MMS sensitivity of these lines were determined. Under a heat shock condition, the survival rates of hsp26-IRrad51 larvae were greatly reduced compared to that of the yw control group. In addition, the transcript level of Rad51dm in wild-type flies increased 2- to 3-fold after X-ray or MMS treatment. These results suggest that Rad51dm is involved in repair of DNA damage.

To study if Rad51dm is involved in meiotic recombination, we determined non-disjunction rates of sex chromosomes and frequencies of recombination using hsp26-IRrad51 females. Under heat shock, hsp26-IRrad51 females showed elevated frequency of X-X non-disjunction and reduced meiotic recombination events compared to control groups without heat shock, implying that IR Rad51dm RNA may inhibit the function of Rad51dm in meiotic recombination. However, non-disjunction rates of X-Y chromosomes of hsp26-IRrad51 transgenic males were similar between the heat-shock group and non-heat-shock group, suggesting that Rad51dm may not be involved in male meiosis.

To determine the expression level and the distribution of Rad51dm protein, we generated a polyclonal antibody. Immunoblot analysis revealed that Rad51dm protein was highly expressed in embryos, but hardly detectable in any other tissues. Immunostaining of embryos showed that the Rad51dm foci associated with unknown materials stained with 4'6-diamidino-2-phenylindole (DAPI), but they did not interact with mitotic chromosomes. To investigate a role of Rad51dm in meiosis, we examined spermatocytes. Although Rad51dm foci were not detected in wild-type spermatocytes, overexpressed Rad51dm proteins exclusively localized in the nucleus during prophase I and rapidly disappeared throughout the subsequent stages as observed for mammalian RAD51 proteins. Since the meiotic recombination occurs only in female of *Drosophila*

melanogaster, it will be interesting to study the distribution of Rad51dm protein in oocytes.

To investigate the overexpression effect of Rad51dm protein, a heat-shock inducible promoter and the UAS-GAL4 binary expression system were used to generate hsp26p-rad51 and UAS-rad51 transgenic flies. Both transgenic lines showed that the overexpression of Rad51dm protein results in lethality regardless of heat shock treatment. Immunocytochemistry revealed that Rad51dm foci in wing and eye imaginal discs are colocalized with foci stained by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, suggesting that the overexpression of Rad51dm protein induces apoptosis. In addition, crosses of UAS-rad51 and UAS-reaper with various GAL4 lines showed that the phenotypes caused by overexpression of Rad51dm protein are similar to those of Reaper protein, a proapoptotic protein, suggesting that Rad51dm might function in apoptosis. To determine if Rad51dm affects cell cycle progression, eye imaginal discs were stained with the mitotic-specific anti-phospho-histone H3 antibody. The result revealed that overexpression of Rad51dm protein disrupts the normal mitotic pattern in the second mitotic wave in eye discs. In conclusion, it is likely that Rad51 may act as a 'sensor' molecule that monitors DNA damage and determine cell survival or cell death.

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PART 1

GENERAL INTRODUCTION

CHAPTER 1

RECA/RAD51 FAMILY: AN OVERVIEW

Homologous recombination is an essential biological function required not only for genetic recombination but also for chromosomal segregation, DNA replication, and DNA repair. Recombination occurs between two homologous DNA sequences in all organisms and the exchange of information is critical for the survival of the species. Homologous recombination provides an effective means of generating genomic diversity that is very important for the evolution of a species. In higher organisms, chromosome pairing and recombination events occur in meiosis and the failure of these processes leads to abnormal chromosomal segregation. Although the precise molecular mechanism of recombination is not fully understood, it has been extensively studied in *E. coli*. In *E. coli*, among the proteins involved in homologous recombination, RecA, which has a molecular mass of 38,500 Da, is a central enzyme both in recombination and in various SOS responses to DNA damage (Ogawa *et al.*, 1992; Alexseyev *et al.*, 1996). It also participates in the initiation of pairing and strand exchange leading to genetic recombination (Radding, 1991; Rao and Radding, 1993).

Rad52 epistasis group

The genes involved in recombination in yeast were identified from isolation of several groups of mutations. Among them, the rad mutations were obtained by their sensitivity to ionizing radiation and referred to as the Rad52 epistasis group (Rad50-Rad57). These genes are involved in double-strand break repair. Rad51 is a member of Rad52 epistasis group and sequence analysis revealed that its product has significant homology to the *E. coli* RecA protein (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). Yeast Rad51 mutants exhibit mitotic and meiotic phenotypes. In mitotic cells, the mutants fail to repair double-stranded breaks (DSB) and in meiosis sporulation is reduced (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). Like RecA protein, *S. cerevisiae* Rad51 forms nucleoprotein filaments on DNA, and promotes homologous pairing and strand exchange reactions *in vitro* (Sung, 1994; Sung and Robberson, 1995). These results suggest that Rad51 is an important component in the repair of DSBs by homologous recombination.

In addition to Rad51 protein, two more homologs of RecA , Rad55 and Rad57, have been identified in the Rad52 epistasis group (Kans and Mortimer, 1991; Lovett, 1994). Both Rad55 and Rad57 are involved in repair of double-strand DNA damage and recombination. Both proteins exist as a heterodimer and appear to act as a cofactor for the assembly of Rad51 protein onto single-stranded DNA (Sung, 1997) (Fig. 1).

Other proteins in the Rad52 epistasis group, such as Rad52 and Rad54, which are not members of the RecA/Rad51 family, also interact with Rad51 and

Figure 1. Diagram of protein interactions in Rad52 epistasis group and homologous recombination for repair of a DNA double-strand break. The double-strand break DNA with exposed 3' ends is shown at the top (gray), which associates with an unbroken homologous molecule (black), and undergoes strand exchange followed by resynthesis (dashed lines) and resolution. Left panel shows the recombination proteins associated with the strand exchange step. Arrows indicate known physical associations between proteins. Adopted from Thacker, 1999.

Rad54
Rad55
Rad57
Rad51
Rpa
Rad52
5'
3'
3'
5'

promote the early stages of homologous recombination and repair (New *et al.*, 1998; Petukhova *et al.*, 1998) (Fig 1). Yeast Rad52 interacts with a single-stranded DNA-binding protein (replicating protein A), and facilitates the formation of nucleofilament (Hays *et al.*, 1998; Gasior *et al.*, 1998). Rad54 is structurally related to a member of the SNF2/SWI2 family of DNA-dependent ATPases (Clever *et al.*, 1997). Members of this family have been implicated in various aspects of DNA metabolism, such as transcription, recombination, and DNA repair. The Rad54 null mutants of mouse and chicken cell lines show severely reduced frequencies of homologous recombination and are extremely sensitive to agents causing severe damage to DNA (Essers *et al.*, 1997; Bezzubora *et al.*, 1997). Based on the functions of SNF2/SWI2 family members, Rad54 may play a role in chromatin remodeling during homologous recombination, but its precise function is not fully understood.

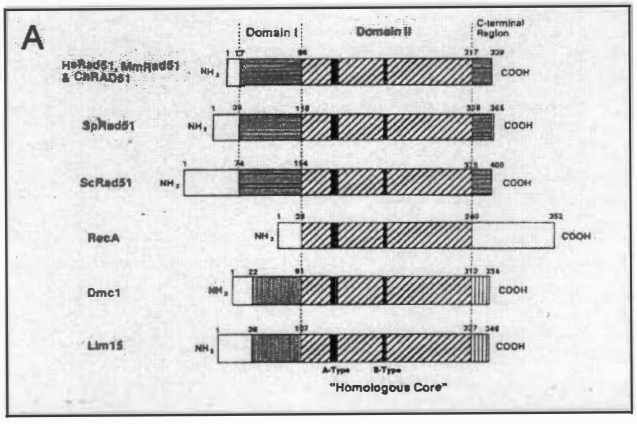
RecA/Rad51 family

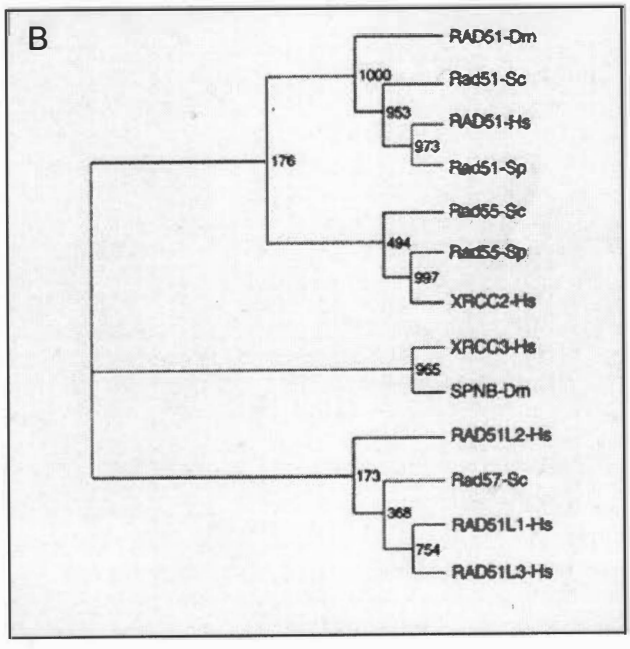
In addition to *Rad51*, *Rad55*, and *Rad57*, another yeast gene, *Dmc1* (disrupted meiotic cDNA), was isolated (Bishop *et al.*, 1992). Dmc1 belongs to RecA/Rad51 family because its amino acid sequence shares homology to the bacterial RecA (Bishop *et al.*, 1992). Dmc1 is expressed only during meiosis and the null mutants block meiotic recombination, disrupt synaptonemal complex formation, and cause a meiotic cell cycle arrest (Bishop *et al.*, 1992). These results indicate a crucial role of Dmc1 during meiosis. However, Dmc1 appears to play no role in mitotic DNA metabolism whereas Rad51 is essential for mitotic

as well as meiotic recombination and repair. The mouse and human Dmc1 homologs have also been isolated and the primary amino acid sequences showed 54 % identity with yeast Dmc1 protein (Habu *et al.*, 1996). The high expression level of Dmc1 in testis and the localization of the protein in spermatocytes suggest a functional similarity to yeast Dmc1 protein (Bishop *et al.*, 1994; Habu *et al.*, 1996; Tarsounas *et al.*, 1999).

In addition to yeast, Rad51 homologs have also been identified in Tetrahymena (Campbell and Romero, 1998), *Arabidopsis* (Doutriaux *et al.*, 1998), *Xenopus* (Maeshima *et al.*, 1995), *C. elegans* (Rinaldo *et al.*, 1998), chicken (Bezzubora *et al.*, 1993), mouse (Morita *et al.*, 1993; Shinohara *et al.*, 1993), and human (Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993), indicating that members of RecA/Rad51 family are highly conserved from bacteria to mammals. Amino acid sequence analysis reveals that all Rad51 proteins consist of two domains, domain I and domain II (Fig. 2A). The domain I is found in all eukaryotic Rad51 proteins, but not in the prokaryotic RecA protein. This region is divergent among species, suggesting species-specific roles in protein interactions. The domain II is the common homologous region for all RecA/Rad51 proteins, thus, this region is referred as the homologous core region. There are two nucleotide-binding consensus sequences (Walker type-A and type-B) within the homologous core region (Ogawa *et al.*, 1993). Therefore, this region is probably involved in functions universal for all recombination proteins, such as searching and pairing of homologous sequences.

Figure 2. Diagram of RecA/Rad51 protein family. (A) The amino acid homology between Rad51 protein family. Obliquely striped bars represent the homologous core region. Horizontally striped bars represent the region homologous to all Rad51 homologs and vertically striped bars represent the region homologous to Dmc1 homologs. The white bars represent extra residues and the black bars indicate ATP-binding consensus sequences. Adopted from Ogawa *et al.*, 1993. (B) The phylogenetic tree derived from the sequence comparisons in Rad51 family. The numbers indicate the frequency with which a specific branch of the tree is found. Dm, Hs, Sc, and Sp represent *Drosophila melanogaster, Homo sapiens, Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe,* respectively. Adopted from Thacker, 1999.


A
Domain I
Domain II
C-terminal Region
HsRad51, MmRad51 & GgRAD51
SpRad51
ScRad51
RecA
Dmc1
Lim15
NH2
COOH
A-Type
B-Type
"Homologous Core"


B
RAD51-Dm
Rad51-Sc
RAD51-Hs
Rad51-Sp
Rad55-Sc
Rad55-Sp
XRCC2-Hs
XRCC3-Hs
SPNB-Dm
RAD51L2-Hs
Rad57-Sc
RAD51L1-Hs
RAD51L3-Hs
1000
953
973
176
494
997
965
173
368
754

Recently, several genes which show the sequence similarity to Rad51 have been reported in mammals. X-ray repair cross complementing (XRCC) 2 and XRCC3 were isolated in rodent and found to be members of the RecA/Rad51 family (Tambini *et al.*, 1997; Liu *et al.*, 1998; Cartwright *et al.*, 1998). These genes are able to complement cellular defects of two rodent cell lines showing genetic instability and sensitivity to a variety of DNA-damaging agents, indicating their role in DNA maintenance and repair. In addition, at least three human *Rad51*-like genes, *Rad51B* (L1), *Rad51C* (L2), and *Rad51D* (L3), were cloned (Albala *et al.*, 1997; Dosanjh *et al.*, 1998; Kawabata *et al.*, 1999). The phylogenetic tree shows that the XRCC2 protein is most closely related to the Rad55 proteins and the three Rad51-like proteins are most similar to Rad57 in yeast (Fig. 2B). It is possible that these proteins function as cofactors during recombination like yeast Rad55 and Rad57 proteins. Recent data have shown that there are direct interactions between Rad51 protein and Rad51-like proteins or XRCC3 protein (Fig. 3). Interestingly, RAD51 and XRCC3 proteins are found in human cell nuclei following DNA damage. Thus, these proteins are closely related to one another structurally and functionally. However, the precise functions of RAD51-like proteins are still unknown.

The roles of Rad51 protein in DNA repair and recombination

Numerous studies by using genetic, biochemical, and cytological methods have shown that Rad51 protein is involved in DNA repair and recombination. In yeast, the Rad51 mutants show deficiencies in the repair of DNA damage

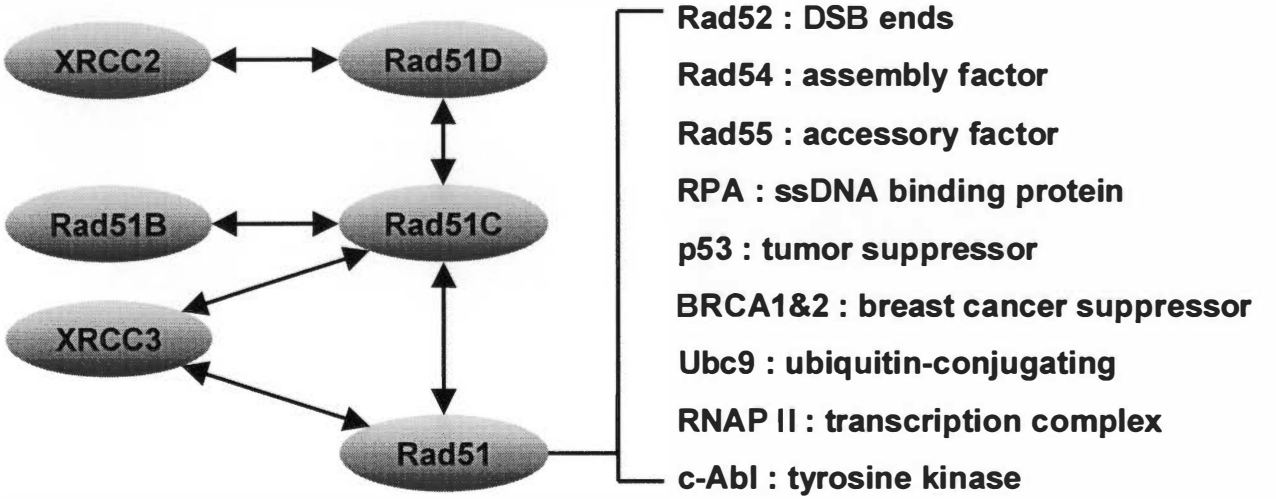


Figure 3. Diagram of the protein interactions between Rad51-like proteins. This diagram also shows various proteins interacting with Rad51.

induced either by treatment with methyl methanesulfonate (MMS) or by irradiation with UV light (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). In addition, the mutants show defects in mitotic recombination as well as meiotic recombination. In contrast to the yeast mutant that is viable, mouse Rad51 mutants are embryonic lethal (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Analysis of the homozygous mutant embryos reveals that cell death occurs after 7.5 days of embryonic development, following a rapid decrease in cell proliferation and increased cell death. These results suggest that mammalian Rad51 protein is essential for cell proliferation. Recently, a chicken cell line was generated in which Rad51 was placed under the control of a repressible promoter (Sonda *et al.*, 1998). Inhibition of Rad51 expression in the chicken cell line caused chromosome breaks, cell-cycle arrest and cell death, indicating that Rad51 plays an essential role in cell proliferation and genome maintenance.

The evidence that Rad51 functions in DNA repair and recombination also comes from Rad51 expression studies. It has been reported using Western blotting analysis that the expression of yeast Rad51 protein was specifically induced 3-fold after MMS treatment over the level present without the treatment (Sinohara *et al.*, 1992), suggesting a role of Rad51 protein in DNA repair. The expression of Rad51 transcript has been studied in the mouse (Morita *et al.*, 1993) and the chicken (Bezzubova *et al.*, 1993). The high level of expression of the Rad51 transcript in the reproductive organs, such as testis and ovary, indicates that the Rad51 is involved in the meiotic recombination. The Rad51 gene is also expressed at a high level in immune organs, such as thymus and

spleen, suggesting the possibility that it could be involved in lymphoid cell-specific reactions, such as recombination during antibody formation and class switching (Bezzubova *et al.*, 1993; Morita *et al.*, 1993). In *Xenopus laevis*, Rad51 is expressed at high levels in the reproductive organs and at low-levels in somatic tissues (Maeshima *et al.*, 1995), suggesting that Rad51 is involved not only in meiosis but also in recombinational repair and DNA maintenance.

Lastly, immunocytological studies have revealed roles of Rad51 protein in DNA repair and meiotic recombination. In yeast, the kinetics and timing of appearance and disappearance of Rad51 foci have been reported to coincide with the occurrence of double-strand breaks, suggesting a function of yeast Rad51 protein in DNA repair (Bishop, 1994). Yeast Rad51 is required for heteroduplex formation, implicating a possible role of Rad51 protein in searching for homology and synaptic initiation during zygonema (Bishop, 1994). It was also demonstrated that Rad51 protein is required to establish or stabilize axial associations between homologous chromatids (Rockmill *et al.*, 1995). These results indicate that yeast Rad51 may function in meiotic recombination.

It has been reported that human RAD51 foci were increased significantly after treatments that induced DNA damage, suggesting a role of mammalian RAD51 in DNA repair (Haaf *et al.*, 1995). It was demonstrated using an antibody against human Rad51 protein that Rad51 protein was localized on meiotic chromatin in mouse spermatocytes and oocytes as well as chicken oocytes during sequential stages of meiosis (Ashley *et al.*, 1995; Barlow *et al.*, 1997). The results suggest that the RAD51 protein is functionally involved in multiple

steps of meiosis from before synaptic initiation through metaphase I although the precise role and mechanism remain to be determined.

Protein interactions and regulation of Rad51

Due to its central role in recombination, Rad51 is likely to be a target for regulatory factors that coordinate DNA repair, transcription, replication, and cell-cycle progression (Fig. 3). The tumor suppressor p53 protein is one of the factors that may interact directly with human Rad51 protein (Buchhop *et al.*, 1997). The p53 protein has a well-established role in linking cell cycle progression with genome integrity (reviewed in Giaccia and Kastan, 1998; Prives and Hall, 1999). This function is likely to require contact with the DNA-repair machinery, such as Rad51. However, there is no indication that the presence of p53 affects the activities of Rad51 protein. Recently, it has been revealed that there is a link between Rad51 and the tumor suppressors Brca1 and Brca2, which have been implicated in breast cancer (Chen *et al.*, 1999). The immunofluorescence staining patterns of human RAD51 and BRCA1 in mitotic S-phase cells and in spermatocytes during meiotic prophase showed significant, albeit not complete, colocalization of the two proteins (Scully *et al.*, 1997). Furthermore, a small fraction of RAD51 was found to co-immunoprecipitate with endogenous BRCA1 (Scully *et al.*, 1997). Interactions between human RAD51 and BRCA2 have been demonstrated using yeast two-hybrid analysis (Sharan *et al.*, 1997). Both Brca1 and Brca2 null mutant mice result in embryonic lethality like Rad51 null mice (Liu *et al.*, 1996; Hakem *et al.*, 1997; Sharan *et al.*, 1997).

Brca1 and Brca2 null strains are defective in cell proliferation during embryonic development. The homozygous mutant blastocytes from Brca2 null mice are sensitive to gamma irradiation. Although the functions of Brca1 and Brca2 in homologous recombination and recombinational repair are not fully understood, it is possible that they have a role in monitoring genome integrity or in regulation of certain DNA-repair processes.

Rad51 also interacts with a ubiquitin-conjugating enzyme (Ubc9) (Kovalenko *et al.*, 1996), a ubiquitin-like protein 1 (Ubl1) (Li *et al.*, 2000), and a tyrosine kinase (c-Abl) (Yuan *et al.*, 1998; Chen *et al.*, 1999). Further studies will be necessary to investigate the roles of Rad51 in the interactions with these molecules.

CHAPTER 2

DROSOPHILA RAD51 (RAD51*DM*)

The *Drosophila* homolog of RecA (Rad51dm) has been isolated (Akaboshi *et al.*, 1995; McKee *et al.*, 1996). Rad51dm consists of two exons (exon 1 is 78 bp and exon 2 is 924 bp) separated by a 63 bp intron (Fig. 4). The deduced protein sequence is most closely related to the yeast and vertebrate Rad51 proteins, and thus, it is classified as a member of the Rad51 family. Transcription of the Rad51dm gene was studied by Northern analysis, RNase protection assay (RPA), and reverse-transcribed polymerase chain reaction (RT-PCR). It was demonstrated that Rad51dm is expressed widely throughout development, albeit at rather low levels (McKee *et al.*, 1996). It has also been shown that the transcript levels were much higher in ovary than in testis or any other adult tissues, suggesting a role of Rad51dm in meiotic recombination (McKee *et al.*, 1996). *In situ* hybridization of Rad51dm to polytene chromosomes from larval salivary glands revealed that the Rad51dm gene is located near the tip of chromosome arm 3R in subregion 99D (McKee *et al.*, 1996). Southern blot analysis under low-stringency conditions revealed several bands hybridized with Rad51dm probe (McKee *et al.*, 1996). This result suggests the presence of a multi-gene family related to Rad51dm.

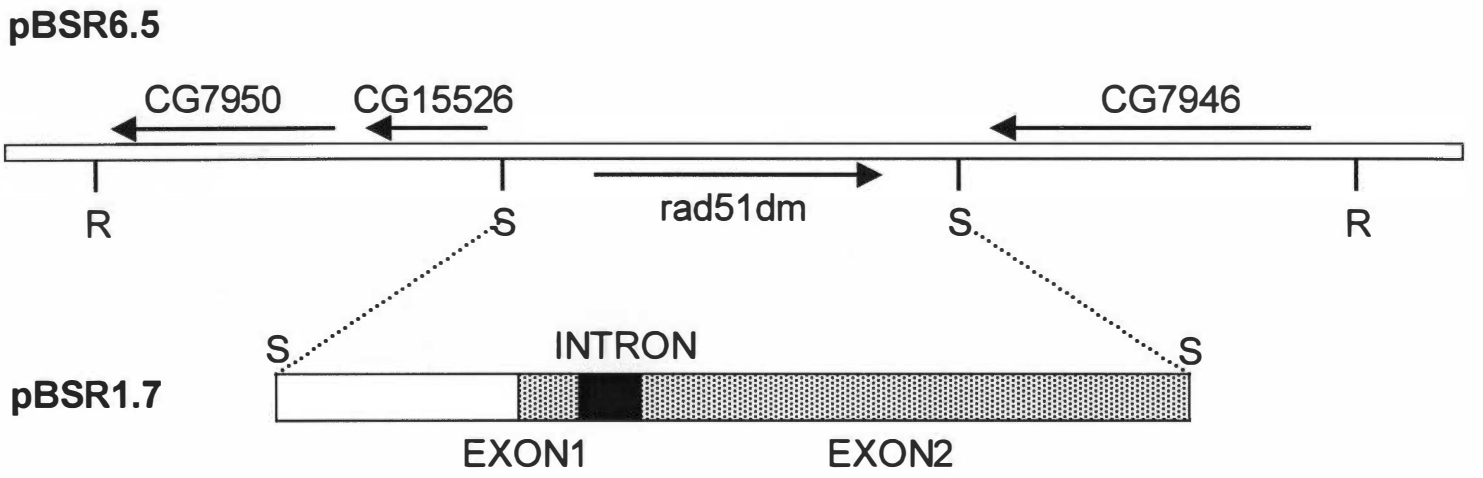


Figure 4. Schematic diagram of the Rad51dm gene organization. pBSR6.5 represents pBluescript plasmid carrying 6.5 Kb genomic fragment containing Rad51dm gene and three conceptual gene (CG). pBSR1.7 represents pBluescript plasmid carrying 1.7 Kb fragment containing Rad51dm gene. Rad51dm gene consists of two exons (gray boxes) separated by a short intron (63 bp) (a black box). R and S stand for *EcoR*I and *Sst*I, respectively.

The objectives of the research described in Part II are to investigate the roles of Rad51dm protein in DNA repair, cell cycle progression, and apoptosis by overexpression of Rad51dm protein using a heat-shock inducible promoter and the UAS/GAL4 binary expression system. The objectives of the research described in Part III are to study the roles of Rad51dm protein in DNA repair and recombination by the down-regulation of Rad51dm using anti-sense (AS) and inverted-repeat (IR) Rad51dm transgenic animals. The results will help elucidate the *in vivo* functions of Rad51dm protein particularly in cell cycle regulation and cell death.

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PART 2

OVEREXPRESSION OF *DROSOPHILA* RAD51 PROTEIN DISRUPTS CELL CYCLE PROGRESSION AND LEADS TO APOPTOSIS

* This part is intended to be submitted for publication as Yoo, Siuk and B. D. McKee (2001).

CHAPTER 1

INTRODUCTION

The Rad51 protein, a homolog of *E. coli* RecA protein, is a central enzyme in both meiotic recombination and recombinational repair of double-strand DNA breaks caused by ionizing radiation or chemical mutagens (reviewed in Baumann and West, 1998). It is a highly conserved protein among eukaryotes and possesses DNA-dependent ATPase activity, both double strand and single strand DNA binding activities, and homologous DNA pairing and strand exchange activity (reviewed in Edelmann and Kucherlapati, 1996).

The functions of Rad51 protein in DNA repair and recombination have been extensively studied in yeast and mammals by biochemical, cytological and genetic approaches. *In vitro* studies with yeast and human Rad51 proteins revealed a crucial role of Rad51 protein in the process of recombination (Sung, 1994; Baumann and West, 1997). Rad51 interacts with DNA to form a DNA-protein complex and catalyzes pairing and exchange of strands between two homologous DNA molecules in the presence of cofactors such as ATP, single strand DNA binding protein, and Rad52 epistasis group proteins which are Rad52, Rad54, Rad55, and Rad57 (Benson *et al.*, 1998; Petukhova *et al.*, 1998; Sung, 1997). Several immunocytochemistry studies have revealed that Rad51 forms discrete nuclear foci with components of the synaptonemal complex

(lateral element proteins) at early prophase I, suggesting that RAD51 is involved in meiotic recombination (Ashley *et al.*, 1995; Plug *et al.*, 1996; Moens *et al.*, 1997; Barlow *et al.*, 1997). It has been also shown that RAD51 foci increase significantly in somatic cells after MMS treatment or ultraviolet irradiation (Haaf *et al.*, 1995). In addition, mutations in the yeast Rad51 gene caused an increase in X-ray and MMS sensitivities, the inability to repair double-strand DNA breaks, a decrease in meiotic recombination, and an increase in chromosome loss (Shinohara *et al.*, 1992; Ogawa *et al.,* 1993). These results indicate the central functions of Rad51 in DNA repair and recombination. Yeast Rad51-deficient cells have been shown to be viable. The homozygous null mutation of murine Rad51, however, results in very early embryonic lethality, following a rapid decrease in cell proliferation and increase of cell death, indicating the importance of Rad51 protein in cell growth (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). A similar observation has been made for a Rad51 deficient chicken cell line (Sonoda *et al.*, 1998). The inhibition of Rad51 expression in the Rad51 deficient chicken cell line led to chromosome breaks, cell-cycle arrest, and cell death, suggesting an essential function of mammalian Rad51 in cell proliferation and maintenance of chromosome integrity.

In contrast to the numerous studies of Rad51 in DNA repair and recombination, few studies have addressed its role in cell cycle regulation and apoptosis. It has been reported that after release of G_1-synchronized cells, the transcript level of yeast Rad51 increases at the G_1/S transition period (Basile *et al.*, 1992). This cell-cycle dependent expression pattern was also observed in

the m5S non-malignant mouse cell line (Yamamoto *et al.,* 1996). The abundance of Rad51 transcript and protein from late G_1/S to G_2 stage suggests that Rad51 is involved in DNA replication. However, it remains unclear whether Rad51 is directly involved in cell cycle regulation. Recently, several lines of evidence indicate a possible role of Rad51 protein in apoptosis. It has been shown that human Rad51 protein is proteolytically cleaved in T-lymphocyte cell lines during apoptosis induced by camptothecin, topoisomerase I inhibitor, or anti-Fas monoclonal antibody (Flygare *et al.*, 1998). It was also demonstrated that overproduced Rad51 protein in the U-937 cell line is cleaved by a caspase-mediated mechanism after induction of apoptosis by ionizing radiation (Huang *et al.*, 1999). According to a model proposed, the proteolytic inactivation of Rad51 by a caspase contributes to the cell death response induced by DNA damage. In addition, it has been shown that after gamma irradiation, most Rad51 foci are sequestered into micronuclei or assembled into Rad51-coated DNA fibers that display genome fragmentation that are typical of apoptotic cell death (Haaf *et al.*, 1999). This result suggests that mammalian Rad51 protein associates with damaged DNA that is temporarily or irreversibly unable to replicate and these foci may subsequently be eliminated from the nucleus. Collectively, although no direct evidence has been reported, these results suggest that Rad51 might contribute directly or indirectly cell cycle progression and apoptosis as a sensor of DNA damage that arrests the cell cycle to allow DNA repair or cell death to occur.

There are functional interactions among the Rad52 epistasis group proteins during homologous recombination. It is believed that Rad55-Rad57 heterodimers stabilize a complex that is involved in the initial stages of recombination (Johnson and Symington, 1997) and Rad54 stimulates Rad51-mediated homologous pairing reaction by promoting a heteroduplex joint formation (Petukhova *et al.*, 1998). The Rad52 protein also facilitates filament formation by binding to ss DNA ends (Gasior *et al.*, 1998; New *et al.*, 1998). Studies have shown that overexpression of Rad51 rescues the Rad55 and Rad57 null mutant phenotypes and partially suppresses defects caused by Rad52 mutations (Hays *et al.*, 1995; Schild *et al.*, 1995; Asleson *et al.*, 1999). Additionally, overexpression of hamster Rad51 protein in CHO cells stimulated the homologous recombination and enhanced resistance to ionizing radiation (Vispé *et al.*, 1998). A similar result was also observed when human Rad52 protein was overexpressed in monkey cells (Park, 1995). Recently, the overexpression of human Rad51 protein in human cells increases the efficiency of gene targeting and enhances resistance to gamma rays (Yanez *et al.*, 1999).

Previously, the *Drosophila* homolog of RecA (Rad51dm) has been isolated (Akaboshi *et al.*, 1995; McKee *et al.*, 1996). The preliminary molecular analyses of Rad51dm have shown that the Rad51dm gene is located in the 99D region of the third chromosome right arm (3R) and is present at one copy per haploid genome (McKee *et al.*, 1996). Its expression level is dramatically higher in ovaries than testes or other adult tissues, suggesting a role in female meiosis.

In this study, we have demonstrated that Rad51dm is involved in the repair of damaged DNA and its transcript level is increased 2-3 fold after X-ray or MMS treatment. It was also shown that the Rad51dm protein has DNA-binding activity and is exclusively localized in the nucleus of spermatocytes during prophase I stage. These results suggest that the properties of Rad51dm are identical to other Rad51 proteins. To understand functions of Rad51dm protein *in vivo*, we examined the consequences of overexpression of Rad51dm protein on DNA repair, cell cycle regulation, and apoptosis. We generated transgenic flies using both a heat shock inducible promoter and the UAS-GAL4 binary expression system. Interestingly, it was demonstrated that overexpression of Rad51dm protein leads to lethality, in contrast to the results observed from other studies using yeast and mammals that showed decreased sensitivity to MMS. In addition, immunocytochemistry studies showed that Rad51dm protein alters the pattern of mitosis and induces apoptosis in imaginal discs. Overexpression of N-terminal deleted Rad51dm protein, however, did not affect viability. These findings contribute to the understanding at the roles of Rad51 protein in cell cycle regulation and apoptosis.

CHAPTER 2

MATERIALS AND METHODS

Fly stocks and culture

Df(3R)X3F/TM3, Dp(3;1)92, and various GAL4 lines were obtained from the Bloomington Stock Center. Df(3R)X3F/TM3 carries a deficiency in a distal region of third chromosome at 99D region and Dp(3;1)92 contains the terminal portion of distal 3R appended to the right arm of the X chromosome (Frisardi and McIntyre, 1984). C96-GAL4 expresses the GAL4 in prospective wing margin in imaginal discs (Gustafson and Boulianne, 1996). glass-GAL4 provides strong expression of GAL4 in all cells behind the morphogenetic furrow in eye discs (Freeman, 1996). MVD1-GAL4 expresses GAL4 in primordial germ cells (Doren *et al.*, 1998). UAS-p35(2), UAS-p35(3), and UAS-reaper lines were kindly provided by J. Park. Flies were reared on standard cornmeal medium at 22° C with the exception of heat-shock treatment in a 37° C incubator.

Mutagen treatments

To test sensitivity to methyl methanesulfonate (MMS) (Boyd *et al.*, 1976), males from Dp(3:1)92 stock carrying 3 copies of the Rad51dm gene were crossed with Df(3R)X3F virgin females carrying 1 copy of the Rad51dm gene. After 3 days, the parents were discarded and developing cultures were treated with MMS at various concentrations (0.001% - 0.01%). Control cultures were left

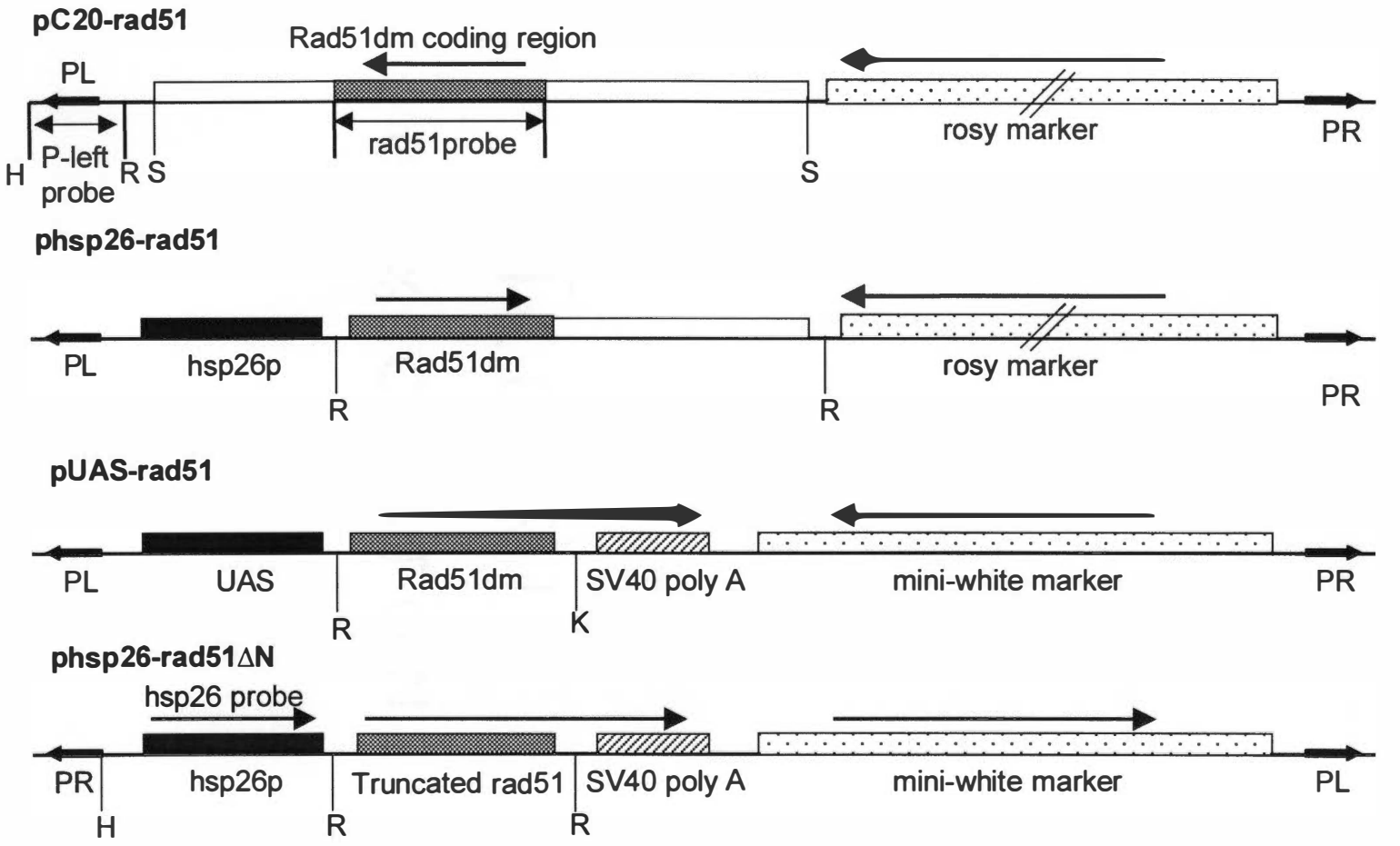
untreated. Eclosed F1 adults were scored up to day 21 to determine the survival rate. To measure the transcript levels of the Rad51dm gene after induction of DNA damage, adult flies aged 3-5 days post-eclosion were either administered 0.1% MMS in 1% sucrose solution for 12 hr or irradiated with 30 kR X-rays. The flies were transferred into a fresh medium for 12 hr (MMS treatment) or 2 hr (X-ray irradiation) for recovery. Poly $(A)^+$ RNA from adult flies was prepared according to the manufacturer's protocol (5 prime-3 prime). RNA slot blots were carried out by standard protocol (Sambrook *et al.* 1989).

Plasmid constructs for transgenic animals

To overexpress the Rad51dm protein *in vivo*, pC20-rad51, phsp26-rad51, phsp26-rad51ΔN, and pUAS-rad51 constructs were generated (Fig. 1). To generate the pC20-rad51 plasmid, the Rad51dm gene was inserted into Carnegie 20 vector (Rubin and Spradling, 1983). From the Rad51dm clone in pBluescript KS vector (pBS-rad51), a 6.5 Kb genomic fragment containing full-length Rad51dm gene was excised by *EcoR*I digestion, end-filled by Klenow fragment, and ligated with *EcoR*I (*Not*I) adaptor containing *Sal*I site (GIBCO/BRL). After *Sal*I digestion, the fragment was cloned into the *Sal*I site of Carnegie 20 to produce pC20-Rad51.

For the phsp26-rad51 construct, a polymerase chain reaction (PCR) was performed to obtain the 0.9 Kb heat shock protein 26 (hsp26) promoter region using genomic DNA as a template and H26F (5'GTTAACCAAGTGAAGACTG

Figure 1. Schematic diagram of plasmid constructs for transgenic animals. Carneigie 20 vector carrying rosy marker was used for pC20-rad51 and phsp26-rad51 constructs and CaSpeR4 vector containing mini-white gene was used for pUAS-rad51 and phsp26-rad51ΔN constructs. The arrows indicate the orientation of genes. The probes used for Southern blot analysis are shown at the top of constructs indicated by thin arrows. Gray boxes; Rad51dm coding region, black boxes; promoter regions, hatched boxes; poly A region, dotted boxes; marker genes, PL;P-element left end, PR; P-element right end, UAS; upstream activating sequence, S; *Sal*I, R; *EcoR*I, K; *Kpn*I, and H; *Hind*III.


pC20-rad51
Rad51dm coding region
PL
rad51probe
rosy marker
PR
H
P-left
probe
R S
S
phsp26-rad51
PL
hsp26p
Rad51dm
rosy marker
PR
R
R
pUAS-rad51
PL
UAS
Rad51dm
SV40 poly A
mini-white marker
PR
R
K
phsp26-rad51ΔN
hsp26 probe
PR
hsp26p
Truncated rad51
SV40 poly A
mini-white marker
PL
H
R
R

AACTA3'; *Hpa*I site is underlined) and H26R (5'TATGTTCCTTTTGCGAGATT3') as primers. The PCR fragment was cloned into the *EcoR*V site of pBluescript KS(-) to produce pBS-hsp26. To delete the upstream of the start codon of the Rad51dm gene in pC20-rad51, a *Hpa*I-*Swa*I fragment of pC20-rad51 was excised and replaced with the *Hpa*I-*Sma*I fragment of hsp26 promoter region from pBS-hsp26 to produce phsp26-rad51. pC20-rad51 and phsp26-rad51 constructs were microinjected into ry embryos.

For the phsp26-rad51ΔN construct, an inverse-PCR was carried out to delete the N-terminal region corresponding to amino acid 18-99 using RAFM1 (5'GGACGCGTGTGCCTCTGGGCTTCCTTAGT3'; *Mlu*I site is underlined) and RARM1 (5'GGACGCGTTTCTTCCTCCTCCTCTTCCTG3'; *Mlu*I site is underlined) as primers and pBS-rad51 as a template. The linearized PCR fragment was digested with *Mlu*I enzyme and self-ligated to produce pBS-RΔN. The upstream region of the start codon of Rad51dm gene was deleted by digestion with *Swa*I/*EcoR*I. After end-filling, this Rad51dm fragment was cloned into the *Sma*I site of pBS-hsp26 to produce pBS-HRΔN. PCR was performed using H26F and K-Stop-R (5'GGGGGATCCCTAGCTCTCCCTGGCGTCTCC3'; *Kpn*I site is underlined) as primers and pBS-HRΔN as a template. The PCR fragment was digested with *Kpn*I enzyme and cloned into *Stu*I/*Kpn*I sites of pCaSpeR4-polyA vector to produce phsp26-rad51ΔN.

For the pUAS-rad51 construct, reverse-transcribed PCR (RT-PCR) was carried out. Briefly, poly $(A)^+$ RNA was isolated from adult flies and cDNAs were

obtained by reverse transcription using an oligo dT primer and Superscript II reverse transcriptase (GIBCO/BRL). Amplification of Rad51dm cDNA was performed using R-Start-F primer (5'GGGAATTCATGGAGAAGCTAACGAA TGTT3'; *EcoR*I site is underlined) and K-Stop-R primer. The PCR fragment was digested with *EcoR*I and *Kpn*I enzymes and cloned into the *EcoR*I and *Kpn*I sites of pUAST vector to produce pUAS-rad51. These constructs, phsp26-rad51ΔN and pUAS-rad51, were microinjected into yw embryos. All transgenic lines were confirmed by Southern blot analysis, and the expression levels of Rad51dm were determined by Northern blot and Western blot analyses.

Generation of polyclonal antibody

Anti-Rad51dm polyclonal antibody was produced by standard protocols using a full-length Rad51dm protein containing 6 additional histidine residues at the C-terminus as an antigen (Harlow and Lane, 1988). To delete the intron of the Rad51dm, an inverse-PCR was carried out using pBS-rad51 as a template. RAFM2 (5'GGACGCGTGGTCGCAGCATCACGGCCAAG3'; *Mlu*I is underlined) and RARM2 (5'GGACGCGTTATTAACTTAGTCACGCTGAG3'; *Mlu*I is underlined) were used as primers. The PCR fragment was digested with *Mlu*I and self-ligated to produce pBS-RΔIn. The full-length coding region of Rad51dm gene was amplified by PCR using RAFM4 (5'GGCATATGGAGAAGCTAACGA AT3'; *Nde*I site is underlined) and RARM4 (5'GGCTCGAGGCTCTCCCTGGCGT CTCC3'; *Xho*I site is underlined) as primers and pBS-RΔIn as a template. After

*Nde*I and *Xho*I digestion, the PCR fragment was cloned into pET30b expression vector to generate pRPNX and transformed into BL21(DE3) strain of *E. coli*.

The recombinant Rad51dm (rRad51dm) protein was expressed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 hr and purified by affinity chromatography using a nickel-chelating column according to manufacturer's protocol (Novagen). For immunization, the purified rRad51dm protein was dialyzed against phosphate buffered saline (PBS) at pH 7.4 containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl and injected subcutaneously into rabbits every 4-6 weeks. To measure titer of the antibody, the immunized rabbit serum was tested by Western blot analysis.

DNA-binding assays

The rRad51dm protein was prepared as described above with some modifications. After IPTG induction, the cells were harvested and lysed in Tris-buffered saline (TBS) at pH 7.5 (25 mM Tris base, 3 mM KCl, and 140 mM NaCl), 1 mM dithiothreitol (DTT), 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride (PMSF) and sonicated. After elution of the protein from a nickel column, the protein was dialyzed against storage buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 0.5 mM DTT, and 5% glycerol) and frozen at -80° C.

For agarose gel mobility shift assay (Benson *et al.*, 1994; Zaitseva *et al.*, 1999), 50 ng of either double-stranded (ds) circular ϕX 174 DNA or single-stranded (ss) ϕX 174 DNA was mixed with varying concentrations of the

rRad51dm protein in DNA binding buffer (30 mM Tris-HCl, pH 7.5, 2 mM ATP, 2 mM DTT and 10 mM $MgCl_2$) to make final 30 μl of reaction mixture and incubated for 10 min at 37° C. After 5 μl of sample loading buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA, 50% glycerol, and bromophenol blue) were added to each reaction, the DNA-protein complexes were analyzed by electrophoresis on an agarose gel in Tris-acetate/EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 4° C.

For restriction enzyme protection assay (Adams *et al.*, 1994), 30 μl of reaction mixture containing 40 μg of the protein and 50 ng of ds ϕX 174 DNA was incubated for 40 min and the enzyme digestion was performed for 20 min at 37° C using 5u of *BssH*I, *Pst*I, and *Xho*I. To inactivate the restriction enzymes, the stop solution (2 μl of 0.5 M EDTA and 1 μl of 10% SDS) was added. After incubation for 10 min at 65° C, the mixture was treated with 1 μl of proteinase K (10 mg/ml) for 10 min at 37° C and run on an agarose gel in Tris-borate/EDTA (TBE) buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0).

Immunoblot analysis

Total proteins were extracted from embryos, larvae, testes, ovaries, male carcass (without testes), and female carcass (without ovaries). Each sample was mixed with approximately equal volume of 6 M urea in TBS buffer containing 1 mM DTT, 1% Triton X-100, 2% sodium dodecyl sulfate (SDS) and 1 mM PMSF, homogenized with 0.1 mm glass beads, and incubated on ice for 1 hr.

After centrifugation, the soluble fraction was mixed with equal volume of SDS sample buffer (Laemmli, 1970) and boiled for 10 min. 10 μg of proteins were electrophoresed on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose by electroblotting.

Rad51dm proteins were detected by chemiluminescent method according to the manufacturer's protocol (Tropix). Briefly, the membrane was soaked in PBS buffer containing 0.2% casein and 0.1% Tween-20 (blocking buffer), incubated with anti-Rad51dm polyclonal antibody (1:1000 dilution in blocking buffer) for 12 hrs and then with the secondary Ab [alkaline phosphatase (AP) conjugated goat anti-rabbit immunoglobulin (Ig) G] diluted 1:5,000 with blocking buffer for 1 hr. Following incubation with CDP-star™, a chemiluminescent substrate for AP, the membrane bound Rad51dm proteins were visualized on X-ray film.

Immunofluorescent staining

Embryos were collected on grape-agar plates, dechorionated with 50% bleach for 5 min, and placed in an equal volume of heptane and 3.7% formaldehyde in PBS solution for 30 min (Ashburner, 1989). After removing the lower phase (fixative), an equal volume of methanol was added and the samples were shaken vigorously for 1 min for devitellization. After rehydration with 3.7% formaldehyde in PBS solution for 30 min, the samples were incubated for 30 min in Detergent/Tween-20 solution containing 1% SDS, 0.5% Twen-20, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl. After blocking in 10% normal goat

serum for 1 hr, the samples were incubated with anti-Rad51dm antiserum diluted 1: 100 with PT solution (PBS containing 0.2% Triton X-100) for 12 hr, and then with the secondary Ab [fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG] diluted 1:150 with PT solution for 1 hr. The samples were washed with PT solution and counterstained with 4'6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) for 5 min.

For immunofluorescent staining of spermatocytes, adult testes were dissected in testis buffer (Ashburner, 1989) and transferred to a clean microscope slide, squashed gently by covering with a siliconized cover slip, and frozen in liquid nitrogen. After removal of the cover slip, the samples were fixed in methanol at –20° C for 5 min, then in acetone at –20° C for 1 min, and finally in PBS containing 1% Triton X-100 and 0.5% acetic acid for 10 min at room temperature (Pisano *et al.*, 1993). The antibody staining and DAPI staining were performed as described above.

For immunofluorescent staining of imaginal discs, third instar larval wing and eye discs were prepared and stained by the method described by Ollmann *et. al.* Briefly, the imaginal discs were dissected in PBS, fixed in 2% formaldehyde for 30 min, permeabilized in 0.5% Triton X-100 for 15 min, blocked in 10% goat serum for 1 hr, and incubated with primary Ab (1:100 dilution). After incubation with secondary antibody (Taxas Red-labeled goat anti rabbit IgG) for 1 hr, the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was performed to detect apoptotic cells in imaginal discs using the ApoAlert DNA fragmentation Assay kit (Clonetech). The samples were equilibrated with equilibration buffer

(200 mM Potassium cacodylate, pH 6.6, 25 mM Tris-HCl, 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM Cobalt chloride) for 10 min. After adding nucleotide mixture and terminal deoxynucleotidyl transferase (TdT), the samples were further incubated at 37° C for 1 hr and washed twice with 2X SSC for 15 mim to terminate the reaction.

To detect mitotic cells in eye imaginal discs, anti-phospho-histone H3 staining was performed using an Anti-phospho-histone H3 Mitosis Marker according to the manufacturer's protocol (Upstate Biotechnology). After fixing and washing described above, the samples were incubated with primary antibody diluted 1:500 with PT solution and counterstained with propidium iodide (0.5 μg/ml) for 5 min. Discs were mounted in anti-fade reagent (Vector Laboratories, Inc.), and images were obtained on a Leica confocal microscope.

CHAPTER 3

RESULTS

The roles of Rad51dm in response to DNA damaging agents

One of the important roles of Rad51 protein is to repair DNA damaged by X-ray irradiation or chemical mutagens such as MMS. To test whether Rad51dm is involved in the DNA repair process, larval sensitivity to MMS was measured. First, we determined the copy number of Rad51dm gene by Southern blot analysis using various stocks either deleted or duplicated for the distal portion of the right arm of the third chromosome where Rad51dm is located (Frisardi and McIntyre, 1984; Kongsuwan *et al.*, 1986) (Fig. 2). We found that Df(3R)X3F/TM3 which is deficient for 99D1-D2 to 99E1, carries 1 copy of the Rad51dm gene, whereas Dp(3;1)92 carries an extra Rad51dm gene on the X chromosome. To generate flies carrying 1, 2, or 3 copies of Rad51dm gene, Df(3R)X3F/TM3 females were crossed with Dp(3;1)92 males (Fig. 3A). After 3 days of egg laying, developing larvae were treated with MMS and the survival rate was determined. At the lowest concentration of MMS (0.001%), almost no significant difference was observed among progeny groups (Fig. 3B). As MMS dose increases, however, the survival rate of the Sb^+ male flies carrying 1 copy of the Rad51dm gene dramatically decreases compared to those of Sb males carrying 2 copies of the Rad51dm gene (Fig. 3B). Additionally, the survival rate of the Sb^+ females

Figure 2. Southern blot analysis of deficiencies to determine the copy number of Rad51dm gene. Genomic DNA digested with *EcoR*I was hybridized with rad51dm (A) and rosy (B) probes. Rosy probe was used for normalization. The band intensity was measured by an Instant-*Imager*. The copy number of the Rad51dm gene was determined by the band intensity of Rad51dm gene divided by the band intensity of rosy gene indicated by the arrows. The arrow heads indicate extra copies of the rosy gene in TM3 balancer chromosome.

lane 1;Oregon R

lane 2; Dp(3;1)124P;Df(3R)B81,P[rp49]/TM3 (male),

lane 3; Dp(3;1)124P;Df(3R)B81,P[rp49]/TM3 (female),

lane 4; Df(3R)B81,P{ry[+}=rp49}F2-80A e[1]/TM3;Dp(3;1)67A(male)

lane 5; Df(3R)B81,P{ry[+}=rp49}F2-80A e[1]/TM3;Dp(3;1)67A(female)

lane 6; Df(3R)R133,B[S]/TM3;Dp(3;1)124P (male)

lane 7; Df(3R)R133,B[S]/TM3;Dp(3;1)124P (female)

lane 8; Df(3R)X3F, P{ry[+}=rp49}A3-84F e[1?]/TM3 (male)

lane 9; Dp(3;1)92/C(1)DX,y[1];ca[1],bv[1] (male)

lane 10; Dp(3;1)R16/C(1)DX,y[1];ca[1],bv[1] (male)

lane 11; Dp(3;1)67N;ca[1],bv[1] (male)

lane 12; Dp(3;1)67N;ca[1],bv[1] (female)

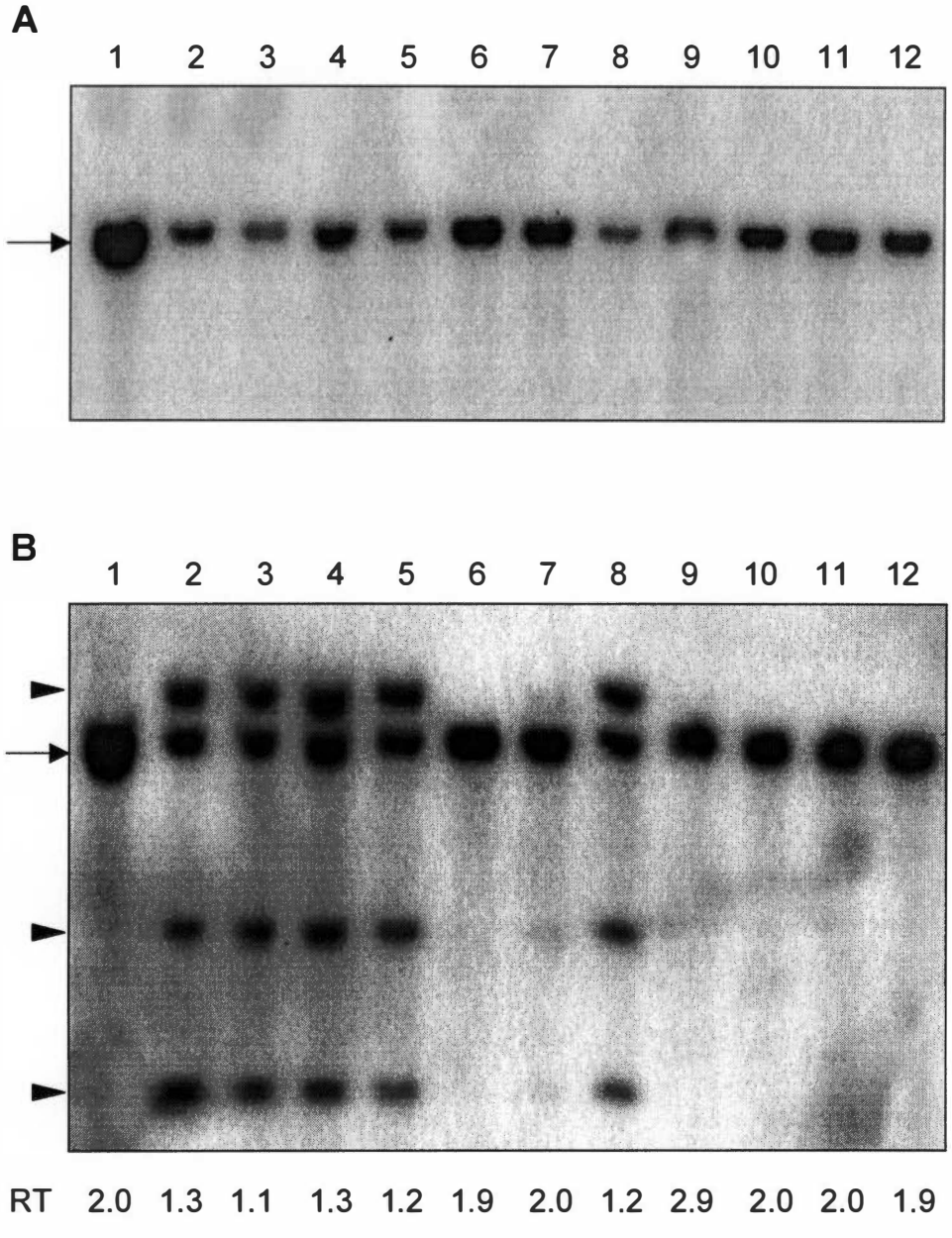
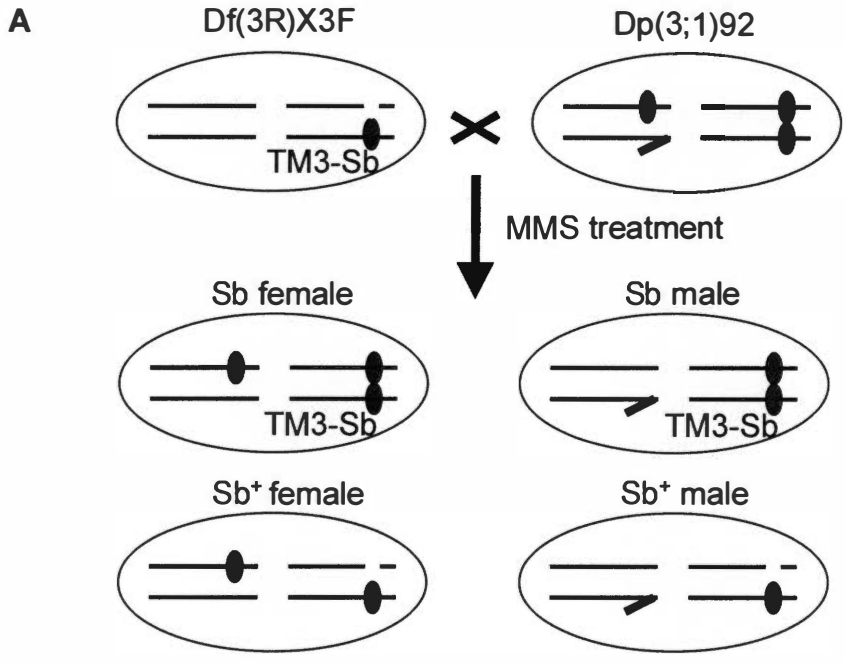
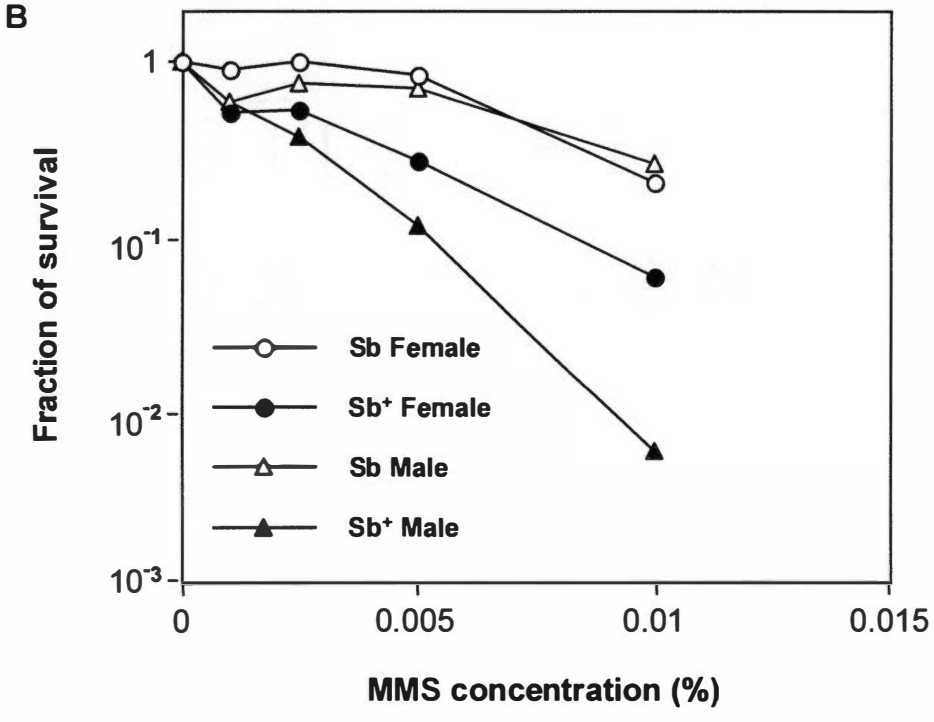

A
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11 12
RT 2.0 1.3 1.1 1.3 1.2 1.9 2.0 1.2 2.9 2.0 2.0 1.9

Figure 3. Analysis of survival rate of larvae after treatment with MMS. (A) Cross scheme for MMS sensitivity experiment. Males from the Dp(3;1)92 stock carrying 3 copies of Rad51dm were crossed with Df(3R)X3F/TM3 virgin females carrying 1 copy of the Rad51dm gene. The parent flies were allowed to lay eggs for 3 days and discarded. At that time 0.1% MMS (V/V) was added to the medium (experiment group). Progeny were classified by their sex and the Sb marker on TM3 balancer chromosome and counted. (B) Survival rates of larvae after MMS treatment. Final concentrations of MMS were calculated by the volume of 0.1% MMS injected into medium divided by the weight of medium (V/W). The survival rates were determined by the average number of progeny per vial in each MMS group divided by the average number of progeny per vial in control group.


A
Df(3R)X3F
Dp(3;1)92
TM3-Sb
MMS treatment
Sb female
Sb male
TM3-Sb
TM3-Sb
Sb+ female
Sb+ male


B
Fraction of survival
1
10-1
10-2
10-3
Sb Female
Sb+ Female
Sb Male
Sb+ Male
0
0.005
0.01
0.015
MMS concentration (%)

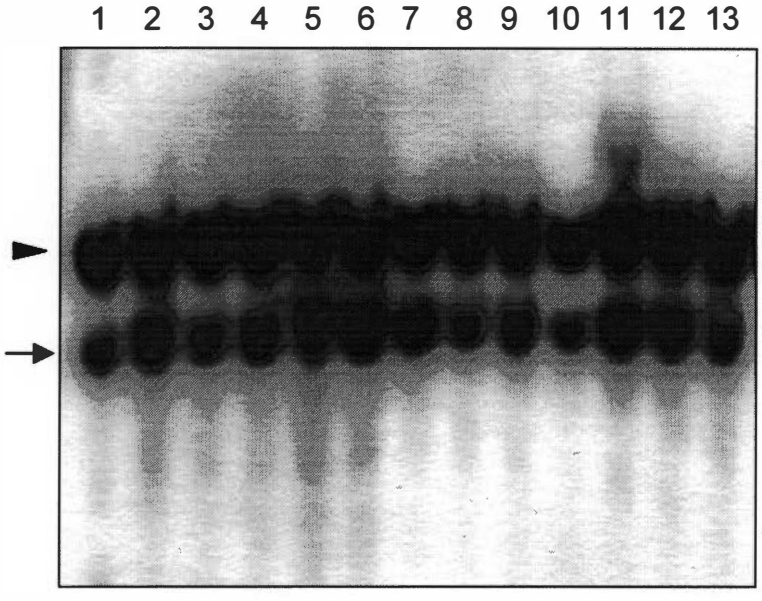
carrying 2 copies of Rad51dm gene was significantly lower than that of the Sb females carrying 3 copies of Rad51dm gene.

To determine if larvae carrying extra copies of the Rad51dm genes show more resistance to MMS, we generated transgenic flies carrying the 6.5 Kb genomic fragment containing the Rad51dm gene (pC20-rad51) (Fig. 4). Homozygote lines were crossed with each other to produce transgenic flies carrying four Rad51dm transgenes and the sensitivity of these flies to MMS was measured. The survival rate of Oregon R (wild type) larvae was 0.1, in contrast, the transgenic flies carrying four Rad51dm genes (two from endogenous gene and two from transgene) showed 0.22-0.55 survival at 0.005% MMS dose. Moreover, the transgenic flies carrying six Rad51dm genes (two from endogenous gene and four from transgene) showed more resistance to MMS (survival rate: 0.35-0.90) (Fig. 5). Although there are the variations of survival rates among transgenic lines due to position effects, these results suggest a role of Rad51dm in repair of DNA damage.

To investigate whether the expression of Rad51dm is induced by DNA damaging agents, poly $(A)^+$ RNA was isolated from adult flies either treated with MMS or irradiated by X-rays and analyzed by RNA slot blot and Northern blot. The results showed that the expression of Rad51dm gene increases 2- to 3-fold by treatment with MMS or irradiation of X-rays (Fig. 6). The results shown in Figs. 3, 5, and 6 suggest that Rad51dm is involved in DNA repair like other Rad51 homologs.

Figure 4. Southern blot analysis of transgenic lines carrying a 6.5 Kb genomic DNA fragment including Rad51dm gene. (A) Genomic DNA digested with *Sal*I was hybridized with rad51 probe. The arrow head represents endogenous Rad51dm gene and the arrow indicates Rad51dm transgene. (B) Genomic DNA digested with EcoRI was hybridized with P-left probe. The different size of DNA fragments indicate that the transgene is inserted into genome at different locations. The probes are shown in Fig. 1 and the numbers indicate each transgenic line.

A



B

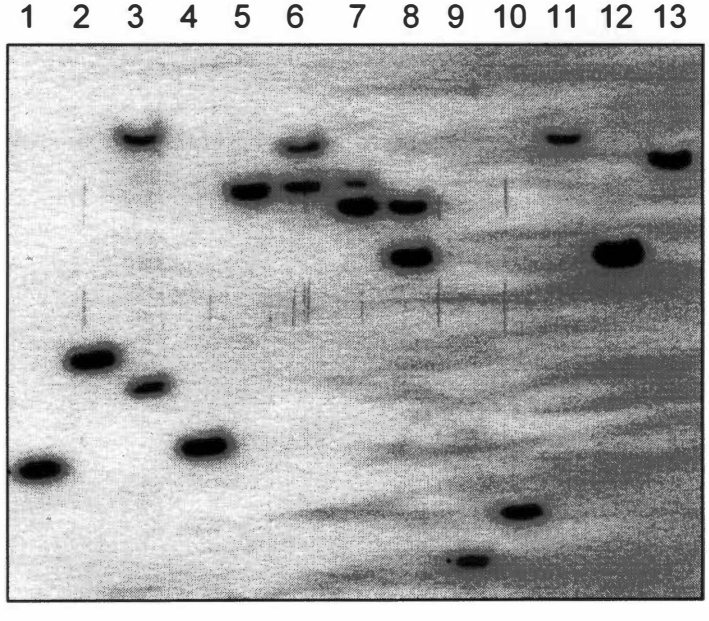


Figure 5. Survival rate of transgenic larvae carrying Rad51dm transgenes after MMS treatment. Each homozygous transgenic line was allowed to lay eggs for 3 days (control), transferred into a fresh medium for another 3 days (experiment), and discarded. The experimental group was treated with MMS at the final concentration of 0.005% (the volume of MMS injected into the medium divided by the weight of the medium), while the control culture was left untreated. The survival rates were determined by the total number of progeny in MMS group divided by the total number of progeny in control group. R(X), R(2), and R(3) represent homozygous transgenic lines carrying two transgenes and R(X+2), R(2+3), and R(X+3) represent transgenic lines carrying four transgenes. The number and X in parenthesis represent the chromosome where transgene is inserted.

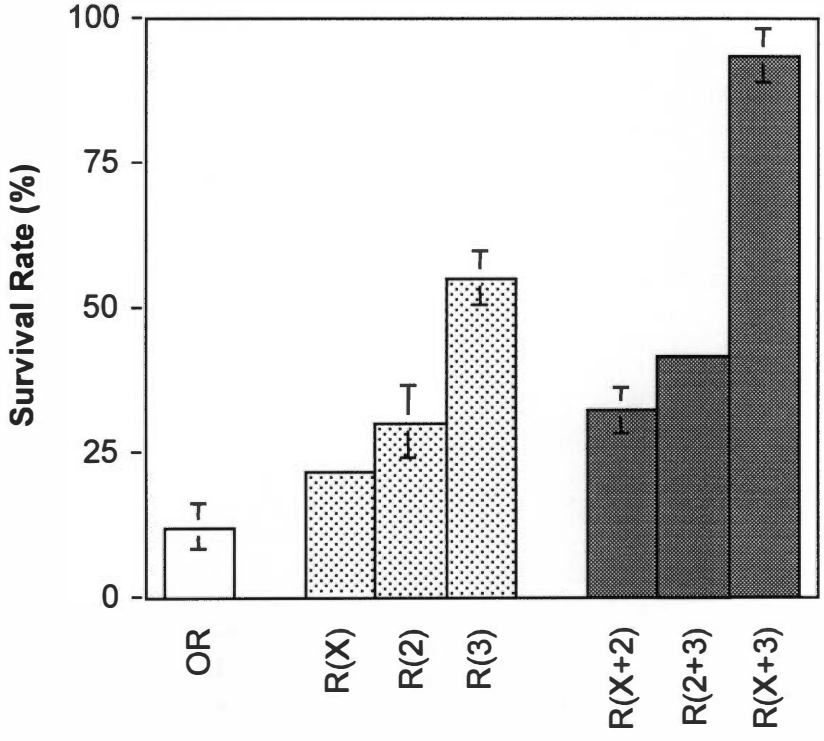
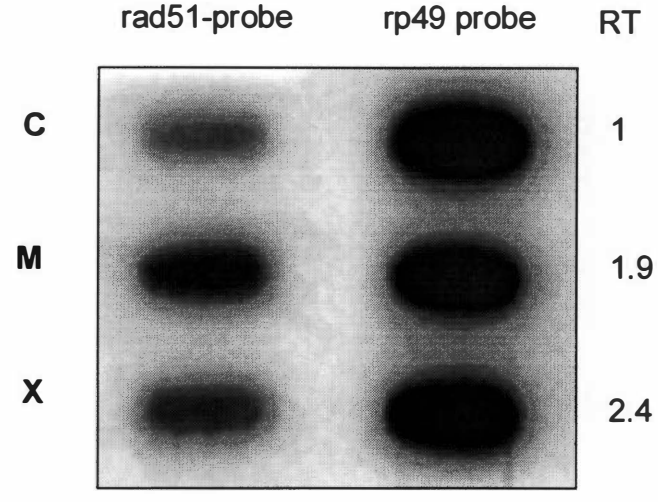
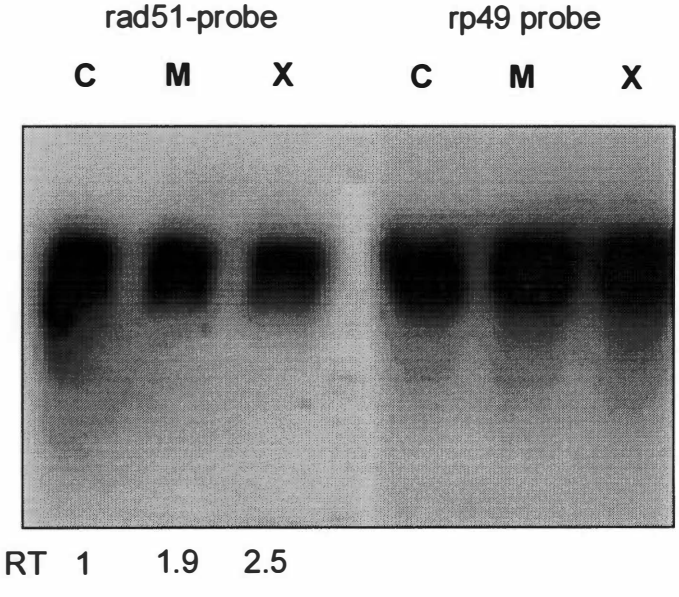

Survival Rate (%)
100
75
50
25
0
OR
R(X)
R(2)
R(3)
R(X+2)
R(2+3)
R(X+3)

Figure 6. Relative Rad51dm transcript levels after treatment with MMS or X-rays as determined by RNA slot blot (A) and Northern blot (B). Poly (A)$^+$ RNA was prepared from adult flies either treated with 0.01% MMS for 12 h or irradiated by 30 kR X-ray. For Rad51dm probe, 20 μg of RNA were analyzed and for rp49 probe, 1 μg of RNA were analyzed. Relative transcript (RT) levels were measured by Rad51 band intensities divided by rp49 band intensities using an Instant-*Imager*. C, M, and X represent Control, MMS, and X-ray groups, respectively.

A


rad51-probe
rp49 probe
RT
C
M
X
1
1.9
2.4

B


rad51-probe
rp49 probe
C
M
X
C
M
X
RT
1
1.9
2.5

DNA-binding activity of Rad51dm protein

DNA-binding activity of the Rad51dm protein was investigated by both agarose gel mobility shift assay (GMSA) and restriction enzyme protection assay (REPA). The Rad51dm protein containing 6 histidine residues at the C-terminus was overexpressed in *E. coli.* This recombinant protein (rRad51dm) was purified by a nickel chelating column chromatography and analyzed by SDS-PAGE (Fig. 7A). An agarose GMSA was used to demonstrate that the rRad51dm protein has DNA-binding activity. As the concentration of rRad51dm protein was increased, both supercoiled and relaxed ds ϕX 174 DNAs were decreased and DNA/protein complexes appeared at the top of the gel (Fig. 7B, upper panel). The recombinant protein also bound ss circular ϕX 174 DNA (Fig. 7B, lower panel). The binding activity of rRad51dm protein to DNA is shown to be non-specific since we obtained the same result when different plasmid DNA, pBlueScript KS was used (data not shown). The DNA binding of Rad51dm protein was confirmed by REPA (Fig. 7C). In the presence of the rRad51dm protein, the cleavage sites of restriction enzymes were protected. Restriction enzymes, however, cleaved ds DNA in the absence of the protein. The binding of rRad51dm protein to DNA required the presence of Mg^{2+}, but did not depend upon a nucleotide cofactor since DNA/protein complexes were observed without ATP (Fig. 7D and E). These results demonstrated that the rRad51dm protein possesses DNA-binding activity.

Figure 7. Binding of the recombinant Rad51dm (rRad51dm) protein to double (ds) or single-stranded (ss) DNA. (A) Purified rRad51dm protein analyzed by SDS-PAGE. The gel was stained with coomassie blue. Each lane represents rRad51dm protein of different fraction from a nickel chelating column. (B) Agarose gel mobility shift assay. 50 ng of ds ϕX 174 DNA (upper gel) or ss ϕX 174 DNA (lower gel) were incubated with various concentrations of the rRad51dm protein as indicated above the gel for 10 min at 37° C. The mixtures were analyzed on a 0.9% (ds DNA) or a 1.5% agarose gel (ss DNA) at 4 °C. Bovine serum albumin (BSA) was used as a control.

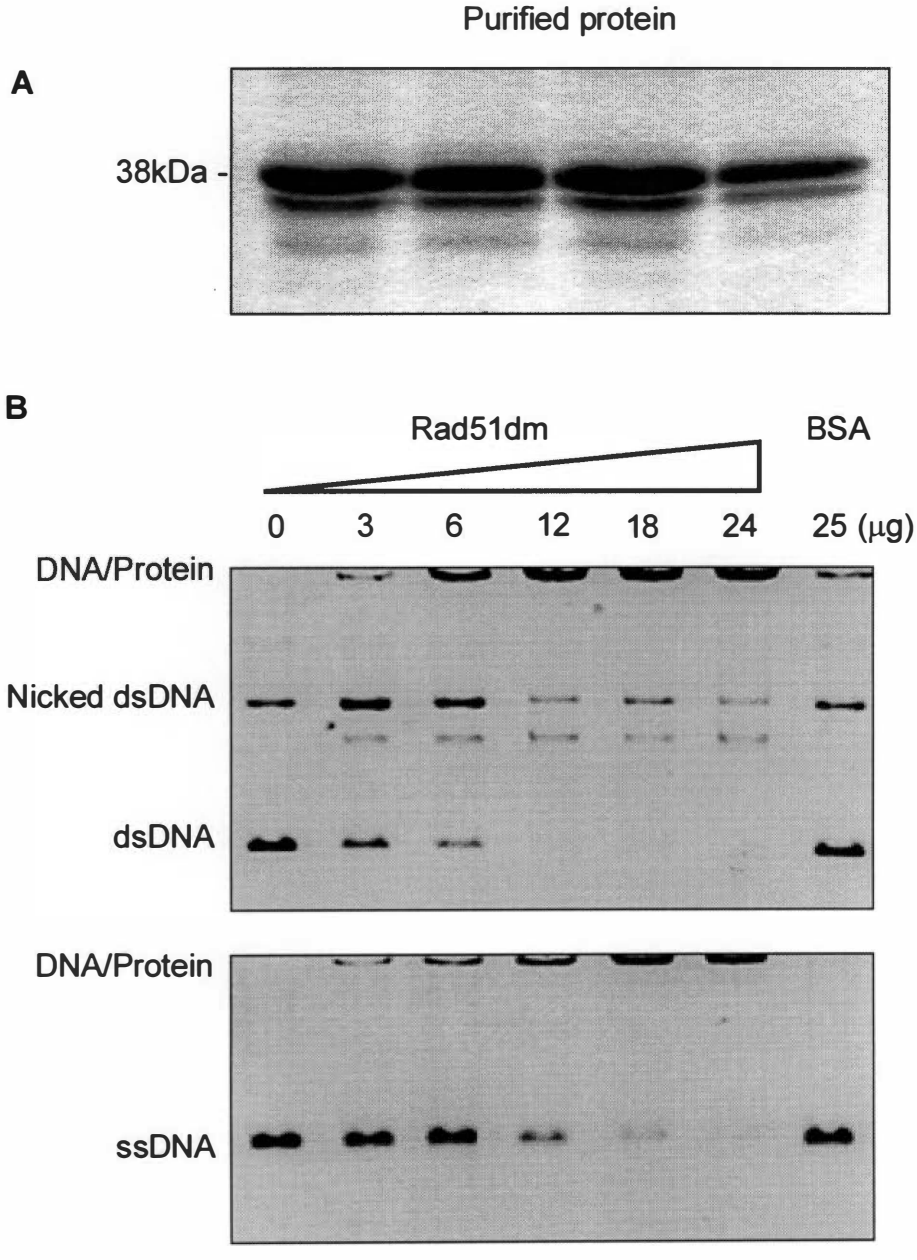
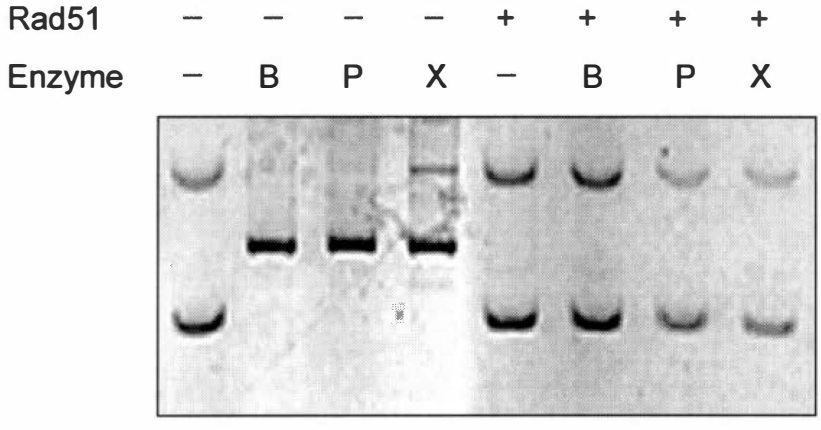
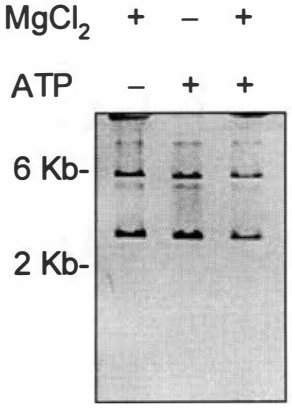

Purified protein
A
38kDa -
B
Rad51dm
BSA
0
3
6
12
18
24
25 (μg)
DNA/Protein
Nicked dsDNA
dsDNA
DNA/Protein
ssDNA

Figure 7 Continued. (C) Restriction enzyme protection assay. 50 ng of ds ϕX 174 DNA was treated with 5 u of *BssH*I (B), *Pst*I (P), or *Xho*I (X) after incubation with or without 40 μg of rRad51dm protein for 40 min at 37° C. To inactivate restriction enzymes, SDS and EDTA were added, and incubated for 10 min at 65° C. Following deproteinization by proteinase K as described in the Materials and Methods, DNA products were analyzed on a 0.8% agarose gel and visualized by ethdium-bromide staining. (D) and (E) Effects of $MgCl_2$ and ATP on binding activity of rRad51dm to dsDNA (D) and ssDNA (E), respectively. 30 μg of rRad51dm protein was mixed with 50 ng of DNA. The mixture was incubated for 5 min at 37° C and analyzed on an agarose gel stained with ethidium bromide.

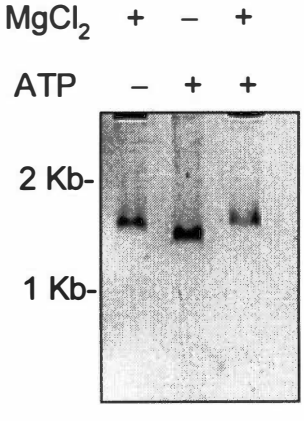
C



D



E



Distribution of Rad51dm protein in embryos and spermatocytes

To study the expression level and the distribution of Rad51dm protein, a polyclonal antibody against Rad51dm protein was generated. Figure 8 shows the interaction of the polyclonal antibody with rRad51dm protein. Using the polyclonal antibody, the expression levels of Rad51dm protein were determined at major developmental stages. Rad51dm protein was highly expressed in embryos, but barely detectable in any other tissues (Fig. 9). To investigate a role of Rad51dm protein in embryos, the localization was examined by an immunostaining. Since the nuclei are mitotically synchronized before the cellularization stage, early embryos are an excellent model system to study mitosis. We observed a number of Rad51dm foci around the nuclei of embryos. During mitosis, Rad51dm proteins did not interact with mitotic chromosome, however, many foci associated with unknown DAPI-staining material around mitotic chromosomes (Fig. 10A and B).

To study a role of Rad51dm protein during meiosis, testes from young males were gently squashed to release the spermatocytes. Since there are no distinct markers to reveal the developing stages of primary spermatocyte, we first observed Y-chromosome fertility factors, ks-1, kl-3, and kl-5, under the phase-contrast microscope to identify prophase cells during meiosis I (Pisano *et al.*, 1993). These factors are giant lampbrush-like loops present only in the male germ line and restricted to the primary spermatocyte at prophase I. The primary spermatocytes from Oregon R were observed, but we failed to detect any Rad51dm signals by immunofluorescent staining. Thus, we overexpressed

Figure 8. Purified rRad51dm protein analyzed by SDS-PAGE (A) and immunoblot (B). Lane 1 contains the molecular marker. Lane 2 shows total proteins extracted from BL21 (DE3) without being transformed with pRPNX expression plasmid carrying full-length Rad51dm coding region. Lane 3 and lane 4 show total proteins before and after IPTG induction, respectively, of BL21 (DE3) transformed with pRPNX plasmid. Purified rRad51dm protein in lane 5 is indicated by the arrow head. (B) Immuno blot of the same protein samples as in (A). Polyclonal anti-serum reacts specifically rRad51dm protein.

Purified
+IPTG
-IPTG
BL21
Purified
+IPTG
-IPTG
BL21
Marker
67KD -
45KD -
29KD -
14KD -

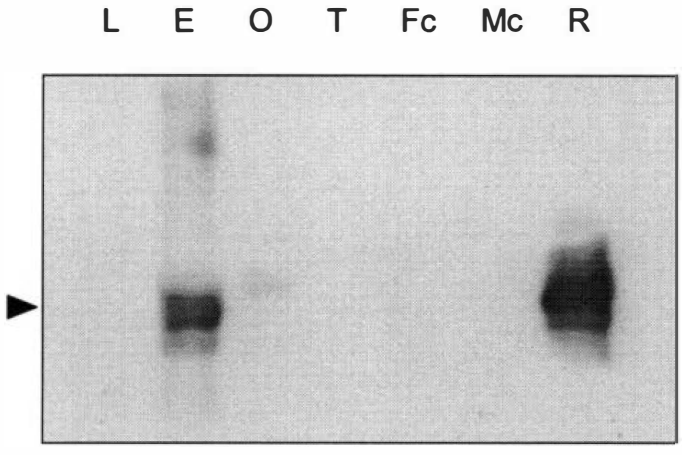
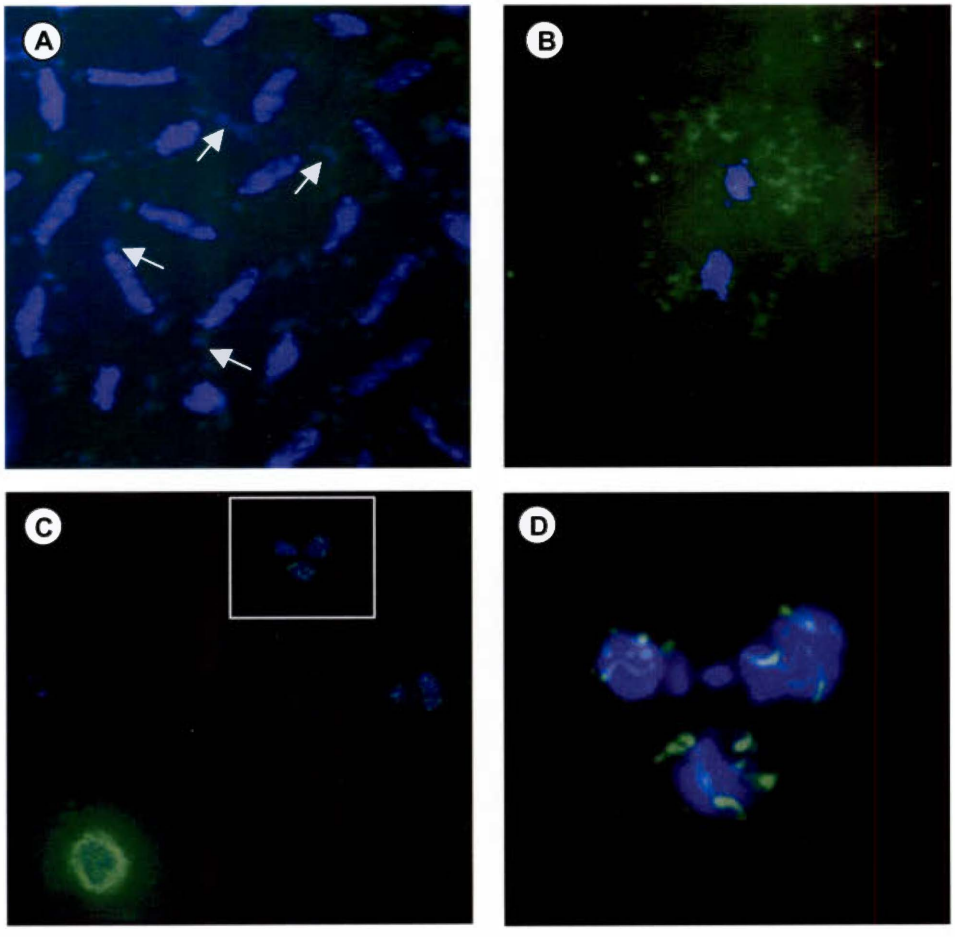


Figure 9. Immunoblot analysis of total proteins at major developmental stages. Total proteins were extracted as described in the Materials and Methods and approximately 10 μg of the proteins were analyzed by SDS-PAGE. After transferring onto a nitrocellulose membrane, Rad51dm protein was detected using chemiluminiscent method. The arrow head indicates the 38 kDa Rad51dm protein. L; larvae, E; embryos, O; ovaries, T; testes, Fc; female carcass (without ovaries), Mc; male carcass (without testes), and R; purified recombinant Rad51dm protein.

Figure 10. Immunolocalization of Rad51dm in wild-type embryos and primary spermatocytes of hsp26-rad51 transgenic flies. For (A) and (B), embryos were stained with anti-Rad51dm polyclonal antibody (green) and then counterstained with the DNA-specific DAPI stain (blue). (A) The distribution of Rad51dm protein at anaphase of mitotic nuclei. Many Rad51dm proteins were localized with unknown DAPI-staining molecule as indicated by the arrows. (B) Rad51dm staining at telophase of mitosis. The proteins were located around the mitotic chromosomes. For (C) and (D), testes were dissected from hsp26-rad51 transgenic flies after 1 hr heat-shock treatment at 37° C following a 12 hr recovery. Primary spermatocytes were stained with anti-Rad51dm polyclonal antibody (green) and DAPI (blue). (C) Distribution of Rad51dm protein at prophase I stage. Rad51dm protein is exclusively localized in the nucleus. (D) High magnification of the late prophase I spermatocytes marked in (C). Rad51dm protein is associated with prophase I chromatin. Note that most fluorescent signals have disappeared. All images were obtained on a Leica confocal microscope.


A
B
C
D

Rad51dm protein in the primary spermatocytes by heat shock treatment of hsp26-rad51 transgenic flies that carry Rad51dm gene driven by the hsp26 promoter. The Rad51dm protein was exclusively localized in the nucleoplasm at the early prophase I stage, but absent in the nucleoli and cytoplasm. The proteins were more concentrated at mid-prophase I stage forming thread-like structures in the nucleus (Fig. 10C, lower left) and rapidly disappeared in subsequent stages (data not shown). High magnifications of late prophase I spermatocytes showed that some Rad51dm proteins bind to chromatin although most signals have disappeared (Fig. 10D).

Overexpression of Rad51dm results in lethality

It has been reported that the overexpression of Rad51 protein increases resistance to DNA damaging agents such as MMS and gamma rays (Vispé *et al.*, 1998). To determine whether overexpression of Rad51dm increases resistance to DNA damaging agents, we generated transgenic flies carrying the full-length Rad51dm coding region driven by the hsp26 promoter (Fig. 11). However, we observed that the transgenic larvae were extremely sensitive to the heat shock treatment. Thus, different heat shock conditions were applied to the hsp26-rad51 transgenic larvae everyday to measure the sensitivity (Fig. 12). Wild-type larvae and hsp26-lacZ larvae carrying the hsp26 promoter driving the β-galactosidase gene were used as controls. With heat shock treatment for 5 min daily, the survival rate of hsp26-rad51 larvae was approximately 50% compared to those of the control groups. No survival was observed when heat shock treatment

Figure 11. Analysis of an hsp26-rad51 transgenic line. (A) Southern blot of the hsp26-rad51 transgenic line. Genomic DNA digested with *EcoR*I was hybridized with the rad51dm probe. The 6.5 Kb fragment represents the endogenous Rad51dm gene and the 3.8 Kb fragment represents the transgene. TMS/Dr and ry were used as controls. (B) Transcript level of Rad51dm transgene determined by RNA slot blot. Total RNA was extracted from the hsp26-rad51 transgenic line treated with heat shock at 37^0 C, subjected to RNA slot blot, and hybridized with rad51dm probe and rp49 probe. rp49 probe was used as an internal control. (C) Western blot analysis showing Rad51dm protein after heat shock treatment. The hsp26-rad51 transgenic line was treated with heat shock for 1 hr at 37^0 C and recovered for 12 hr. Total protein was prepared as described in the Methods and Materials and run on 10% SDS polyacrylamide gel. After transferring the protein onto nitrocellulose membrane, the 38 kDa Rad51dm protein was detected using polyclonal antibody.

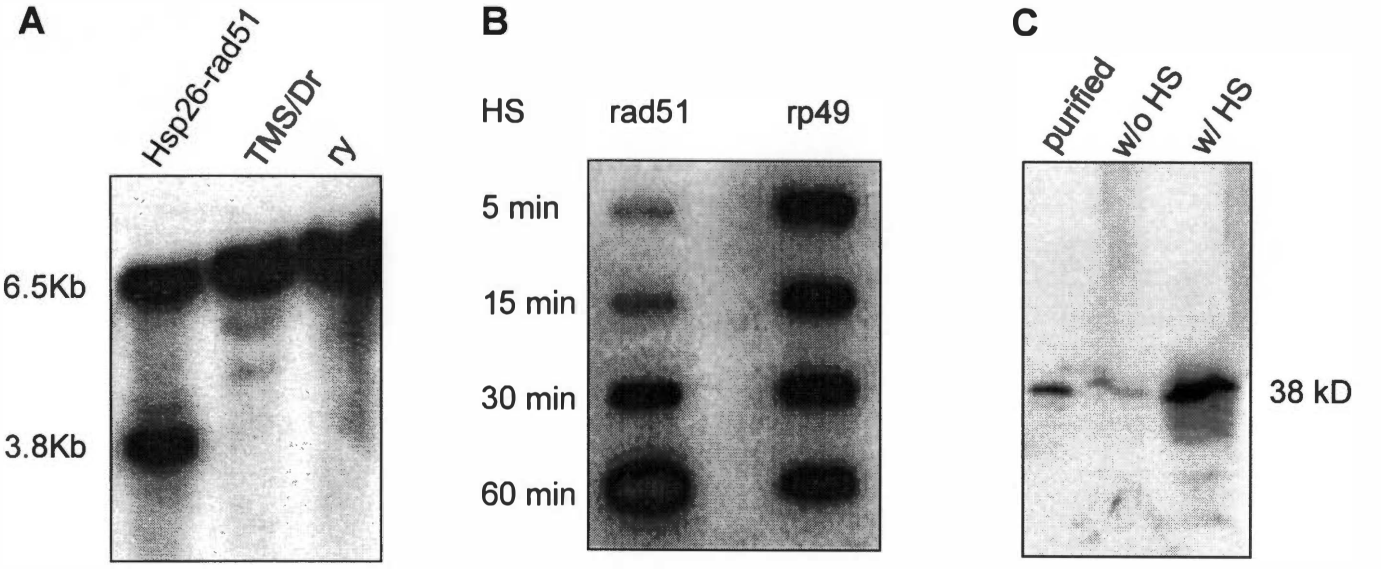
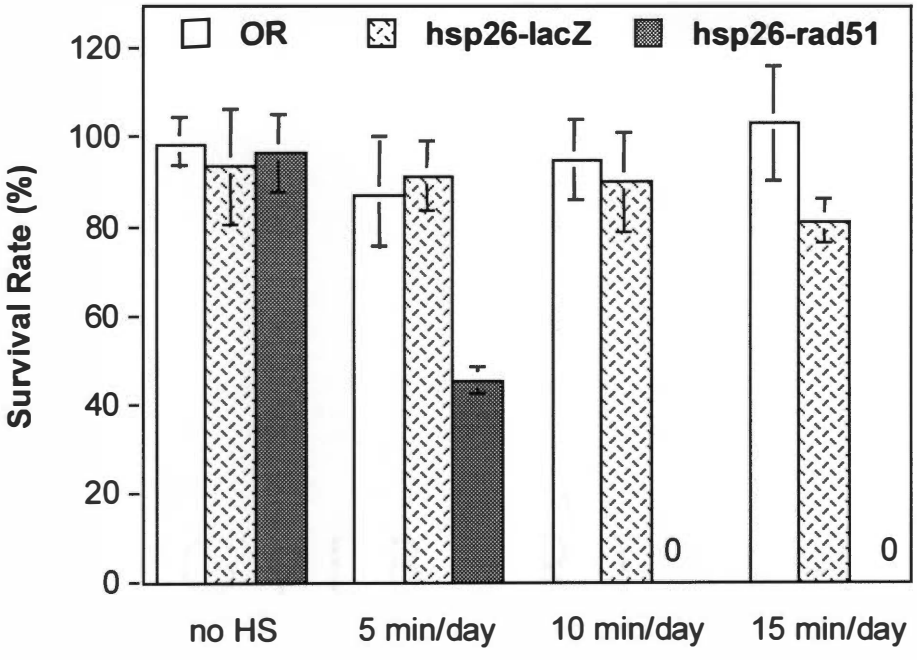

A
Hsp26-rad51
TMS/Dr
ry
6.5Kb
3.8Kb
B
HS
rad51
rp49
5 min
15 min
30 min
60 min
C
purified
w/o HS
w/ HS
38 kD

Figure 12. Survival rate of hsp26p-rad51 larvae after heat-shock treatment. Adult flies were allowed to lay eggs for 3 days (control groups), transferred to fresh vial for another 3 days (experiment groups), and discarded. The experiment groups were heat-shocked in a 37° C incubator for 5 min, 10min, or 15 min everyday. Oregon R (wild-type) and transgenic flies carrying β-galactosidase gene driven by the hsp26 promoter (hsp26p-lacZ) were used as controls. The survival rate was measured by the total number of progeny with heat-shock treatment (experiment) divided by the total number of progeny without heat-shock treatment (control).


OR
hsp26-lacZ
hsp26-rad51
Survival Rate (%)
120
100
80
60
40
20
0
0
0
no HS
5 min/day
10 min/day
15 min/day

lasted more than 5 min. In contrast, the control groups showed nearly normal survival rates even with heat shock treatments lasting for 15 min (Fig. 12).

To eliminate any effects of heat shock on the survival rate, we generated UAS-rad51 transgenic flies to overexpress Rad51dm protein using the UAS-GAL4 binary expression system. Five out of ten transgenic lines were crossed with an Act5C-GAL4 line that expresses the GAL4 protein ubiquitously. None of the Act5C-GAL4;UAS-rad51 flies developed beyond the larval stage (Table 1). Next, we investigated whether this lethal effect depends on developmental stages. hsp26-rad51 and hsp70-GAL4;UAS-rad51 animals were exposed to heat shock at larval, pupal, and adult stage. It was observed that embryos and larvae were much more sensitive to the overexpression of Rad51dm than pupae and adults, suggesting that the overexpression of Rad51dm protein severely interferes with an essential function for cell viability during early development (data not shown).

Rad51dm overexpression induces apoptosis and alters mitosis

To further investigate the effect of overexpression of Rad51dm protein, we induced tissue-specific expression of Rad51dm using transgenes that express GAL4 protein under the control of various tissue-specific promoters. After crossing each line of transgenic flies expressing GAL4 with five different UAS-rad51 transgenic flies, the viability and the mutant phenotypes caused by the overexpression of Rad51dm protein were examined. In four of the GAL4 lines, induction of Rad51dm expression resulted in lethality during embryonic or larval

Table 1. Summary of the progeny phenotypes from the cross of various GAL4 lines with UAS-rad51, UAS-reaper, and UAS-p35.

GAL4 line	Expression of GAL4	UAS-rad51[a]	UAS-reaper	UAS-p35[b]
hsp70-GAL4	ubiquitous	lethal	lethal	ND[c]
Act5C-GAL4	ubiquitous	lethal	lethal	lethal
twi-GAL4	early embryos	lethal	lethal	survive
T80-GAL4	all imaginal discs	lethal	lethal	lethal
24B-GAL4	embryonic mesoderm	lethal	lethal	lethal
ey.H-GAL4	ey+ pattern	lethal	lethal	survive
C96-GAL4	wing margin in wing disc	wg-margin nicks	wg-margin nicks	normal[d]
glass-GA:4	posterior region of morphogenetic furrow in eye disc	small and rough eye	small and rough eye	normal[d]
MVD-GAL4	primordial germ cells	(semi)sterile	fertile	fertile

[a]Five different UAS-rad51 lines were tested and all of them showed similar phenotypes.

[b]Two UAS-p35 lines inserted into the second and the third chromosome were tested and the same results were obtained.

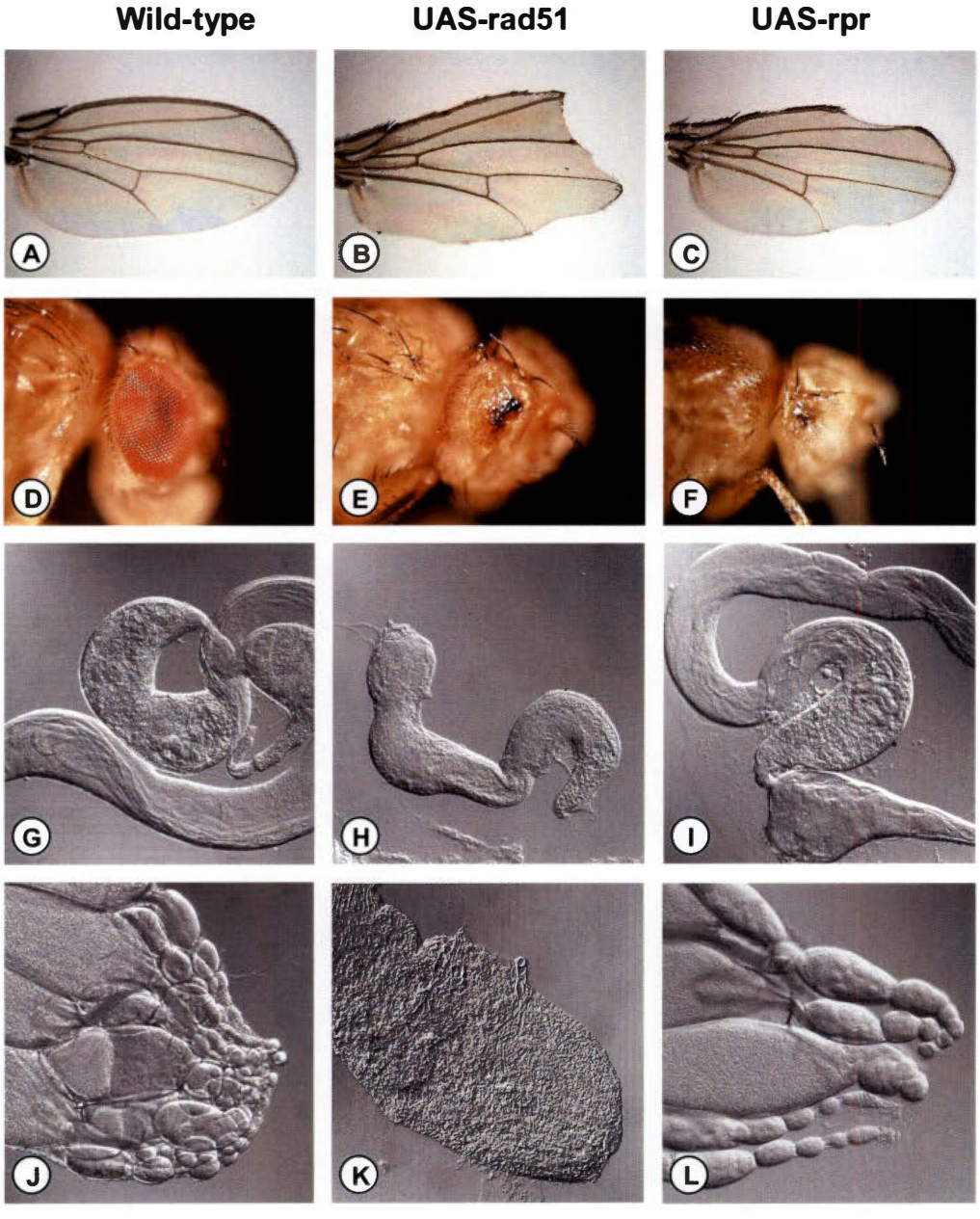
[c]Not determined

[d]The phenotype was observed under a dissecting microscope.

development (Table 1). These lines were twi-GAL4 that expresses GAL4 protein in early embryos, T80-GAL4 that expresses GAL4 protein ubiquitously in the third instar imaginal discs, 24B-GAL4 that expresses GAL4 in embryonic mesoderm, and ey.H-GAL4 that expresses GAL4 strongly in eye imaginal discs. The same lethal effect was also observed as a result of induced expression of Reaper, a suicide protein that regulates programmed cell death (White *et al.*, 1996), by the same GAL4 lines (Table 1). However, the induction of Rad51dm expression by C96-GAL4 which expresses GAL4 in the prospective wing margin in the wing imaginal disc, glass-GAL4 that expresses GAL4 in cells posterior to the morphogenetic furrow in the eye imaginal disc, or MVD-GAL4 that expresses GAL4 in primordial germ cells did not affect viability (Table 1). Instead, they showed severe mutant phenotypes in the wing margin (bristle loss and margin nicks), eye tissue (reduced size and rough shape), or reproductive organs (lack of spermatocytes and oocytes) (Fig. 13B, E, H, and K, respectively) although some variations observed between the UAS-rad51 lines were due to position effects. The expression of Reaper by C96-GAL4 and glass-GAL4 also exhibited similar phenotypes to the overexpression of Rad51dm (Fig. 13C and F, respectively). However, the expression of Reaper in primordial germ cells did not affect the ovary or the testis (Fig. 13I and L, respectively). Collectively, these results demonstrate that the overexpression of Rad51dm protein induces cell death at levels similar to that caused by the Reaper protein.

To determine whether the overexpression of Rad51dm protein induces apoptosis, a TUNEL assay was performed in the wing and eye imaginal discs.

Figure 13. Overexpression effects of Rad51dm and Reaper in various tissues. (A-F) Photographs of wing (A-C) and eye tissues (D-F). Wild-type wing and eye tissues from Oregon R are shown in (A) and (D), respectively. The C96-GAL4 enhancer trap line that expresses GAL4 in prospective wing margin in imaginal disc was crossed with either UAS-Rad51 (B) or UAS-reaper (C). The glass-GAL4 line that expresses GAL4 strongly in cells posterior to the morphogenetic furrow of eye imaginal disc was crossed with either UAS-rad51 (E) or UAS-reaper (F). Note the notches and loss of margin bristles of the wing and the reduced size and roughness of the eye caused by the overexpression of Rad51dm or Reaper. (G-L) Differential Interference Contrast (DIC) micrographs of testis (G-I) and ovary (J-L). The MVD-GAL4 enhancer trap line that expresses GAL4 in primordial germ cells was crossed with either UAS-rad51 (H and K) or UAS-reaper (I and L). DIC micrographs of wild type testis and ovary are shown in (G) and (J), respectively. Note the reduced sizes and lack of sperm bundles or oocytes caused by the overexpression of Rad51dm.


Wild-type
UAS-rad51
UAS-rpr
A
B
C
D
E
F
G
H
I
J
K
L

Dissected wing imaginal discs containing one copy each of C96-GAL4 and UAS-rad51 were stained with anti-Rad51dm antibody (red) and then the TUNEL assay was performed to detect apoptotic cells (green). ,Cells expressing Rad51dm and apoptotic cells were mostly co-localized in the distal tip along the dorsal-ventral wing margin (Fig. 14B) where the C96-GAL4 driver is expressed (Helms *et al.*, 1999). Similar results were obtained from the eye imaginal discs of transgenic larvae containing glass-GAL4 and UAS-rad51. Glass is a transcriptional factor expressed in all cells behind the morphogenetic furrow which progresses across the eye disc from posterior to anterior during cellular differentiation (Moses and Rubin, 1991). Antibody staining (red) showed that the expression of Rad51dm begins in the morphogenetic furrow and is higher in more posterior regions of the disc, and is colocalized with a cluster of apoptotic cells (green) (Fig. 14D). These results, together with those shown in Fig. 12 and Fig. 13, indicate that overexpression of Rad51dm protein induces apoptosis. To determine whether Rad51dm-induced apoptosis can be suppressed by Baculovirus p35, a universal antiapoptotic protein that can block apoptosis by inactivation of caspases (reviewed in Rodriguez *et al.*, 1998), Rad51dm and p35 proteins were co-expressed under the control of either C96-GAL4 or glass-GAL4. Table II shows that expression of p35 can partially suppress Rad51dm-induced apoptosis.

To ascertain whether overexpression of Rad51dm protein affects cell cycle progression, we examined the mitotic cells in the second mitotic wave that cells synchronously undergo posterior to the morphogenetic furrow (de Nooij and Hariharan, 1995). In wild-type discs, antiphospho-histone H3 antibody staining,

Figure 14. Overexpression of Rad51dm induces apoptosis and alters the pattern of mitosis. Confocal micrographs of wing discs (A and B) and eye discs (C-F) from a wild-type fly (OR) (A, C, and E) or a transgenic fly carrying one copy of a UAS-rad51 transgene and C96-GAL4 (B) or glass-GAL4 (D and F). (A-D) Wing and eye discs were dissected from the third instar lava, stained with anti-Rad51dm antibody (red) and then counterstained by the TUNEL assay (green). Note that the apoptotic cells are specifically localized in the wing marginal portion and the region posterior to the morphogenetic furrow in which Rad51dm is overexpressed. A dashed line indicates the location of the dorsal-ventral compartment boundary of wing disc (A) and anterior-posterior compartment boundary of eye disc (C). (E and F) Eye imaginal discs were stained with anti-phospho-histone H3 antibody (green) to detect the mitotic cells and counterstained with PI specific for DNA (red). Note that the overexpression of Rad51dm alters the mitotic pattern in cells of the second mitotic wave (arrow heads). Arrows indicate the morphogenetic furrow (C-F).

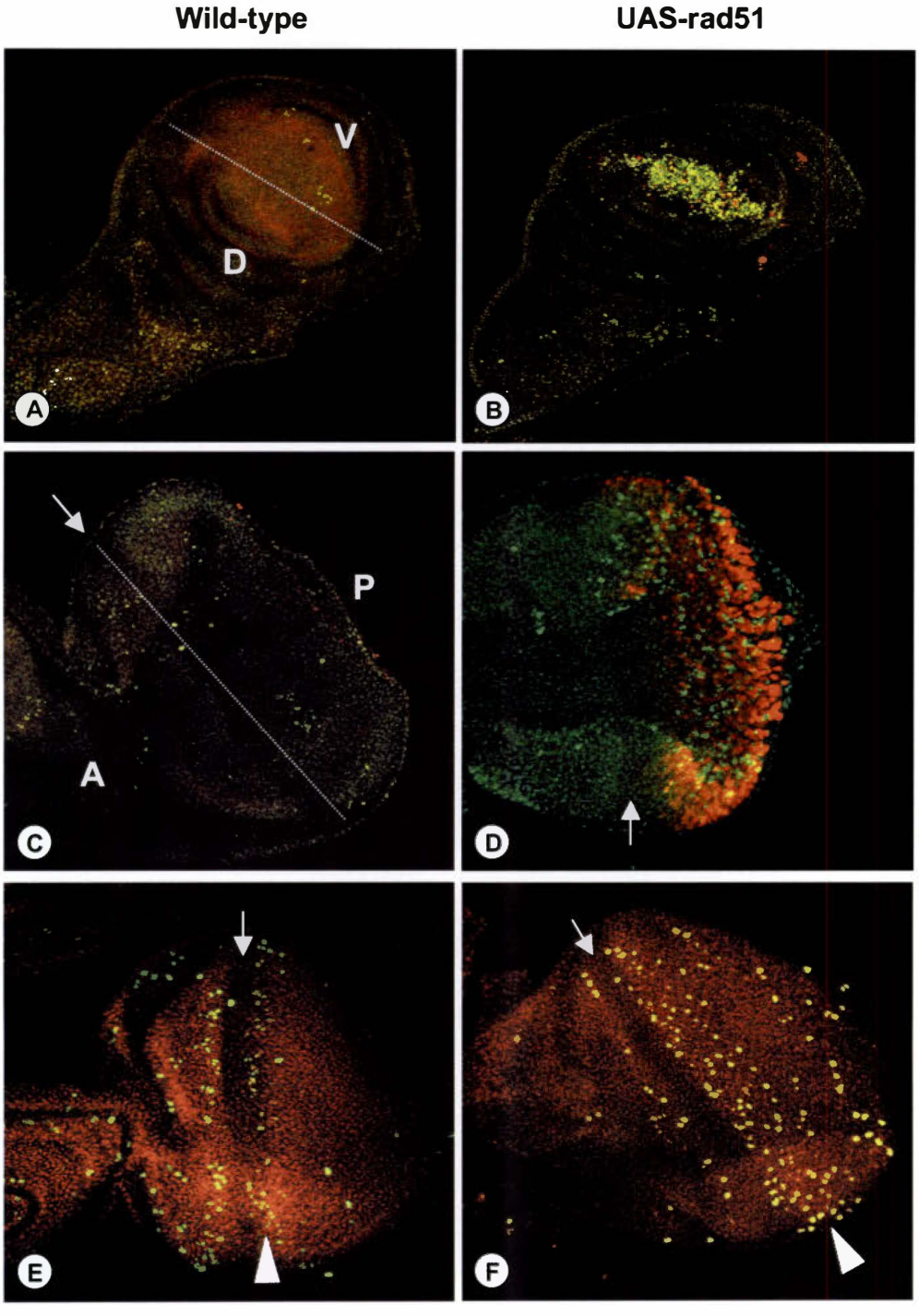

Wild-type
UAS-rad51
V
D
A
B
P
A
C
D
E
F

Table 2. The phenotypes caused by the overexpression of Rad51dm are partially suppressed by the co-overexpression of p35.

Genotype[a]		No. of flies	
GAL4 line	UAS line	wild type	mutant[b]
C96-GAL4(3)[c]	UAS-rad51(2)	0	105
	UAS-p35(3)	241[d]	0
	UAS-rad51(2);UAS-p35(3)	203	88
Glass-GAL4(2)	UAS-rad51(1)[e]	0	99
	UAS-p35(2)	46[d]	0
	UAS-rad51(1);UAS-p35(2)	84	15

[a]Each genotype was produced by the cross of the GAL4 line with the UAS line.

[b]The mutant phenotypes in wing (C96-GAL4) and eye (glass-GAL4) are the same as presented in Fig. 13.

[c]The number in parenthesis represents the chromosome where the transgene is inserted.

[d]The phenotype caused by overexpression of p35 was observed under a dissecting microscope.

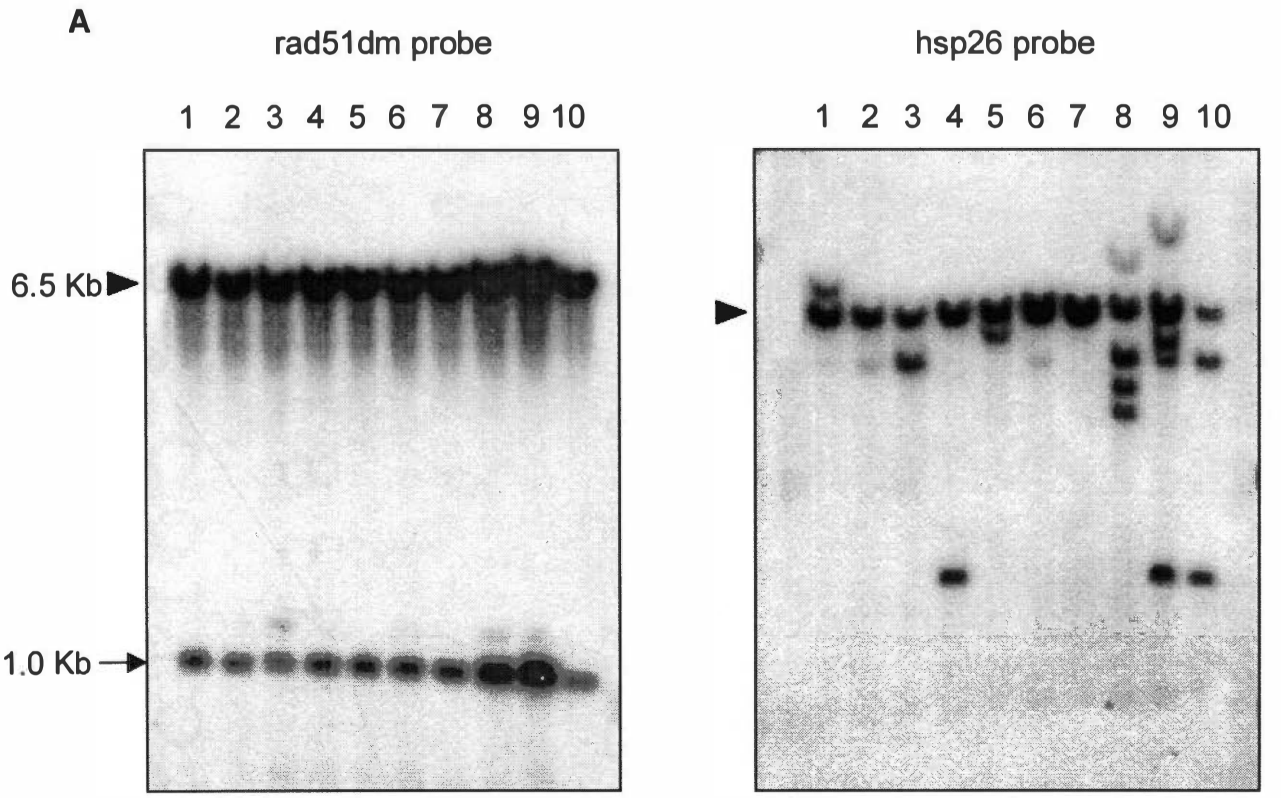
[e]Only female progeny were scored since the UAS-rad51(1) mutant phenotype is female specific (sexual dimorphism).

which is specific for M phase, revealed a distinct and narrow band of cells in the second mitotic furrow (Fig. 14E). However, glass-GAL4;UAS-rad51 eye discs showed a much broader and diffused band (Fig. 14F), suggesting that the overexpression of Rad51dm protein interferes with the normal mitotic pattern in the second mitotic wave.

N-terminal truncated Rad51dm protein

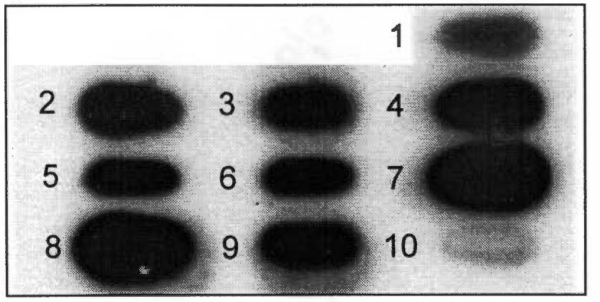
The Rad51 protein consists of two domains, a less conserved N-terminal domain (domain I) and a highly conserved core domain containing two nucleotide-binding sites (domain II). It is believed that the N-terminal domain is involved in species-specific interactions and the homologous core domain (domain II) is responsible for functions of DNA repair and recombination (Ogawa *et al.*, 1993). It has been reported that N-terminal truncated RecA protein severely interferes with all functions of the native RecA protein (Yancey and Porter, 1984; Horii *et al.*, 1992; Lauder and Kowalczykowski, 1993). Recently, an NMR study showed that the N-terminal domain of the human RAD51 protein binds to DNA (Aihara *et al.*, 1999). To investigate whether the N-terminal truncated Rad51dm protein interferes with the role of wild-type protein, we generated transgenic flies carrying the Rad51dm gene with a deletion of amino acids 18-99 driven by the hsp26 promoter (hsp26-rad51ΔN) (Fig. 15). To examine if the N-terminal truncated Rad51dm protein can induce apoptosis, the transgenic larvae were subjected to heat shock treatment. Interestingly,

Figure 15. Southern blot and RNA slot blot analyses of hsp26-rad51ΔN transgenic lines. (A) Genomic DNA digested with *EcoR*I was hybridized with rad51dm probe or hsp26 probe. The regions of probes are shown in Fig. 1. The endogenous Rad51dm and hsp26 genes are indicated by the arrow heads and Rad51dm transgene is indicated by the arrow. (B) Transcript level of Rad51dm transgene determined by RNA slot blot. Total RNA was extracted from hsp26-rad51ΔN transgenic lines treated with heat shock at 37^0 C for 1 hr, subjected to RNA slot blot, and hybridized with the rad51dm and rp49 probes. rp49 probe was used as an internal control. The numbers represent each transgenic line.

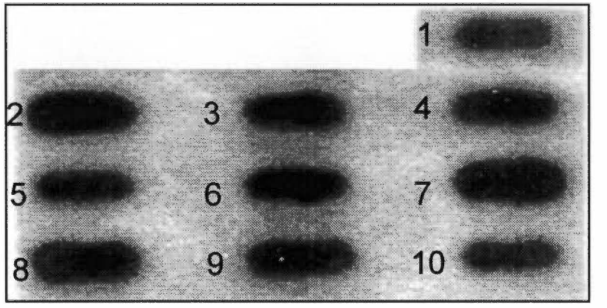

A
rad51dm probe
hsp26 probe
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
6.5 Kb
1.0 Kb

B

rad51dm probe

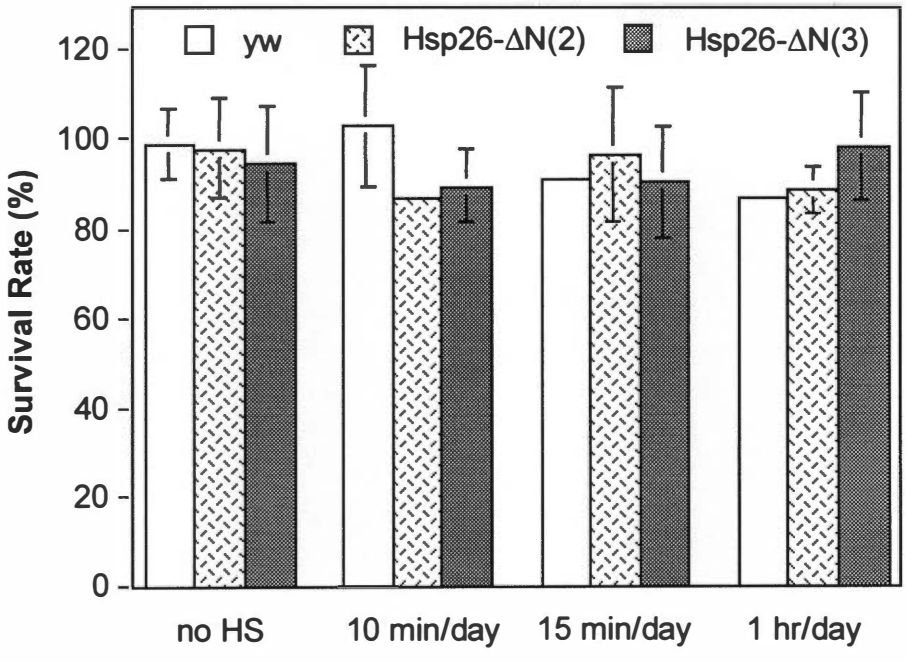


rp49 probe



overexpression of Rad51ΔN did not affect larval viability under severe heat shock conditions (1 hr heat shock daily) (Fig. 16). Since similar results were obtained with six different lines, the possibility of the position effects of transgenes was eliminated.

Figure 16. Survival rate of hsp26-rad51ΔN larvae after heat-shock treatment. Adult flies were allowed to lay eggs for 3 days (control groups), transferred to a fresh vial for another 3 days (experiment groups), and discarded. The developing larvae of experimental groups were treated with heat-shock in a 37° C incubator for 10 min, 15 min, or 1 hr everyday. yw was used as a control. The survival rate is represented as a fraction of the total number of progeny with heat-shock treatment (experiment) divided by the total number of progeny without heat-shock treatment (control).


yw
Hsp26-ΔN(2)
Hsp26-ΔN(3)
Survival Rate (%)
120
100
80
60
40
20
0
no HS
10 min/day
15 min/day
1 hr/day

CHAPTER 4

DISCUSSION

In this study, we demonstrate that Rad51dm is involved in the repair of damaged DNA induced by MMS, and that it possesses ds and ss DNA-binding activities similar to other Rad51 proteins. Immunoblot analysis shows that Rad51dm is highly expressed in embryos, but barely detectable in any other tissues. Immunostaining reveals that under conditions of high expression, Rad51dm protein is exclusively distributed in the nucleus of spermatocytes at the early prophase I stage and disappears rapidly at the late prophase I stage. Furthermore, we demonstrate that the overexpression of Rad51dm protein alters the progression of the cell cycle and eventually causes cell death.

The roles of Rad51dm protein in DNA repair

One of critical roles of Rad51 protein is to repair damaged DNA by homologous recombination in concert with many other factors including the Rad52 epistasis group proteins, p53, Brca1, Brca2, single strand DNA binding protein, cAbl, Ubc9, and Ubl1 (Kovalenko *et al.*, 1996; Baumann and West, 1998; Chen *et al.*, 1999; Li *et al.*, 2000). It has been shown that Rad51 protein is concentrated in the nucleus of somatic cells when or where DNA is damaged (Haaf *et al.*, 1995). In this study, we show that the sensitivity of larvae to MMS varies inversely with the copy number of the Rad51dm gene. Additionally, the

expression levels of Rad51dm RNA increase 2- to 3-fold after treatment with DNA damaging agents. These results strongly suggest that Rad51dm functions in DNA repair.

Rad51 protein has been demonstrated to possess DNA binding-activity, ATPase activity, and strand transfer activity. These properties are essential to homologous recombination since the primary function of Rad51 protein is to bring two homologous DNA molecules into close proximity to facilitate the formation of heteroduplex DNA and to mediate strand exchange between them. Although recent findings are still controversial in terms of the requirement of ATP hydrolysis and the formation of the tertiary structure on the DNA filament (Morrison *et al.*, 1999 and Passy *et al.*, 1999), it is believed that Rad51 acts to conjoin DNA during a repair event. In this context, it is not surprising that the recombinant Rad51dm protein containing 6 additional histidine residues at the C-terminus has both ds- and ss-DNA binding activities in a Mg^{2+}-dependent but ATP-independent manner.

Rad51 protein is also involved in meiotic recombination. In yeast, Rad51 mutants show very low ability in spore formation, accumulation of double-strand breaks, and proficiency in commitment to gene conversion (Shinohara *et al.*, 1992; Basile *et al.*, 1992). The highest expression of Rad51 is observed in testis of various animals such as *Xenopus* (Maeshima *et al.*, 1995), chicken (Bezzubora *et al.*, 1993), and mouse (Morita *et al.*, 1993). Imunocytochemistry studies in vertebrates also show that RAD51 foci begin to localize in the nucleus at late leptotene to early zygotene stage of prophase I, along with synaptonemal

complex and rapidly disappear at the pachytene stage. These results suggest a role of RAD51 in the interhomologue interactions during meiotic recombination (Ashley *et al.*, 1995; Barlow *et al.*, 1997, and Moens *et al.*, 1997). In this study, we observe the distribution of Rad51dm protein in spermatocytes during meiosis using a polyclonal antibody. Although we could not detect any Rad51dm foci in the wild type spermatocytes, under high expression conditions, foci were observed only in the nucleus of early prophase I spermatocytes from transgenic fly. The Rad51dm foci are dramatically reduced and undetectable after prophase I. Immunoblot anaylsis shows that Rad51dm protein is expressed at the highest level in embryos, but barely detectable in any other tissues, including testes. These results indicate that Rad51dm may play an important role during embryogenesis, but not be needed during meiosis of male since meiotic recombination, synaptonemal complex, and chiasmata have not been observed in *Drosophila* male (Cooper, 1964).

Overexpression effects of Rad51dm

We demonstrate that overexpression of Rad51dm protein by both hsp26-rad51 and hsp70-GAL4;UAS-rad51 results in lethality in embryos and/or larvae. However, adult flies are more resistant to the overexpression of Rad51dm. Overexpression of Rad51 in wing or eye imaginal discs results in severe mutant phenotypes reflecting cell lethality in the domains of Rad51 expression. This lethal effect is not tissue-specific since we obtained identical results from all GAL4 lines tested driving GAL4 in various tissues. Consistent with these

observations, the immunocytochemical study of imaginal discs also shows colocalization of anti-Rad51dm antibody staining and TUNEL staining (Fig. 14A-D). Collectively, these results suggest a possible role of Rad51dm protein in apoptosis. In *Drosophila*, the induction of apoptosis requires the products of the *reaper, hid*, and *grim* genes encoded by the 75C1, 2 region of the third chromosome (White *et al.*, 1994). It has been reported that ectopic expression of Reaper, a 65-amino acid protein, induces cell death in different tissues of transgenic animals and cultured cells (White *et al.*, 1996; Pronk *et al.*, 1996). Apoptosis is negatively regulated by the inhibitor of apoptosis proteins (IAPs) that can interact with caspases. Reaper induces apoptosis by binding directly to and preventing *Drosophila* IAPs (DIAPs) from inhibiting caspase activation (Goyal *et al.*, 2000). To determine whether the phenotypes caused by overexpression of the Reaper protein are similar to those caused by overexpression of the Rad51dm protein, UAS-reaper lines were crossed with various GAL4 lines. All phenotypes observed from UAS-reaper;GAL4 were similar to those from UAS-rad51;GAL4 with the exception of the primordial germ cells which are resistant to Reaper activity (Fig. 13). It is conceivable that Reaper is not active in early dividing germ cells or a caspase induced by Reaper may be absent in primordial germ cells (Kondo *et al.*, 1997). Nonetheless, the phenotypic similarities shared by Reaper and Rad51dm strongly suggest that Rad51dm is involved in apoptosis. p35, another class of baculovirus antiapoptotic protein, can block apoptosis induced by a variety of stimuli in insects (Clem *et al.*, 1994; Hay *et al.*, 1994). It binds to and inactivates members of the family of interleukin-1 β-

converting enzyme (ICE)-like cysteine proteases or caspases. We demonstrate using the transgenic flies carrying each copy of UAS-rad51, UAS-p35, and GAL4 driving genes that Rad51-induced apoptosis is partially suppressed by coexpression of p35, indicating that it is not solely a p35-dependent pathway. This result implies that Rad51dm may be involved in another apoptotic pathway besides the p35-dependent apoptotic pathway.

While a myriad of studies have been performed on the function of Rad51 in DNA repair and recombination, much less is known about the cell cycle control of the Rad51 protein. The cell-cycle dependent expression pattern of Rad51 has been reported in yeast and mouse (Basile *et al.*, 1992; Yamamoto, 1996). It is believed that the Rad51 transcript and protein are expressed during the period of late G_1 through G_2, but not during the G_0 phase. Consistently, mammalian Rad51 foci are observed during S phase (Chen *et al.*, 1999), suggestive of a role in DNA replication or cell cycle regulation. In this study, we observe that the overexpression of Rad51dm protein disrupts the cell cycle progression. Although there is no evidence that Rad51 directly interacts with cell cycle proteins such as cyclins, it has been reported that Rad51 binds to several proteins involved in cell cycle progression including p53, Brca1, and Brca2, (Buchhop *et al.*, 1997; Scully *et al.*, 1997; Chen *et al.*, 1998). Recently, it was shown that the overexpression of human Rec2/Rad51B, a Rad51-like protein containing protein kinase activity, causes a G_1 delay (Havre *et al.*, 1998; Havre *et al.*, 2000). Therefore, Rad51 might regulate the cell cycle indirectly through communication with these molecules.

In summary, these studies suggest that Rad51dm has features similar to other Rad51 proteins. In addition, the overexpression of Rad51dm protein disrupts cell cycle progression, and leads to cell death. Rad51 may influence the cell cycle and apoptosis in several ways. First, it is reasonable to speculate that increased amounts of Rad51 protein might disrupt the stoichiometry of the components needed for DNA repair/recombination or cell proliferation. Alternatively, overexpressed Rad51 protein might sequester an essential molecule whose function is crucial for cell viability, such as single strand DNA-binding protein, Brca1, or Brca2, thus leading to cell death. Secondly, it is conceivable that Rad51 may act as a 'sensor' molecule that monitors DNA damage and decides either survival or cell death. If this is the case, Rad51 may function as a connector between a survival signal and a death signal by interacting with various regulators. The conflicting results between our study and others which demonstrated that the overexpression of mammalian Rad51 in immortalized tumor cell lines are more resistant to MMS, might be due to the different expression levels of Rad51 protein. It is possible that the moderate expression level increases repair ability to a degree, but the higher expression level might induce apoptosis. However, we can not rule out the possibility that the regulation of Rad51 activity is different between *Drosophila* and mammals. Although Rad51 plays important roles in DNA repair and recombination, its regulation is not understood yet. The regulation should be orchestrated with many other proteins involved in DNA repair and recombination as well as cell cycle regulation and apoptosis. Further work is required to define the precise

mechanism of Rad51 regulation, which will lead us to understand the functions of Rad51 in DNA-damage induced cell cycle arrest and apoptosis.

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PART 3

DOWN-REGULATION OF *DROSOPHILA* RAD51 RESULTS IN INCREASED MMS SENSITIVITY AND FREQUENCIES OF X-X NON-DISJUNCTION, AND DECREASED MEIOTIC RECOMBINATION.

* This part is intended to be submitted for publication as Yoo, Siuk and B. D. McKee (2001).

CHAPTER 1

INTRODUCTION

The function of a specific gene is traditionally characterized by selecting or creating a mutation in the gene of interest followed by detailed analysis of the phenotype. *Drosophila* is an excellent model organism for genetic approaches to analyze gene function *in vivo* since many genetic techniques such as ethyl methanesulfonate (EMS) mutagenesis, P-element-mediated mutagenesis, enhancer trapping, P-element insertion, and X-ray-induced mutagenesis are well established.

It has been reported that anti-sense RNA interferes with endogenous RNA and down-regulates gene function in some organisms such as *C. elegans* (Guo and Kemphues, 1995). This phenomenon is very close to the posttranscriptional gene silencing (PTGS) in plants described first as 'cosuppression' which involves a sequence-specific RNA degradation process that inactivates a homologous host gene and transgene (Grant, 1999). Numerous systems of PTGS have been studied extensively and there have been a variety of models proposed for the induction and operation of PTGS. However, the precise mechanism of PTGS is poorly understood. One possible model is that the aberrant RNA, which might be double-strandedness, premature transcriptional termination, or other unidentified features of the activator RNA, activates PTGS. To account for the sequence

specificity of PTGS, the model proposed has suggested that the degradation mechanism could be specifically mediated by short complementary RNAs (cRNAs) synthesized from the transgene RNA by a cellular RNA-dependent RNA polymerase (RdRP). These sense RNA and cRNA may then interact to lead to the formation of double-stranded RNA (dsRNA) that can be recognized and degraded by dsRNA-specific RNase (reviewed in Montgomery and Fire, 1998). It was demonstrated using a *Drosophila in vitro* system that dsRNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals (Tuschl *et al.*, 1999; Zamore *et al.*, 2000).

Recently, RNA-mediated interference (RNAi) using dsRNA has been reported in a number of organisms including planarians (Alvarado and Newmark, 1999), *C. elegans* (Montgomery *et al.*, 1998; Fire *et al.*, 1999), zebrafish (Li *et al.*, 2000), *Drosophila* (Kennerdell and Carthew, 1998 and Misquitta and Paterson, 2000), and mice (Wianny and Zernicka-Goetz, 2000). In these experiments, injection of dsRNA corresponding to a specific gene into organisms silences expression of the specific gene probably by rapid degradation of mRNA in affected cells. Although RNAi is a potent and specific inhibitor to interfere with endogenous gene function, the main problem is that the effect of dsRNA is transient and not stably inherited. Therefore, it is difficult to study a role of genes in later developmental stages. To bypass this problem, a method to express dsRNA endogenously as an extended hairpin-loop RNA has been recently reported in *Drosophila* (Kennerdell and Carthew, 2000; Martinek and Young, 2000) and *C. elegans* (Tavernarakis *et al.*, 2000). It has been demonstrated in

Drosophila that an inverted-repeat RNA sequence of *period* was expressed in the tissue of interest under the control of the GAL4/UAS binary expression system and interferes with endogenous gene function, producing a hypomorphic *period* phenotype (Martinek and Young, 2000).

Rad51, a highly conserved protein among eukaryotes, is an essential enzyme in DNA repair and recombination. It has been reported that inhibition of the mouse Rad51 gene by anti-sense oligonucleotides significantly reduces the RNA and protein levels of Rad51, and enhances the radiosensitivity of mouse malignant gliomas (Ohnishi *et al.*, 1998). Previously, a *Drosophila* homolog of Rad51 (Rad51dm) has been reported (McKee *et al.*, 1996 and Akaboshi *et al.*, 1995). We have focused on generating Rad51dm defective mutants using both EMS-based chemical mutagenesis and P-element-mediated mutagenesis; however, a Rad51dm mutant has yet to be obtained. To study Rad51dm *in vivo*, we employed a method of RNA-mediated gene silencing. We generated both anti-sense (AS) and inverted-repeat (IR) transgenic animals carrying the Rad51dm coding region in a reverse orientation or inverted-repeat orientation, respectively. These transgenes are expressed by inducible promoters such as heat-shock 26 (hsp26) protein and heat-shock 70 (hsp70) protein promoters.

In this study, we demonstrate that hsp26-IRrad51 transgenic flies are more sensitive to MMS. This result suggests that the transgene (IR Rad51dm RNA) may interfere with endogenous Rad51dm function in repair of DNA damage. In addition, hsp26-IRrad51 females show elevated frequency of X-X non-disjunction and reduced meiotic recombination events, implying that IR

Rad51dm RNA may inhibit the function of Rad51dm in meiotic recombination. However, AS and hsp70-IR transgenic animals did not show any defects in these experiments.

CHAPTER 2

MATERIALS AND METHODS

dsRNA interference

To produce double-stranded (ds) RNA, anti-sense and sense Rad51dm RNA were synthesized *in vitro*. The Rad51dm coding region was amplified by polymerase-chain reaction (PCR) from pBS-rad51 which contains a 6.5 Kb genomic fragment that includes the Rad51dm gene. RAFM3 (5'GGGAATTCA-TGGAGAAGCTAACGAATGTT3'; *EcoR*I site is underlined) and RARM4 (5'GGCTCGAGGCTCTCCCTGGCGTCTCC3'; *Xho*I site is underlined) were used as primers. The PCR fragment was digested with *EcoR*I and *Xho*I enzymes and cloned into the *EcoR*I and *Xho*I sites of pBluescript SK and pBluescript KS to produce pBSK-R and pBKS-R, respectively. For anti-sense Rad51dm RNA, pBSK-R was linearized with *Xba*I enzyme and for sense RNA, pBKS-R was linearized with *Xho*I enzyme. The transcription reaction was performed at 37° C for 1 hr in the mixture containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 0.5 mM NTPs, 1 μg linearized plasmid DNA, 50 units bacteriophage T7 RNA polymerase, and 20 units RNasin (Promega). After RNA synthesis, the templates were digested with DNase I for 30 min at 37° C. To anneal the sense and anti-sense RNA, the reactions were pooled, boiled for 10 min, and cooled down to room temperature

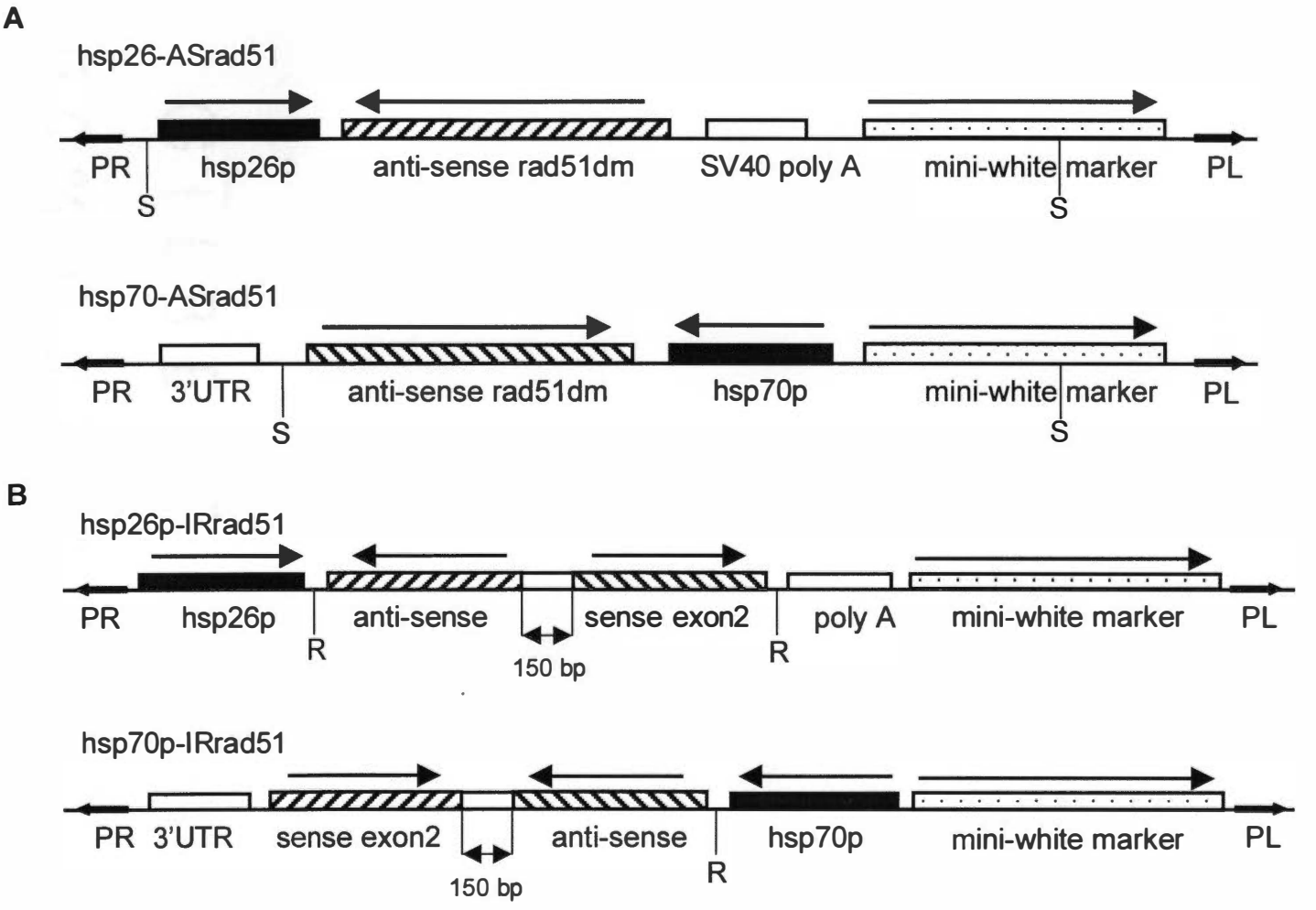
for 12 hr. dsRNA was extracted by using phenol/chloroform, precipitated with ethanol and resuspended in injection buffer containing 5 mM KCl and 0.1 mM PO_4, pH 7.8. dsRNA was confirmed by native agarose gel electrophoresis in TBE buffer (45 mM Tris-borate, 1 mM EDTA).

To microinject dsRNA into embryos, needles were baked for 12 hrs at 120^0 C to remove RNase. Oregon R embryos prior to the syncytial blastoderm stage were collected every 30 min period, dechorionated, and injected. The embryos were kept in an 18^o C incubator for 24 hr and hatched larvae were transferred into a fresh medium to determine survival rate. For controls, injection buffer (mock) and the plasmid carrying Rad51dm gene were injected.

Plasmid constructs for transgenic flies

To down-regulate the endogenous Rad51dm gene, anti-sense (AS) or inverted-repeat (IR) Rad51dm gene driven by heat shock 26 (hsp26) or heat shock 70 (hsp70) promoter was introduced into chromosomes by P-element mediated germ-line transformation. For anti-sense constructs, Rad51dm coding region was amplified by PCR using pBS-rad51 as a template and RAF4 (5'GGCATATGGAGAAGCTAACGAAT3') and RARM4 as primers. The PCR fragment was cloned into the *Sma*I site of a pBS-hsp26 plasmid containing hsp26 promoter to generate pBS-H-AS. After *Hpa*I and *Spe*I digestion, a fragment containing hsp26 and reverse orientation of Rad51dm coding region was cloned into the *Stu*I and *Spe*I sites of pCaSpeR4-poly A vector to generate phsp26-ASrad51 (Fig. 1A). To generate a phsp70-ASrad51 construct, the Rad51dm

Figure 1. Schematic diagram of the plasmid constructs for anti-sense (AS) and inverted-repeat (IR) Rad51dm transgenic animals. CaSpeR4 vector containing mini-white gene was used for both AS (A)and IR (B) constructs. IR constructs were designed to produce dsRNA with a 150 bp hairpin structure within exon two of Rad51dm. The arrows indicate the orientation of genes. Hatched boxes; Rad51dm coding region, black boxes; promoter region, dotted boxes; mini-white marker gene, PL;P-element left end, PR; P-element right end, S; *Sal*I, and R; *EcoR*I.


A
hsp26-ASrad51
PR
hsp26p
anti-sense rad51dm
SV40 poly A
mini-white marker
PL
S
S
hsp70-ASrad51
PR
3'UTR
anti-sense rad51dm
hsp70p
mini-white marker
PL
S
S
B
hsp26p-IRrad51
PR
hsp26p
anti-sense
sense exon2
poly A
mini-white marker
PL
R
150 bp
R
hsp70p-IRrad51
PR
3'UTR
sense exon2
anti-sense
hsp70p
mini-white marker
PL
150 bp
R

coding region was excised by digestion of pBS-H-AS with *EcoR*I and *Not*I and cloned into the *EcoR*I and *Not*I sites of pCaSpeR-hs vector containing the hsp70 promoter (Fig. 1A).

For the inverted-repeat constructs, the exon 2 region of Rad51dm gene was amplified by PCR using pBS-rad51 as a template and Spe-F (5'GGGACTAGTGGCGGCAGCATCACGGCC3'; *Spe*I site is underlined) and Kpn-R (5'GGGGGTACCGCTCTCCCTGGCGTCTCC3'; *Kpn*I site is underlined) as primers. The PCR product was digested with *Spe*I and *Kpn*I enzymes and cloned into the *Spe*I and *Kpn*I sites of phsp26-ASrad51 to generate phsp26-IRrad51 (Fig. 1B). To generate the phsp70-IRrad51 construct, the PCR product was digested with *Spe*I and cloned into the *Spe*I and *Stu*I sites of phsp70-ASrad51 (Fig. 1B). These constructs were microinjected into yw embryos by standard protocols.

Southern blot and RNA blot analyses

To confirm the insertion of transgene, Southern blot was carried out. For AS transgenes, genomic DNA was isolated from hsp26-ASrad51 and hsp70-ASrad51 transgenic animals and digested with *Sal*I. After agarose gel electrophoresis, the DNA fragments were transferred to Nylon membrane (Schleicher and Schuell) and hybridized with ^{32}P-labeled rad51dm probe. For IR transgenes, genomic DNA of hsp26-IRrad51 and hsp70-IRrad51 transgenic animals were digested with *EcoR*I and hybridized with ^{32}P-labeled rad51dm probe. To measure the expression levels of AS or IR Rad51dm RNA, an RNA

slot blot was performed by a standard protocol (Sambrook *et al.* 1989). Total RNA was isolated using Tri-Reagent (Sigma) from transgenic animals after treatment with heat shock for 1 hr at 37° C and subjected to slot blot analysis. The RNA was hybridized with ^{32}P-labeled rad51dm probe. The expression level was normalized using rp49 probe.

Analysis of MMS sensitivity

To test the sensitivity of larvae to methyl methanesulfonate (MMS) (Boyd *et al.*, 1982), the homozygous transgenic flies were allowed to lay eggs for 3 days (control), transferred into a fresh medium for another 3 days (experiment) and discarded. The experimental group was treated with MMS at the final concentration of 0.005% (volume of MMS injected into the medium/weight of the medium), while the control culture was left untreated. During development, heat shock was treated in a 37° C incubator for 1 hr daily. After eclosion, progeny were scored up to day 21 and the survival rate was calculated as the total number of progeny in the experimental group divided by the total number of progeny in control group.

Determination of sex chromosome non-disjunction rate

The homozygous transgenic flies were allowed to lay eggs for 3 days and discarded. The developing cultures were treated with heat shock in a 37° C incubator for 1 hr daily until hatching out. To measure the frequency of X-X non-disjunction, heat-shocked females were mated with yw/B^{s}Yy^{+};Cy/Sp males. The

parents were removed by day 10 and the progeny were counted until day 21. Nullo-X (XO) and duplo-X (XXY) were recognized as B^+ males and B females, respectively. The percent of non-disjunction was calculated as [2 X (XO males + XXY females)/ total number of progeny] X 100. The sum of XO males and XXY females was multiplied by 2 to account for YO and XXX products. To measure the frequency of X-Y non-disjunction, yw/B^sYy^+;Cy/+;p[hsp26-IRrad51]/+ males from X-X non-disjunction experiment were crossed with homozygous transgenic females. After 3 days, the parents were discarded and the developing cultures were treated with heat shock as described above. Among the progeny, homozygous males were selected according to their bright red eye color and mated singly with two yw virgins. The parents were removed by day 10 and the progeny were counted until day 21. The percent of non-disjunction was calculated as (XO males + XXY females)/ total number of progeny X 100. For a control, heat shock was not treated during crosses.

Recombination frequency

To determine the recombination frequency, homozygous hsp26-IR females were crossed with pn cv m f/Y males. After 3 days, the parents were discarded and heat-shock was treated described as above. Female progeny of the genotypes pn cv m f/y w; p[hsp26-IRrad51]/+ were crossed with pn cv m f/Y males, and the progeny were scored for the recombination events in the cv-m and m-f intervals independently. For a control, heat shock was not treated during crosses.

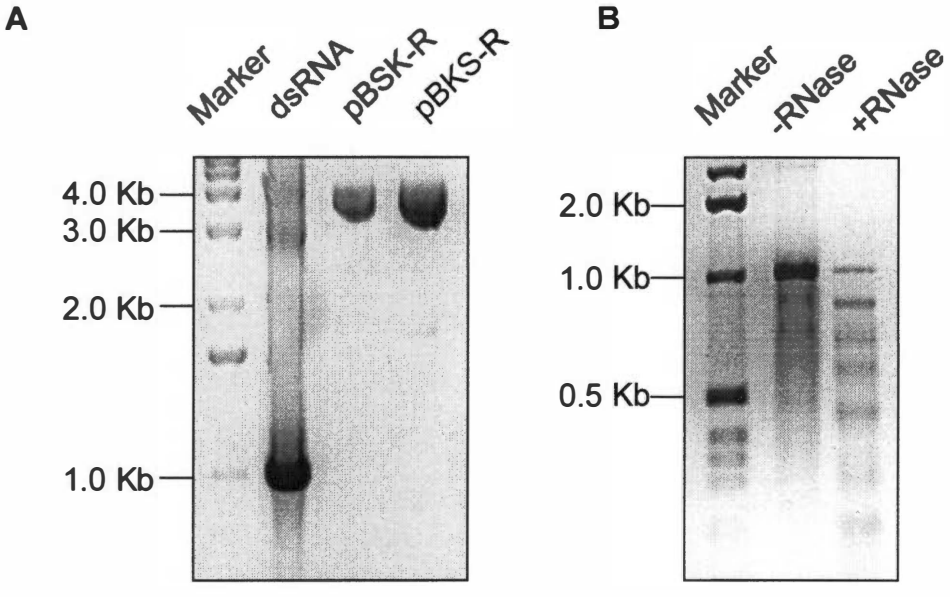
CHAPTER 3

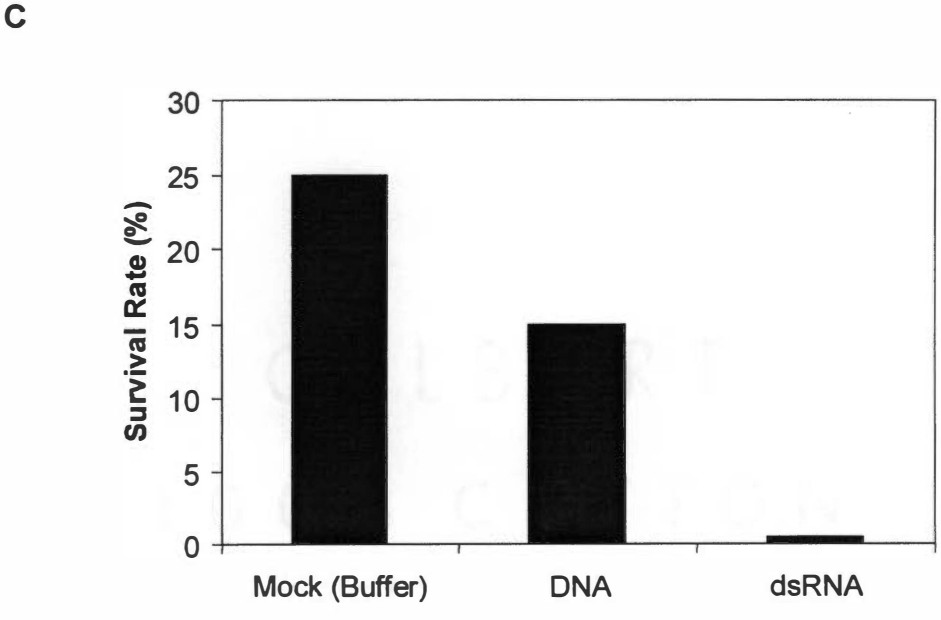
RESULTS

Interference of dsRNA in early embryonic development

It has been demonstrated that double-stranded (ds) RNA effectively blocks gene function in a sequence-specific manner in many animals including *Drosophila* (Kennerdell and Carthew, 1998 and Misquitta and Paterson, 2000). Thus, to study Rad51dm functions in DNA repair and recombination, anti-sense and sense Rad51dm RNA were produced by *in vitro* transcription using T7 RNA polymerase. dsRNA was generated by annealing anti-sense and sense RNA. Agarose gel electrophoresis and an RNase treatment assay confirmed that 1 Kb ds Rad51dm RNA fragment corresponds to the Rad51dm coding region (Fig. 2A and 2B). It was demonstrated by Western blot analysis and immunofluorescence staining that the Rad51dm protein is expressed at high levels in embryos (Fig. 9 and 10 in Part I), although its function during early development is unknown yet. To investigate if dsRNA interferes with normal embryonic development by inhibiting endogenous Rad51dm function, dsRNA was injected into preblastoderm embryos and the survival rate was measured (Fig. 2C). This study shows that injection of dsRNA inhibits normal development compared to injection of plasmid DNA carrying the Rad51dm gene and injection buffer only (mock), suggesting an important role of Rad51dm in early development.

Figure 2. dsRNA-mediated interference during embryonic development. (A) dsRNA was shown by agarose gel electrophoresis (lane 2) after annealing of anti-sense and sense Rad51dm RNA. Anti-sense RNA and sense RNA were generated by in vitro transcription using pBSK-R (lane 3) and pBKS-R (lane 4) as templates, respectively. (B) The dsRNA was confirmed by RNase digestion. (C) Survival rate of embryos after injection of dsRNA. For controls, a plasmid containing the Rad51dm gene and injection buffer (mock) were injected.

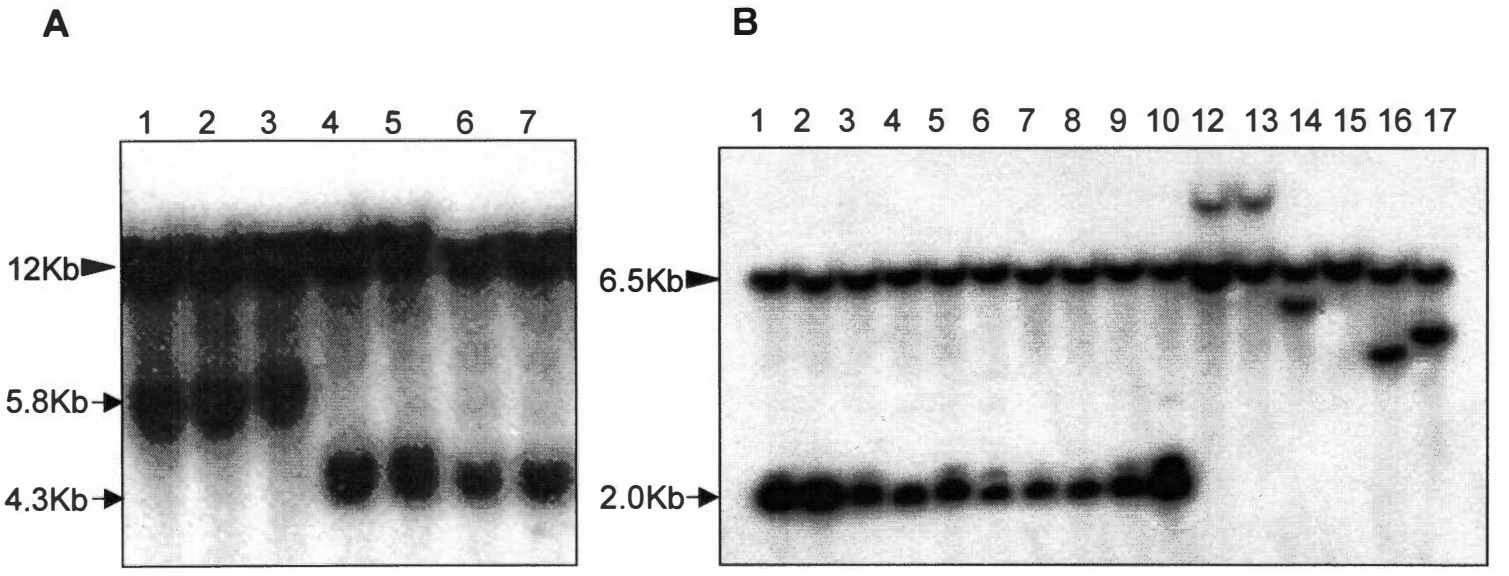

A
Marker
dsRNA
pBSK-R
pBKS-R
4.0 Kb
3.0 Kb
2.0 Kb
1.0 Kb
B
Marker
-RNase
+RNase
2.0 Kb
1.0 Kb
0.5 Kb

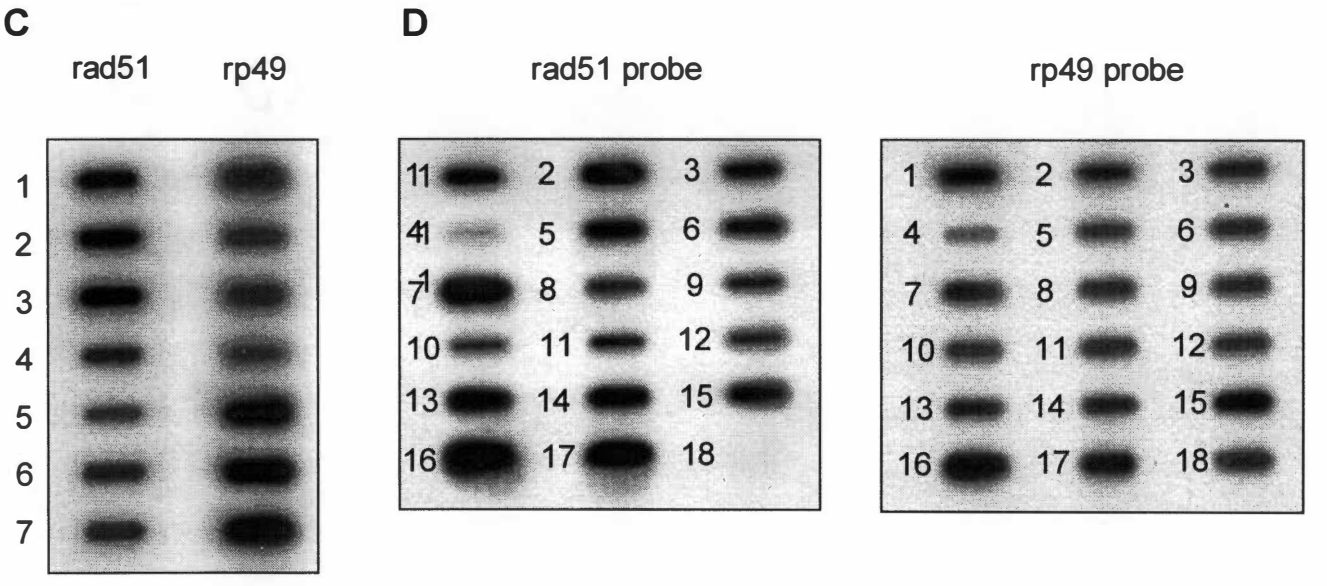

C
Survival Rate (%)
30
25
20
15
10
5
0
Mock (Buffer)
DNA
dsRNA

Generation of AS and IR transgenic flies

Although dsRNA-mediated interference is a powerful method to study the function of a certain gene *in vivo*, there are some limitations. One is that the injection of dsRNA interferes with gene expression transiently and is not stably inherited. A second drawback is that it is difficult to study gene function in late development due to low penetrance. The embryos injected with dsRNA have been shown to fail to develop beyond the larval stage. Therefore, to overcome these problems and to investigate Rad51dm function in later development, we designed a system to express dsRNA *in vivo* using heat-shock inducible promoters. First, we produced plasmid constructs containing the Rad51dm coding region in a reverse orientation (AS) or inverted- repeat orientation (IR) driven by either heat shock 26 (hsp26) or heat shock 70 (hsp70) promoter. These constructs are designed to produce anti-sense Rad51dm RNA or dsRNA as an extended hairpin-loop RNA (Fig. 1). After microinjection of the constructs into the embryos, we obtained seven AS transgenic lines and seventeen IR transgenic lines. Southern blotting analysis showed that the 5.8 Kb and 4.3 Kb *Sal*I fragments hybridized with a ^{32}P-labeled Rad51dm probe represent the transgenes for hsp26-ASrad51 and hsp70-ASrad51 lines, respectively (Fig. 3A). In IR lines, 6.5 Kb *EcoR*I frangments represented the endogenous Rad51dm gene, while others represented the transgenes (Fig. 3B). To determine the expression level of AS and IR Rad51dm, RNA blotting was carried out. Overall, both AS and IR transcripts driven by the hsp26 promoter were expressed at

Figure 3. Southern blot and RNA slot blot analyses of the anti-sense (AS) and inverted-repeat (IR) Rad51dm transgenic lines. (A) Southern blot analysis of the AS transgenic lines. Genomic DNA was digested with *Sal*I, and hybridized with rad51dm probe. hsp26-ASrad51 lines are 1-3 and hsp70-ASrad51 lines are 4-7. (B) Southern blot analysis of the IR transgenic lines. Genomic DNA was digested with *EcoR*I and hybridized with rad51dm probe. hsp26-IRrad51 lines are 1-10 and hsp70-IRrad51 lines are 11-17. The endogenous Rad51dm gene is indicated by the arrow heads and Rad51dm transgene is indicated by the arrows. (C) and (D) RNA slot blot analyses showing the transcript level of Rad51dm transgene. After treatment with heat shock for 1 hr at 37^0 C in adult flies, total RNAs were extracted from AS transgenic lines (C) and IR transgenic lines (D), subjected to RNA slot blot, and hybridized with rad51dm probe. rp49 probe was used as an internal control. yw were used as a control (lane 18).


A
1 2 3 4 5 6 7
12Kb
5.8Kb
4.3Kb
B
1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17
6.5Kb
2.0Kb


C
rad51
rp49
1
2
3
4
5
6
7
D
rad51 probe
rp49 probe
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

higher levels than those of hsp70 promoter after normalization against rp49 levels (Fig. 3C and 3D).

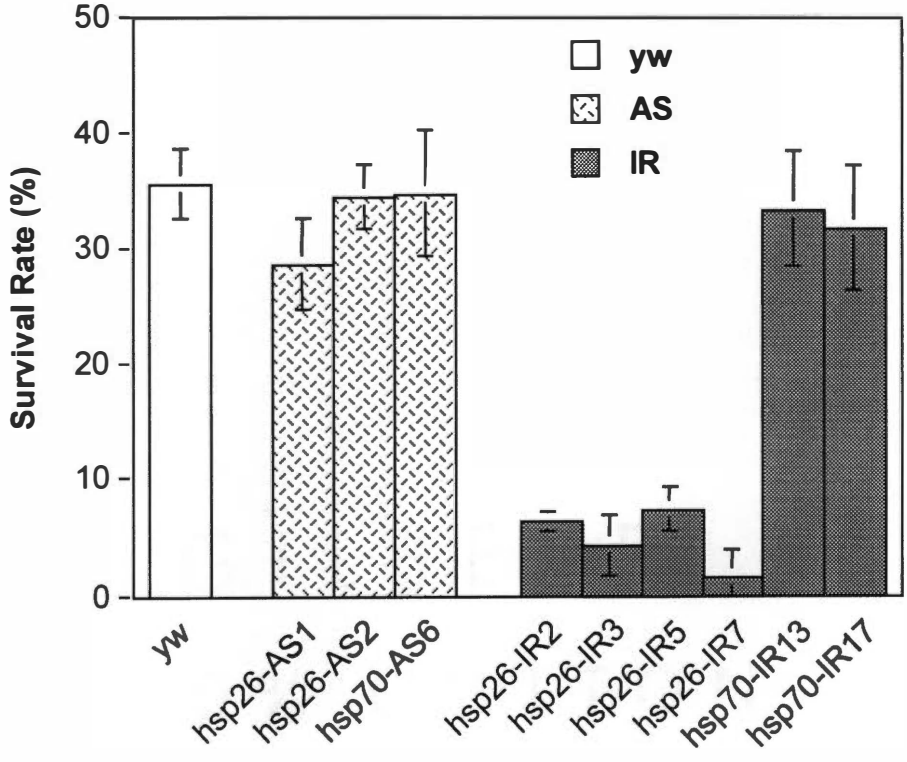
Larval sensitivity to MMS

To study if the AS and IR transgenes interfere with the function of the endogenous Rad51dm gene in the repair of DNA damage, MMS sensitivity of transgenic larvae was measured. From the RNA blotting analysis shown in Fig. 3C and 3D, transgenic lines that express higher levels of AS and IR Rad51dm were chosen for MMS sensitivity. Developing transgenic larvae were administered 0.005% MMS (V/W) and treated with heat shock for 1 hr at 37° C daily and eclosed F1 flies were scored. As a control, larvae were treated with heat shock without MMS administration. The result showed that the survival rates of AS transgenic animals and hsp70-IR lines were similar to that of the yw control. However, all hsp26-IR lines tested showed very low survival rates compared to the yw control (Fig. 4). These results suggest that IR Rad51dm RNA driven by hsp26 promoter may act as a strong inhibitor to block Rad51dm function in DNA repair.

Sex-chromosome non-disjunction

To investigate whether AS and IR Rad51dm RNA affect chromosome separation during meiosis, the frequency of sex-chromosome non-disjunction was measured. For X-X non-disjunction, transgenic females were treated with heat shock during their development and crossed with males carrying B^s marker

Figure 4. Survival rate of AS and IR transgenic larvae after MMS treatment. The homozygous transgenic flies were allowed to lay eggs for 3 days (control), transferred into a fresh medium for another 3 days (experiment), and discarded. The experimental groups were treated with MMS at the final concentration of 0.005 % (the volume of MMS injected into the medium divided by the weight of the medium), while the control culture was left untreated. During development, heat shock was treated in a 37° C incubator for 1 hr daily. After eclosion, the progeny was scored up to day 21 and the survival rate was determined by the total number of progeny in the experiment group divided by the total number of progeny in the control group.


Survival Rate (%)
50
40
30
20
10
0
yw
AS
IR
yw
hsp26-AS1
hsp26-AS2
hsp70-AS6
hsp26-IR2
hsp26-IR3
hsp26-IR5
hsp26-IR7
hsp70-IR13
hsp70-IR17

on the Y chromosome. This B^s marker was used to distinguish exceptional XO male progeny and XXY female progeny from their siblings. Table 1 represents the frequency of X-X non-disjunction in transgenic animals. Both AS and hsp70-IR transgenic flies showed similar frequency of X-X non-disjunction to that of the yw control regardless of heat shock treatment. However, without heat shock treatment, the frequency of X-X non-disjunction of hsp26-IR transgenic animals increased 2- to 6-fold compared to the yw control. Moreover, after heat shock treatment, these animals showed elevated frequency of X-X non-disjunction (4- to 6-fold) compared to no heat shock condition, suggesting that IR Rad51dm RNA inhibits chromosome separation during female meiosis.

To investigate if IR RNA also affects chromosome separation in male meiosis, X-Y non-disjunction was measured (Table 2). Unlike the result from X-X non-disjunction, heat shock treatment did not increase the frequency of X-Y non-disjunction of hsp26-IR transgenic animals, implying that Rad51dm may not be required for male meiosis.

Recombination frequency

It was demonstrated that yeast Rad51 mutant cells are severely defective in meiotic recombination (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). In *Drosophila*, Rad54 mutants have also been shown to be defective in both meiotic and mitotic recombination (Ghabrial *et al.*, 1998; Kooistra *et al.*, 1997; Kooistra *et al.*,1999). To test if IR Rad51dm RNA interferes with meiotic recombination,

Table 1. X-X non-disjunction frequencies of anti-sense (AS) and inverted-repeat (IR) transgenic females

Transgenic lines	HS[a]	N[b]	XX	XY	XXY	XO	%NDJ[c]
A Control							
yw	–	1804	1043	758	0	3	0.33
	+	2502	1323	1176	1	2	0.24
B Anti-Sense							
hsp26-ASrad51(1)	+	645	318	326	0	1	0.31
hsp26-ASrad51(2)	+	1359	718	638	1	2	0.44
hsp70-ASrad51(4)	+	2322	1224	1092	2	4	0.52
hsp70-ASrad51(6)	+	1389	757	629	1	2	0.43
C Inverted-repeat							
hsp26-IRrad51(2)	–	2784	1470	1304	2	8	0.72
	+	2049	1092	926	11	20	3.03
hsp26-IRrad51(7)	–	2401	1272	1106	10	13	1.92
	+	1163	583	504	25	51	13.07
hsp70-IRrad51(13)	–	996	547	448	0	1	0.20
	+	1749	923	825	1	0	0.11
hsp70-IRrad51(14)	–	2161	1082	1077	2	0	0.19
	+	1737	923	810	1	3	0.46
hsp70-IRrad51(17)	–	896	488	408	0	0	0
	+	1766	911	854	1	0	0.11

[a]Heat-Shock was treated in 37^0 C incubator for 1 hr daily.

[b]The total number of progeny scored from a cross of homozygous transgenic females listed above and B^sY^+/yw;Cy/Sp males.

[c]The non-disjunction frequency was calculated as [2 X (XO males + XXY females) /N] X 100.

Table 2. X-Y non-disjunction frequencies of hsp26-IRrad51 transgenic males

Transgenic lines	HS[a]	Sperm Genotype					
		N[b]	XX	XY	XXY	XO	%NDJ[c]
hsp26-IRrad51(2)	–	2946	1624	1311	3	8	0.37
	+	3797	2095	1690	0	12	0.31
hsp26-IRrad51(7)	–	2658	1514	1137	3	4	0.26
	+	2591	1470	1109	2	10	0.46

[a]Heat-Shock was treated in 37^0 C incubator for 1 hr daily.

[b]The total number of progeny scored from a cross of homozygous transgenic males carrying B^sYy^+ with yw females

[c]The non-disjunction frequency was calculated as (XO males + XXY females) /N X 100.

recombination frequency was measured using genetic markers on the X chromosome. Homozygous hsp26-IR females were crossed with pn cv m f/Y males in pairs and one of each pair was treated with heat shock during development; in contrast, the other was left untreated as a control. F1 females which are heterozygous for X chromosomal markers and transgene (pn cv m f/+ + + +;p[hsp26-IRrad51]/+) were crossed with pn cv m f/Y and the recombination events between cv-m and m-f were measured from F2 progeny according to their phenotypes (Table 3). Both hsp26-IR transgenic females of heat shock groups showed a decrease of recombination frequency (10-15%) compared to controls without heat shock treatment. This result suggests that IR Rad51dm RNA might inhibit the role of Rad51dm in female meiotic recombination.

Table 3. Recombination frequencies of hsp26-IRrad51 females

Transgenic lines	HS[a]	N[b]	Recombinants[c]		Frequency[d]	
			cv-m	m-f	cv-m	m-f
Hsp26-IRrad51(2)	–	1341	315	266	23.50	19.83
	+	1335	264	224	19.78	16.78
					P[e]<0.01	
Hsp26-IRrad51(7)	–	1137	255	210	22.43	18.50
	+	861	178	147	20.67	17.07
					P<0.1	

[a]Heat-Shock was treated in 37[0] C incubator for 1 hr daily.

[b]The total number of progeny scored from a cross of pn cv m f/+ + + +;[hsp26-IRrad51]/+ transgenic females listed above and pn cv m f/Y males.

[c]Recombinants for the two intervals included all recombinant progeny between the markers to include the second recombination events.

[d]The recombination frequency for each interval was calculated as f=[recombinants]/N.

[e]P value was determined by chi-square contingency test between HS and non-HS group

CHAPTER 4

DISCUSSION

In this study, the *in vivo* function of the Rad51dm gene was examined using transgenic animals carrying AS and IR Rad51dm gene under control of hsp26 or hsp70 promoters. The results demonstrate that the hsp26-IRrad51 transgenic animals have a much higher sensitivity to MMS than yw control group, suggestive a role of Rad51dm in the repair of DNA damage. In addition, these transgenic females showed a higher frequency of sex chromosome non-disjunction and a lower frequency of meiotic recombination with heat-shock treatment than without heat-shock treatment, suggesting that IR Rad51dm RNA may interfere with the native function of Rad51dm in recombination.

Interference of dsRNA

It has been demonstrated that the injection of dsRNA into adult animals was substantially more effective at producing interference than the injection of either strand individually in *C. elegans* (Fire *et al.*, 1998). This method has also been successfully applied to *Drosophila* embryos to interfere with the function of a native gene (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). Therefore, to investigate the function of Rad51dm, dsRNA was injected into wild type embryos and the survival rate was determined. This experiment resulted in

an extremely low survival of embryos compared to the injection of the plasmid DNA carrying the Rad51dm gene, suggesting the potent inhibitory effect of dsRNA. Although the precise role of Rad51dm has not been determined, these results imply a crucial role of Rad51dm in early embryonic development. It was reported that the disruption of Rad51 gene in mice results in early embryonic lethality (Tsuzuki *et al.*, 1996; Lim and Hasty, 1996). The mutant embryos arrest in the early developmental stage shortly after implantation and exhibit a decrease in cell proliferation followed by programmed cell death (Lim and Hasty, 1996). Recently, Rad51L1, a Rad51-like protein, was disrupted in embryonic stem cells and this mutant also showed lethality in early development (Shu *et al.*, 1999). These results indicate an essential function of mammalian Rad51 in cell proliferation during embryonic development. In contrast to mammalian cells, yeast Rad51 null mutants are viable although they are defective in recombination and DNA repair (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). *Drosophila* Rad51 might perform a similar function to mammalian Rad51 in early development since the embryos injected with dsRNA can't survive beyond larval stage. It will be imperative to obtain Rad51dm null mutants to test if they are viable.

Role of Rad51dm in DNA repair

To overcome the lethal effect of dsRNA on embryos and to study the functions of Rad51dm in late development, we generated transgenic animals carrying an AS and IR Rad51dm coding region driven by the hap26 or hap70 promoters. The sensitivity of transgenic larvae to MMS was measured to

investigate the function of Rad51dm in DNA damage repair. All hsp26-IRrad51 lines tested showed high sensitivity to MMS, in contrast, the AS and hsp70-IRrad51 transgenic animals showed the same survival rates as the yw control group. This result suggests that the function of native Rad51dm in DNA repair was interfered with IR Rad51dm RNA transcribed from hsp26 promoter, but not with AS Rad51dm RNA. It has been reported that injection of anti-sense RNA or sense RNA is not efficient to inhibit a native RNA compared to injection of dsRNA in *Drosophila* (Kennerdell and Carthew, 1998). In addition, transgenic lines that express either anti-sense RNA or sense RNA did not interfere with the function of an endogenous gene (Martinek and Young, 2000). Therefore, the AS Rad51dm RNA might not be efficient to block Rad51dm function in DNA repair. RNA slot blot showed that the expression level of IR Rad51dm RNA driven by hsp70 promoter is lower than its expression driven by hsp26 promoter. This might partly explain the inefficient interference of hsp70-IRrad51 with native Rad51dm.

Role of Rad51dm in recombination

The most important biological processes during meiosis, such as genetic recombination, chromosome pairing, and synapsis of homologous chromosome, occur at prophase I stage. Cytogenetic studies have shown that a proteinaceous core is formed between sister chromatids during leptotene and early zygotene. Later, synaptonemal complexes and recombination nodules are formed during pachytene. It has been reported that enzymes and intermediates involved in recombination may also be involved in chromosome synapsis (Alani *et al.*, 1990).

It is generally thought that meiotic exchange is initiated by the formation of double-strand breaks (DSB), and that repair of DSBs is required for the formation of heteroduplex DNA and chiasmata. The former is a necessary intermediate in the production of recombinant chromosomes, and the latter is necessary for proper disjunction of homologous chromosomes at anaphase of meiosis I. Therefore, analyses of non-disjunction rate and recombination frequency are required for the determination of Rad51dm function during meiosis. It has been demonstrated that the *Drosophila* Rad54 homolog (okra) and Rad51-like protein (spnB) are involved in meiotic recombination (Ghabrial *et al.*, 1998). Both mutant females showed an increase in X-X non-disjunction rates and a decrease in recombination frequencies. However, okra males did not affect X-Y non-disjunction rate, suggesting that Rad54 may not be involved in chromosome separation in *Drosophila* male (McKee *et al.*, 2000). In this study, we demonstrated that X-X non-disjunction rates of hsp26-IRrad51 lines were 4- to 6-fold higher under heat shock treatment than without heat shock treatment. These lines also showed slightly higher X-X non-disjunction rates than the yw control group even when heat shock was not treated, implying basal activity of hsp26 promoter (Glaser *et al.*, 1986). The AS transgenic lines showed the same X-X non-disjunction rates as the yw control group. Taken together with the results from the MMS sensitivity experiment, this result indicates that AS Rad51dm RNA is not efficient to inhibit the native function of Rad51dm. In contrast to high X-X non-disjunction rates of the hsp26-IRrad51 lines, the X-Y non-disjunction rates of the hsp26-IRrad51 lines under heat shock condition were

similar to the controls without heat shock treatment, implying that Rad51dm might not be involved in male chromosome segregation. In *Drosophila*, meiotic recombination is limited to female meiosis, thus, males achieve chromosome pairing and segregation without the benefit of recombination, SC, or chiasmata (Cooper, 1964). Therefore, it is conceivable that the role of Rad51dm during meiosis is confined to the female.

The hsp26-IRrad51 lines showed that the frequencies of recombination under heat shock condition are 10-15% lower than controls without heat shock treatment. One might expect a much lower frequency of recombination since a number of studies revealed that Rad51 is a central enzyme in meiotic recombination. Unlike the non-disjunction experiments which used homozygotes for the transgenes, the recombination experiment was performed using transgene heterozygotes. This may explain why hsp26-IRrad51 showed a slightly reduced recombination frequency. In addition, it is possible that other *Drosophila* recombinases partially compensate for the function of Rad51dm. It is likely that the reduction of the frequency of recombination and increase of sex chromosome non-disjunction in hsp26-IRrad51 females reflect the requirement of Rad51dm in the DSB repair step of meiotic recombination. To better understand the functions of Rad51dm in DNA repair and recombination *in vivo*, the generation of a Rad51dm null mutant and the analysis of its phenotype will be necessary.

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PART 4

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER 1

ROLES OF RAD51*DM* IN DNA REPAIR, RECOMBINATION, AND APOPTOSIS

The main purpose of this research was to determine the function of Rad51dm *in vivo*. A large number of studies were performed in yeast and mammals in order to investigate a role of Rad51 protein; however, little is known about *Drosophila* Rad51 (Rad51dm) protein. In this study, the functional analysis of Rad51dm protein was approached by two different methods. One is determining the effects of overexpression of Rad51dm protein using transgenic animals carrying the Rad51dm coding region driven by the hsp26 promoter or UAS/GAL4 binary expression system. The other is down-regulating Rad51dm at the post-transcriptional level by RNA interference transiently (injection of dsRNA into embryos) or stably (generating transgenic animals carrying Rad51dm gene in a inverted-repeat (IR) orientation under control of heat-shock inducible promoters). This study demonstrated that the Rad51dm functions in DNA repair, meiotic recombination, cell cycle progression, and apoptosis. The Rad51dm protein, like other Rad51 proteins, has functions in DNA repair and recombination. Moreover, Rad51dm is involved in the regulation of cell cycle and cell death. The implications and significances are discussed below.

Role of Rad51dm in DNA repair

Previously, the expression level of Rad51dm transcript was determined by RNase protection assay (RPA) (McKee *et al.*, 1996). The transcripts were detected at major developmental stages, such as embryos, third-instar larvae, and adults, albeit at low levels, suggesting that Rad51dm is constitutively expressed throughout *Drosophila* development. It has been reported that Rad51 is expressed at very low levels in yeast vegetative cells and a variety of somatic tissues in chicken and mouse (Shinohara *et al.*, 1992; Bezzubora *et al.*, 1993; Morita *et al.*, 1993). These results imply that Rad51 might be involved in DNA repair or maintenance of somatic cells. Rad51 mutant analysis in yeast provides direct evidence of the function of Rad51 in DNA repair. The mutants were extremely sensitive to MMS, a DNA damaging agent (Basile *et al.*, 1992; Ogawa *et al.*, 1993). Moreover, in yeast cells, the expression level of Rad51 protein increases 2- to 3-fold after treatment with MMS (Shinohara *et al.*, 1992).

In this study, we demonstrated increased transcript levels of Rad51dm after treatment with DNA damaging agents, such as MMS or X-rays (Fig. 6 in Part I). In addition, it was shown using transgenic lines carrying the Rad51dm gene that the resistance of transgenic larvae to MMS was increased as the copy number of the Rad51dm gene increased (Fig. 5 in Part I). The decrease of Rad51dm dose was obtained from the cross of Df(3R)X3F with Dp(3;1)92 and from transgenic animals that express double-stranded Rad51dm RNA endogenously (Fig. 3 in Part I and Fig. 3 in Part II). The down-regulation of Rad51dm resulted in decreased resistance to MMS. Collectively, these data

indicate the positive relation between the copy numbers of Rad51dm and the resistance to MMS, suggesting a role of Rad51dm in DNA repair.

Role of Rad51dm in recombination

Rad51 protein is an essential enzyme in recombination. This hypothesis has been supported by several lines of evidences. The first evidence came from the study of Rad51 expression. The expression pattern of Rad51 has been reported in vertebrates such as *Xenopus*, chicken, and mouse (Maeshima *et al.*, 1995; Bezzubora *et al.*, 1993; Morita *et al.*, 1993). The expression of Rad51 homologs is strongly induced in immune organs where somatic recombination occurs and in reproductive organs undergoing meiotic recombination. In addition, Rad51-like genes are also highly expressed in testis (Cartwright *et al.*, 1998; Dosanjh *et al.*, 1998; Kawabata and Saeki, 1999). These results suggest Rad51 functions in both somatic and meiotic recombination. In *Drosophila*, the highest level of Rad51dm transcripts was observed in ovaries (McKee *et al.*, 1996), implying that Rad51dm is involved in female meiosis. However, in the present study, the highest level of Rad51dm protein was detected in embryos (Fig. 9 in Part I). Therefore, it is possible that Rad51dm transcripts are maternally stored in oocytes for early embryonic development.

Secondly, Immunocytochemical studies revealed that the Rad51 protein functions during prophase of the first meiotic division. During prophase I, the sister chromatids are associated by synaptonemal complexes and the exchange of genetic material occurs through recombination nodules. In a later stage of

prophase I, when homologues begin to repel each other, the chiasmata become visible, which is important for proper disjunction of homologous chromosomes. It is believed that both meiotic exchange of genetic materials and the formation of chiasmata are closely related to the repair of DSBs. It has been reported that RAD51 foci are observed during prophase I in mouse spermatocytes and oocytes (Ashley *et al.*, 1995; Plug *et al.*, 1996; Moens *et al.*, 1997; Tarsounas *et al.*, 1999). RAD51 foci appear as early as premeiotic S phase before the initiation of synapsis, increase in number and become organized during leptotene. As pachytene progresses, the number of foci drops dramatically. The time course of appearance of RAD51 associated with chromatin and its distribution in spermatocytes suggest that RAD51 plays an important role in the interhomologue interactions that occur during meiotic recombination. We investigated distribution and localization of *Drosophila* Rad51 protein during meiosis. Rad51dm foci were not detected in wild-type spermatocytes probably due to the lack of recombination events in *Drosophila* males. When Rad51dm proteins were overexpressed, however, the foci were observed during prophase I and rapidly disappeared at late prophase I. To investigate a role of Rad51dm protein in meiosis under physiological conditions, the oocytes have been examined, but it is not clear whether Rad51dm foci are associated with synaptonemal complexes (SC) since no marker for the SC is available.

Lastly, yeast Rad51 null mutants are defective in mitotic recombination (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). During meiosis, the mutant accumulates meiosis-specific double-strand breaks at a recombination hot spot

and reduces the formation of recombinants (Shinohara *et al.*, 1992). It has been reported that the *Drosophila* homolog of Rad54 (okra) and a Rad51-like protein (spnB) are involved in meiotic recombination and chromosome disjunction (Ghabrial *et al.*, 1998). Mutations in both genes showed a decrease in meiotic recombination frequencies and a increase in non-disjunction rates of X chromosomes. To study a role of Rad51dm in meiotic recombination, we measured recombination frequencies and X-X non-disjunction rates using transgenic lines carrying the IR Rad51dm gene under the control of the hsp26 promoter (Table 3 and 5 in Part II). Like okra and spnB mutants, the transgenic lines showed low frequencies of recombination and high rates of non-disjunction under heat shock condition compared to control groups without heat shock treatment. These results suggest a possible role of Rad51dm in meiotic recombination in the female. Rad51dm might not play a major role in male meiosis since (1) Rad51dm protein was not detected in testes (Fig. 9 in Part I), (2) Rad51dm foci were not observed in wild-type spermatocytes, and (3) hsp26-IRrad51 transgenic lines showed the same level of X-Y non-disjunction rate as in the yw control (Table 4 in Part II).

Role of Rad51dm in early embryogenesis

It has been reported that the disruption of Rad51 by gene targeting methods results in lethality during early embryonic development, indicating a crucial role of Rad51 in embryogenesis (Tsuzuki *et al.*, 1996; Lim and Hasty, 1996). Since the cells are rapidly dividing during embryogenesis, Rad51 might

be involved in the control of cell cycle, DNA replication, or transcription presumably by interacting with p53, Brca1, or Brca2. However, the precise role of Rad51 protein in embryos is not understood. In *Drosophila*, the embryonic nuclei are mitotically synchronized until the syncytial blastoderm stage (2 hr after egg laying). Our immunostaining data show that Rad51dm protein is localized with unknown materials stained with DAPI around the nuclei or mitotic chromosomes (Fig. 10 in Part I). These unknown materials do not display genome fragmentations caused by apoptosis since cell death begins at stage 11 (7 hours after egg laying) (Abrams *et al.*, 1993). These data differ from immunostaining of meiotic cells which show the association of Rad51dm protein with chromatids during prophase I of hsp26-rad51 spermatocytes. Since this study shows that Rad51dm protein is expressed at the highest level in embryos, it might play an important role during embryogenesis. It is possible that Rad51dm functions in the repair of mis-incorporated nucleotides during DNA replication or in the removal of nucleotide wastes. To inhibit the function of native Rad51dm, dsRNA was injected into embryos (Fig. 2 in Part II). The extremely low survival of embryos in this experiment supports the hypothesis that Rad51dm is an essential component during embryogenesis. Further study will be necessary to determine the role of Rad51dm in embryos.

Roles of Rad51dm in cell cycle progression and apoptosis

Based on the results of the cell-cycle dependent expression pattern in yeast and mouse (Basile *et al.*, 1992; Yamamoto *et al.*, 1996) and the interaction

with molecules that are involved in cell cycle regulation such as p53, Brca1, and Brca2 (Buchhop *et al.*, 1997; Deng *et al.*, 2000; Chen *et al.*, 1998), it is reasonable to hypothesize that the Rad51 protein may function in the regulation of the cell cycle. However, it is not known how Rad51 affects cell cycle progression. Recently, it has been reported in mammalian cells that overexpression of human Rec2/Rad51B, a Rad51-like protein, causes G_1 delay (Havre *et al.*, 1998). In this study, it was demonstrated that the overexpressed Rad51dm protein disrupts the normal mitotic pattern in the second mitotic wave behind the morphogenetic furrow of eye imaginal discs (Fig. 14 in Part I). The broader and more diffused mitotic band in the eye disc might be explained by prolonged cell cycle progression. Moreover, the overexpression of Rad51dm protein in wing, eye discs and primordial germ cells induces apoptosis where Rad51dm is overexpressed (Fig. 13, and Fig 14 in Part I). Since the morphogenetic furrow progresses from the posterior to the anterior region of the eye disc and Rad51dm expression is higher in more posterior region, it is conceivable that relatively low levels of Rad51dm protein in the second mitotic wave inhibit cell cycle progression. If the expression is higher, the Rad51dm protein may lead to cell death. In conclusion, it is likely that Rad51dm is induced by any DNA damage (Fig. 6 in Part I) and delays cell cycle progression to allow time for DNA repair to occur (Fig. 14E and F in Part I). The higher expression of Rad51dm protein above a threshold level might lead to cell death to prevent aberrant cellular function (Fig. 14A-D in Part I). However, it is not clear whether

Rad51dm protein is expressed at high level to cause apoptosis under physiological conditions.

CHAPTER 2

FURTHER STUDY

The direct way to study the *in vivo* function of a gene is to analyze phenotypes of the mutant. Unfortunately, the region of Rad51dm does not contain any known repair or meiotic mutations. We attempted to generate Rad51dm mutants by site-selected mutagenesis using P-element transposons and by chemical mutagenesis such as ethyl methanesulfonate (EMS), but Rad51dm mutants have not been obtained yet. Recently, a method of gene targeting by homologous recombination has been reported in *Drosophila* (Rong and Golic, 2000). This method consists of three parts. The first part is a transgene that expresses a site-specific recombinase. For this purpose, FLP site-specific recombinase that recognizes and cleaves FRT (FLP recombination target) sequence (34 bp) has been used. The second part of the targeting strategy is a transgene that expresses a site-specific endonuclease. The construct carrying I-*Sce*I endonuclease that recognizes and cuts 18 bp sequences has been generated. Both FLP site-specific recombinase and I-*Sce*I endonuclease are driven by the hsp70 promoter. The last part is a transgene donor construct that carries the recognition site of FLP recombinase, FRT sequences, at both ends of the DNA to be targeted and the recognition site of I-*Sce*I endonuclease in the middle of DNA molecule. The basic idea of this

strategy is to enhance the targeting efficiency of the DNA molecule by generating broken ends that is achieved by I-*Sce*I endonuclease and the 18 bp recognition site inserted into DNA.

To produce Rad51 null mutants using this gene targeting method, 6.5 kb genomic fragment including the Rad51dm gene was inserted into *EcoR*I site of pCaSpeR4 vector to generate pGTrad51. The recognition site of I-*Sce*I endonuclease was inserted in the middle of Rad51dm gene. To generate null mutants, the promoter region (200 bp of upstream of start codon), start codon, and Walker type-B ATP binding site were removed. After microinjection of pGTrad51 into yw embryos, at least two X-chromosome insertion lines were obtained. To produce Rad51dm mutants, the cross of GTrad51 lines with a transgenic line carrying both FLP recombinase and I-*Sce*I endonuclease obtained from K. G. Golic is currently being performed.

Once heterozygous Rad51dm mutant is obtained, a homozygous Rad51dm null mutant will be generated. It will be interesting to examine whether the Rad51dm null mutant is viable, since mouse Rad51 mutant is lethal during early embryonic development. If the Rad51dm null mutant shows no defects during development, the experiments described above, such as MMS sensitivity, non-disjunction rate of sex chromosome, and recombination frequency will be performed to investigate the functions of Rad51dm.

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