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Assembly of RecA-like recombinases: Distinct roles for mediator proteins in mitosis and meiosis

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Members of the RecA family of recombinases from bacteriophage T4, *Escherichia coli*, yeast, and higher eukaryotes function in recombination as higher-order oligomers assembled on tracts of single-strand DNA (ssDNA). Biochemical studies have shown that assembly of recombinase involves accessory factors. These studies have identified a class of proteins, called recombination mediator proteins, that act by promoting assembly of recombinase on ssDNA tracts that are bound by ssDNA-binding protein (ssb). In the absence of mediators, ssb inhibits recombination reactions by competing with recombinase for DNA-binding sites. Here we briefly review mediated recombinase assembly and present results of new *in vivo* experiments. Immuno-double-staining experiments in *Saccharomyces cerevisiae* suggest that Rad51, the eukaryotic recombinase, can assemble at or near sites containing ssb (replication protein A, RPA) during the response to DNA damage, consistent with a need for mediator activity. Correspondingly, mediator gene mutants display defects in Rad51 assembly after DNA damage and during meiosis, although the requirements for assembly are distinct in the two cases. In meiosis, both Rad52 and Rad55/57 are required, whereas either Rad52 or Rad55/57 is sufficient to promote assembly of Rad51 in irradiated mitotic cells. Rad52 promotes normal amounts of Rad51 assembly in the absence of Rad55 at 30°C but not 20°C, accounting for the cold sensitivity of *rad55* null mutants. Finally, we show that assembly of Rad51 is induced by radiation during S phase but not during G₁, consistent with the role of Rad51 in repairing the spontaneous damage that occurs during DNA replication.

Homologous recombination reactions promote repair of DNA ends formed by double-strand breaks (DSBs) and by replication fork collapse. Recombinational repair also allows cells to replicate past DNA lesions that block the progress of DNA polymerase. In phage T4, recombination is critical for initiating replication. In eukaryotes, homologous recombination is critical for accurate reductional segregation of chromosomes during meiosis. Finally, in prokaryotes, recombination allows horizontal transfer of alleles among and between bacteria and phage.

At the center of homologous recombination are the recombinases, proteins that promote the formation of heteroduplex DNA. Of particular importance are the recombinases of the RecA family, including RecA in eubacteria, RadA in archaea, Rad51 and Dmcl in eukaryotes, and the bacteriophage T4 UvsX protein.

RecA-Like Recombinases Assemble on Single-Strand DNA (ssDNA).

Several of the RecA recombinases have been shown to act by assembling into filaments on ssDNA (1–5). ssDNA tracts are formed by nucleolytic processing of DNA ends and by stalling of polymerase during DNA replication (6, 7). The nucleoprotein filaments formed by assembly of recombinases on ssDNA are capable of “searching” intact DNA duplexes for homologous regions (8–11). Location of a homologous duplex by the recombinase filament results in formation of a homologous joint

between the ssDNA and the duplex and leads to strand exchange. During strand exchange, the ssDNA contained within the nucleoprotein filament forms Watson–Crick base pairs with the complementary strand of the “target” duplex, displacing the noncomplementary strand in the duplex. The hybrid DNA formed by strand exchange is further processed by repair polymerase and other recombination factors, eventually yielding two intact DNA duplexes (7, 8, 11, 12).

Accessory Factors Act to Promote Assembly of Recombinase. Recombinases are able to promote strand exchange to form hybrid DNA *in vitro* without additional proteins. However, accessory factors can stimulate strand exchange. These factors can be divided into two broad classes: those that act before homology search by promoting assembly of recombinase filaments, and those that act during homology search and strand exchange. Assembly factors can, in turn, be divided into two classes: ssDNA-binding protein (ssb) and assembly “mediators.” Here we focus on the roles of assembly factors. A general model for the mechanism of recombinase assembly is shown in Fig. 1.

ssbs are involved in multiple pathways of DNA metabolism, including replication, recombination, and repair. ssbs are abundant; the abundance of bacteriophage T4 gp32 protein is $1\text{--}3 \times 10^4$ copies/infected cell (13), that of tetrameric *Escherichia coli* SSB about $1\text{--}2 \times 10^3$ /cell (14), and that of the heterotrimeric RPA protein from eukaryotes about $10^4\text{--}10^5$ /cell (15). As their name implies, ssbs bind ssDNA specifically. The relative binding affinity (ssDNA/double-strand DNA) is at least 10^6 for gp32 (16), at least 10^8 for SSB (D. T. Lohman, personal communication), and 70-fold for RPA (17). ssbs can bind ssDNA in a cooperative manner, forming filaments that can readily saturate long stretches of ssDNA (18).

ssbs can stimulate assembly of recombinases on ssDNA, but their ability to do so depends highly on reaction conditions (19–22). If an amount of ssb sufficient to saturate binding sites on ssDNA is added to strand exchange reactions before addition of recombinase, the reaction is inhibited. On the other hand, if recombinase is added first to such reactions, strand exchange activity is often stimulated compared with reactions containing no ssb. Inhibition of recombinase by ssb results from its ability to block initial binding of recombinase to ssDNA; ssb can outcompete recombinases as a consequence of their higher affinity and faster binding kinetics. A model that accounts for these observations is as follows. ssb competes with recombinase for initial binding sites on ssDNA. However, when recombinase

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Abbreviations: ssDNA, single-strand DNA; ssb, ssDNA-binding protein (generic); RPA, replication protein A; DSB, DNA double-strand break; CHO, Chinese hamster ovary; WT, wild type.

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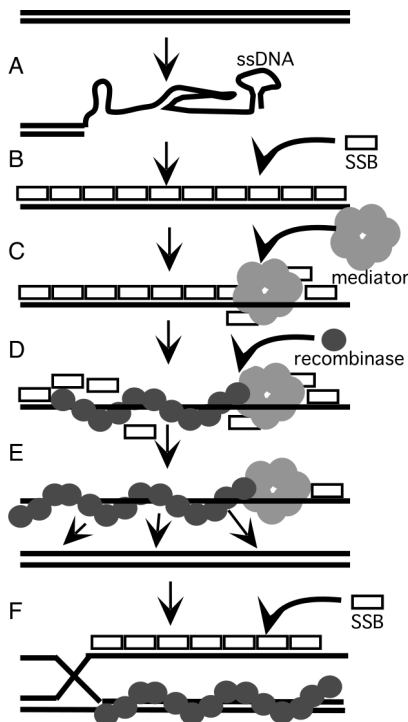


Fig. 1. Generic model for assembly of recombinase on ssb-coated ssDNA. (A) Tracts of ssDNA form because of resection at DSB sites or stalling of polymerase. (B) ssb assembles into oligomeric filaments on tracts of ssDNA, removing secondary structures. (C) Mediator protein binds to ssb-coated DNA, causing a local remodeling of the ssb filament. (D) Recombinase initiates filament formation at sites of mediator-ssb-ssDNA. Recombinase filaments then elongate displacing ssb. (E) The elongated recombinase filament searches for homologous sequences. (F) Strand exchange occurs. The outgoing ssDNA strand is bound by ssb.

filaments are initiated, interactions that occur during recombinase filament elongation displace ssb from DNA. Elongation of recombinase filaments is blocked by DNA secondary structures, whereas assembly of ssb filaments is not. As a result of these properties, adding ssb to reactions after recombinase allows recombinase the opportunity to initiate filaments on ssDNA before ssb is added and then extend past sites of secondary structure with the help of ssb. The recombinase filaments formed as a result of ssb-recombinase interactions are more efficient in strand exchange reactions than filaments formed by recombinase alone because of persisting secondary structures in ssDNA.

RPA Assembles Before Rad51 After Radiation Treatment. Immunostaining techniques can detect assembly of recombination proteins into subnuclear staining foci (23–32). Several lines of evidence suggest that these foci represent protein oligomers assembled at sites of DSBs and possibly daughter strand gaps as well. Double staining shows that multiple proteins required for recombination assemble at the same sites during recombination. Such experiments in yeast and mammalian cells showed that RPA colocalizes with Rad51 (29, 31–33). An important question raised by the biochemical interaction of ssb and recombinase is: Does ssb assemble before or after recombinase *in vivo*? The high efficiency of binding to ssDNA and the abundance of ssb predict that ssb will assemble more rapidly than recombinases on tracts of ssDNA. However, the one previous experiment that addressed the question of the relative timing of assembly of RPA and Rad51 during recombination found that Rad51 foci assembled first, with colocalizing RPA foci appearing at a later stage during meiosis in mouse spermatocytes (33).

To determine the relative timing of appearance of RPA and

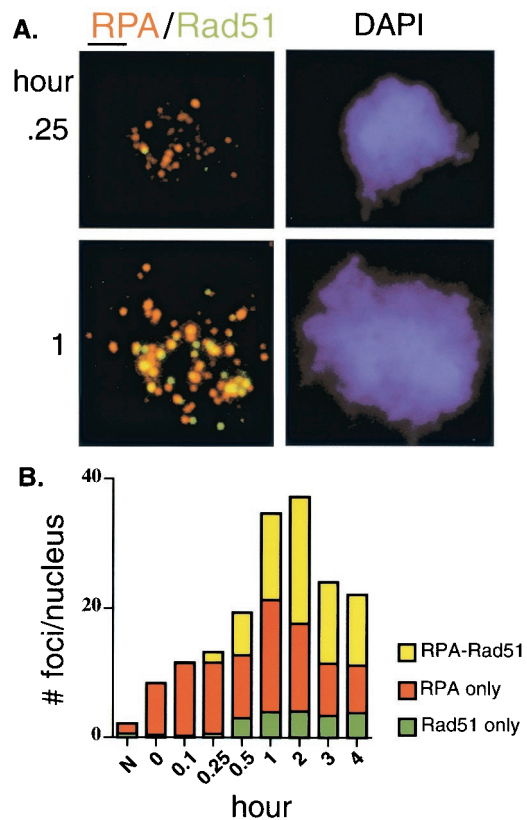


Fig. 2. RPA assembles before Rad51 after γ -irradiation of G_2 arrested *S. cerevisiae* spheroplasts. A diploid yeast strain (NKY1314, SK-1 strain background) was grown in rich medium and arrested in G_2/M by treatment with nocodazole. Cells were spheroplasted by zymolyase treatment, irradiated to a dose of 50 krad with a ^{60}Co source, and resuspended in osmotically stabilized growth medium containing nocodazole. At the times indicated, culture aliquots were processed to obtain surface spread nuclei. Spread nuclei were indirectly immunostained with polyclonal guinea pig anti-Rad51 (green) and rabbit anti-RPA70 (red), counter stained with a DNA-specific dye (DAPI), and examined by epifluorescence microscopy. The number of foci per nucleus was determined by visual examination of digital images. Details of the method have been published (29, 59). (A) Representative nuclei from the times indicated. Overlapping signals are yellow in these pseudocolored images. (B) Average number of foci detected per nucleus at the times indicated.

Rad51 foci after induction of damage by γ -rays, a log phase culture of diploid cells was arrested in G_2/M with the microtubule inhibitor nocodazole. Cells were arrested at the G_2/M phase of the cell cycle to avoid detection of the RPA foci that form in undamaged cells as a normal part of S phase (29). In addition to being arrested in G_2/M , cells were treated with zymolyase to remove the cell wall, which allowed rapid processing of samples. After treatment of arrested cells with 50 krad, the culture was incubated at 30°C for various times and spread nuclei prepared for immunostaining. Fifty unselected surface-spread nuclei were examined from each sample. The data are shown in Fig. 2. Induction of RPA focus positive nuclei was detected immediately after treatment, i.e., at the time point designated $t = 0$ h. The RPA foci seen in these nuclei depend on irradiation, because they did not appear in an untreated control. Induction of Rad51 foci was not detected until $t = 0.25$ h. The average number of induced RPA-containing foci increased to a maximum of 33/nucleus at 2 h. Rad51 containing foci also peaked at 2 h (18/nucleus). At all times, the majority of Rad51-containing foci (67–83%) also stained with RPA. The subset of Rad51 foci that did not contain detectable amounts of RPA could result from assembly of Rad51 at sites devoid of RPA. Alternatively, they could result from displacement of RPA, epitope masking of

RPA by Rad51, or limited detection of RPA by the probe used. The early appearance of RPA foci relative to Rad51 foci, together with the observation that most Rad51-containing foci also contained RPA, strongly suggests that Rad51 can assemble at or near sites of previously assembled RPA.

Recombination Mediator Proteins. Given that *ssb* inhibits the ability of recombinases to bind ssDNA *in vitro* when added to reactions before *ssb*, a mechanism is needed to overcome the inhibitory effect of *ssb* on recombinase assembly. So-called “mediator” proteins appear to play this role. These proteins promote efficient formation of recombinase oligomers on ssDNA that are coated with *ssb* (34, 35). The bacteriophage T4 UvsY was the first protein to be shown to have mediator activity *in vitro* (21, 36, 37). In addition to overcoming inhibitory effects of gp32 on UvsX, UvsY can stabilize UvsX filaments when formed (36, 37).

E. coli appears to have two distinct mechanisms for promoting assembly of RecA. RecO and RecR act together as mediators to overcome *ssb*-dependent inhibition of RecA-mediated strand exchange (38, 39). A third protein, RecF, is likely to function with RecO, and RecR *in vivo* and has been shown to be capable of limiting the assembly of RecA to regions of ssDNA *in vitro* (40). Genetic studies provide evidence that RecF, RecO, and RecR function specifically during the repair of daughter strand gaps caused by the stalling of polymerase (6).

The second RecA assembly mechanism involves the multifunctional recombination protein RecBCD (41). RecBCD has both exonuclease and helicase activity. When a RecBCD complex is engaged in processing duplex DNA and a specific 8-nt sequence called χ is encountered, the 3' to 5' exonuclease activity of RecBCD is attenuated, but the enzyme retains both helicase and 5' to 3' exonuclease activity (42). Attenuation of the 3' to 5' activity leads to formation of ssDNA tracts with 3' ends as the enzyme travels away from χ . In an *in vitro* reaction containing linear duplex, a homologous supercoiled plasmid, SSB, RecA, and RecBCD, χ stimulates the strand exchange between the 3' ssDNA end in the linear DNA created by RecBCD and the supercoiled plasmid (43). In addition to creating the ssDNA substrate for this “coupled” nuclease–recombinase reaction, RecBCD promotes assembly of RecA on the 3' ssDNA molecules it creates (44–48). Thus, *E. coli* uses two different mechanisms to build RecA filaments at sites of DNA damage in wild-type (WT) cells; RecBCD promotes assembly of RecA at DNA ends, whereas RecFOR controls assembly of RecA at single-strand gaps. This functional specialization is not strict however; genetic suppressor studies suggest that RecFOR can substitute for RecBCD in promoting recombination of DNA ends (6).

Mediator Proteins in Eukaryotes. Both the *S. cerevisiae* Rad52 and heterodimeric Rad55/Rad57 have been shown to have mediator function *in vitro* (35, 49–52). Both Rad52 and Rad55/57 can overcome inhibition of Rad51-mediated strand exchange by RPA. Human Rad52 even stimulates Rad51 reactions independent of the effects of RPA (53, 54). Although Rad52 stimulates assembly of Rad51, other observations indicate that it has additional activities that can promote recombination. Rad52 has been shown to promote annealing of complementary ssDNA (55, 56), an activity that may account for the ability of *rad51* mutant strains to carry out limited but significant amounts of recombination. Rad52 forms heptameric ring structures (56, 57) that bind selectively to ssDNA ends (58).

The role of *RAD55*, *RAD57*, and *RAD52* in assembly of Rad51 has been examined *in vivo* by using cytological techniques (29). During meiosis in the budding yeast *S. cerevisiae*, Rad51 foci appear transiently during normal meiotic recombination, but no foci are seen in *rad55*, *rad57*, or *rad52* mutants, suggesting both

Rad52 and Rad55/57 are required for Rad51 assembly in meiosis.

Recombination proteins also influence the assembly of the meiosis-specific RecA homologue, Dmc1. In this case, Rad51 is important for normal assembly of Dmc1 (23, 30). However, assembly of Dmc1 was found to be independent of Rad52 (23, 59). This result was surprising, because formation of Rad52 foci depends on Rad52. Our current interpretation of the lack of dependence of Dmc1 foci on Rad52 is that Rad51's ability to promote Dmc1 assembly is independent of its ability to oligomerize into a cytologically detectable structure. Perhaps Rad55, Rad57, and Rad51 can form a heterotrimer or other lower-order heterooligomer that serves as a mediator for Dmc1. A recent study has also shown that another recombination accessory factor, Tid1 (also known as Rdh54), is required to coordinate the assembly of Dmc1 foci at sites of Rad51 foci (30). Tid1 can bind directly to both Rad51 and Dmc1 (24), raising the possibility that it serves as an intermolecular bridge between these two proteins during assembly.

In higher eukaryotes, mammalian Rad52 is the only protein shown by biochemical methods to promote Rad51 assembly (54). However, cytological studies provide evidence for additional assembly factors. Xrcc3, an orthologue of yeast Rad57 (60), was found to be required for assembly of Rad51 foci after DNA damage in Chinese hamster ovary cells (CHO) (61). Xrcc3 is one of five paralogues of Rad51 found in mammalian cells (Table 1). All five Rad51 paralogues were recently shown, by cytological methods, to be important for Rad51 assembly after x-ray treatment of chicken DT40 cells (82–84). This led Takeda and colleagues to propose that the five may function together as a single mediator complex.

In addition to the Rad51 paralogues, the breast cancer susceptibility genes *BRCA1* (85) and *BRCA2* (86) have been shown to be important for formation of Rad51 foci after DNA damage. The BRCA proteins had been shown previously to associate with Rad51 (75–81) and to colocalize with damage-induced Rad51 foci (87, 88). These results suggested that the BRCA proteins might act directly to promote assembly. Analysis of a cell line that expresses a truncated form of *BRCA2* showed failure of both *BRCA2* and Rad51 to localize to the nucleus (89). Thus, it is possible that *BRCA2*'s role in assembly is limited to nuclear transport of Rad51. Biochemical studies are required to determine which of the many vertebrate proteins required for subnuclear Rad51 assembly *in vivo* are directly involved.

Beginning with the biochemical studies of Alberts and colleagues (62), mediator proteins have been found to interact both with *ssbs* and with recombinases (see Table 1). In the T4 system, as well as in the bacterial and eukaryotic systems, direct interactions have been detected between mediators and *ssb* and/or between mediators and recombinase (reviewed in ref. 35). These interactions have been proposed to remodel *ssb*–ssDNA complexes forming structures more accessible for recombinase filament initiation and to recruit recombinase to the sites of ssDNA–*ssb*–mediator complexes (35, 39, 49–51, 54, 90, 91). Such recruitment of recombinase to ssDNA appears to be particularly important in the case of Rad51, because this protein has no preference for ssDNA over dsDNA *in vitro* (4).

The Role of Mediator Proteins in Rad51 Assembly After DNA Damage.

To determine whether proteins known to have *in vitro* mediator activity are required for formation of Rad51 foci after mitotic DNA damage, exponentially growing WT and mutant cells were γ -irradiated in parallel. At various times after irradiation, cells were spheroplasted, and spread nuclei were prepared. The *rad52* and *rad55* single mutants used were the same as those used previously to examine the role of *RAD52* and *RAD55* in meiosis (29). An isogenic *rad55 rad52* double mutant was also examined. Because the radiation sensitivity of *rad55*-null mutants is more

Table 1. Recombinases and assembly factors

	rec	ssb	Mediator	Mediator interactions*		
				ssb	rec	Indirect
T4	UvsX	gp32	UvsY	(62, 63)	(62)	
<i>E. coli</i>	RecA	SSB	RecO	(39)		
			RecR			RecO (39)
			RecF [†]			RecR (64)
			RecBCD [‡]			
<i>S. cerevisiae</i>	Rad51	RPA	Rad52	(65)	(66–68)	
			Rad55		(67, 69)	
			Rad57			Rad55 (52, 67, 69)
Vertebrates	Rad51	RPA	Rad52	(70)	(71)	
			[Xrcc2] [§]			Rad51D (72)
			[Xrcc3]		(73)	
			[Rad51B]			Rad51C (74)
			[Rad51C]		(72)	Xrcc3 (72)
			[Rad51D]			Rad51C (72)
			[Brca1]		(75) [¶]	Brca2 (76)
			[Brca2]		(77, 81)	

*Evidence for interaction includes biochemical (e.g.; affinity chromatography, immune precipitation, chemical cross-linking), and two-hybrid data.

[†]Although RecF has not been found to contribute to mediator activity directly, it can influence assembly of RecA and associates directly with RecR.

[‡]RecBCD has been shown to promote assembly of RecA, although the mechanism may not involve displacement of SSB from ssDNA. Although there is no showing direct evidence for protein–protein interactions, the properties of certain RecB mutants strongly suggest that it is the subunit of RecBCD most closely involved in RecA assembly.

[§]The proteins listed in parentheses have not been shown by biochemical experiments to be mediators. However, these proteins are considered to be “putative mediators”, based on two properties: they bind directly or indirectly to recombinase via protein–protein interactions, and they are required for assembly of recombinase at sites of damage in vivo. (Xrcc3 is a likely orthologue of yeast Rad57, a known mediator protein.) We do not list proteins that influence recombinase assembly but are likely to act by posttranscriptional modification of recombinase.

[¶]The interactions detected in this paper could have been direct or indirect protein–protein interactions with Rad51.

pronounced at low temperatures, experiments were carried out at both 20°C and 30°C. The results are shown in Fig. 3. All strains examined showed significant induction of Rad51 foci after irradiation. The number of foci continued to increase during the interval examined (up to 5 h). In striking contrast to the previous meiotic experiments, radiation-induced mitotic Rad51 foci were observed in the *rad52* and *rad55* single mutants. Significant induction of foci was detected even in the *rad52 rad55* double mutant, indicating formation of foci after radiation of mitotic cells does not depend strictly on either Rad55 or Rad52.

Although focus induction was observed in *rad55* and *rad52* mutants, there were significant and reproducible differences in the rate of focus accumulation in the mutant strains as compared with WT. At 20°C, the *rad55* mutant accumulated foci at a slower rate than WT; the mutant displayed fewer foci than WT at all times after induction ($P < 0.025$). In contrast to *rad55*, differences between WT and the *rad52* single mutant were not significant except for the 1-hr sample. The number of foci seen in the *rad52 rad55* double mutant was not significantly less than that seen in the *rad55* single mutant at 2, 3, and 5 h. Thus, *RAD55* is required for the normal efficiency of focus induction at 20°C, whereas *RAD52* is largely dispensable. A *rad52 rad55 rad57* triple mutant was also examined and showed the same efficiency of focus induction as a *rad55 rad52* mutant, indicating that *RAD57* is not required for the residual Rad51 focus formation detected in the absence of *RAD55* and *RAD52* (59).

The *rad55* mutant showed a much smaller defect in Rad51 focus induction at 30°C than at 20°C. The rate of focus appearance in the *rad55* mutant was about 2.5 greater at 30°C than at 20°C, and this difference was significant ($P < 0.005$), indicating that focus formation is cold sensitive in *rad55* mutants.

The *rad52* single mutant showed a more pronounced lag in Rad51 focus formation at 30°C than at 20°C; significantly fewer

foci were seen in *rad52* than in WT at 1 and 2 h, indicating that the timing of focus induction is delayed in the mutant. Of particular importance is the observation that the *rad52 rad55* double mutant assembled fewer Rad51 foci than either the *rad55* or the *rad52* single mutant at 30°C ($P < 0.005$ for the 2-, 3- and 5-h time points). These results suggest that *RAD52* and *RAD55* can substitute for one another in promoting focus formation at 30°C. Taken together, the results indicate that *RAD52* can promote nearly normal numbers of Rad51 foci in the absence of *RAD55*, at 30°C but not at 20°C. Lovett and Mortimer originally proposed that “*RAD55* and *RAD57* participate in a process for which a functional substitute exists,” and that “this alternative process may be naturally temperature-dependent” (92). The results presented here suggest that the naturally temperature-dependent process is Rad52-promoted assembly of Rad51.

The results raise the question of which of the two mediators, Rad52 or Rad55/57, acts normally in WT cells to promote Rad51 assembly. It is possible that Rad55/57 is the predominant assembly factor at all temperatures, with Rad52 acting only when Rad55/57 is defective. This seems somewhat unlikely, given that the *rad52* single mutant showed a significant delay in Rad51 assembly at 30°C. A second possibility is that Rad52 promotes most assembly at 30°C, with Rad55/57 acting in repair only when cells happen to be in a cool environment and when they enter the meiotic pathway. Note that *rad55* and *rad57* are defective in meiosis even at 30°C, consistent with observed defect in Rad51 focus formation (29, 92). In this context, it is important to point out that although the *rad52* mutant showed little defect in the efficiency of focus induction at low temperature, it is not possible to conclude from cytological observations that the structures observed in this or any other mutant strain are functionally normal. The biochemical properties of Rad52 suggest one way in which the Rad51 foci that form in *rad52* mutant might be

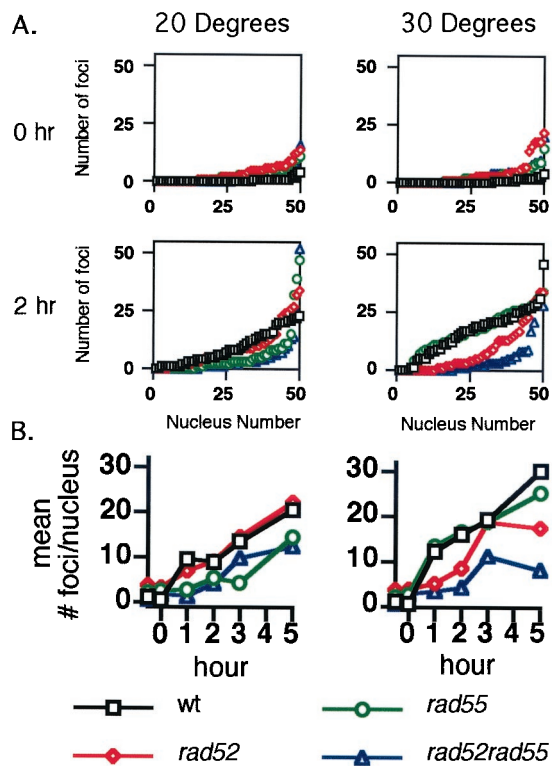


Fig. 3. Role of *S. cerevisiae* mediators in assembly of Rad51 after γ -irradiation. Log phase diploid cells were irradiated to a dose of 100 krads and incubated in rich medium for the times indicated. All strains used were isogenic derivatives of SK-1. Mutants contain homozygous deletion alleles. Wilcoxon sum of ranks tests were used to assess the statistical significance of differences between samples referred to in the text. (A) The left column shows the distribution of focus counts at 0 and 2 h after irradiation from 20°C cultures, the right column from 30°C cultures. Focus counts from 50 unselected nuclei are displayed in ascending order. (B) Average number of foci plotted as a function of time for each of the strains examined. For detailed methods, see ref. 59.

abnormal. Rad52 binds selectively to DNA ends (58). It is possible that Rad52 normally directs initiation of Rad51 filaments to DNA ends created by radiation, and that only filaments that initiate near ends are fully functional in repair. These considerations could provide an explanation for why the radiation sensitivity of *rad52* mutants is greater than that of *rad55* and *rad57* mutants even at cold temperature.

Significant numbers of foci were induced by radiation in a mutant that eliminates all three known mediator proteins, Rad55, Rad57, and Rad52. There are three hypotheses that can account for this observation. First, there could be additional mediator proteins, yet to be identified, that act in the absence of Rad55/57 and Rad52. Second, aberrant structures, which are cytologically indistinguishable from repair-associated structures, may form as a default mechanism when normal assembly is blocked. Third, a fraction of radiation-induced recombination events may involve mediator-independent assembly of Rad51 into functional complexes (or such assembly may occur when mediators are absent). Biochemical and genetic experiments provide support for this last possibility. Residual Rad51 activity is detected in the absence of mediators at high *ssb* concentrations (22, 49–52), and overexpression of Rad51 can suppress the phenotypes of mutations that eliminate mediator function (66, 69, 93, 94) (S.L.G., E. Aelson, D. Livingston, and D.K.B., unpublished results).

Spontaneous Rad51 Foci. To determine whether Rad51 foci were present in undamaged yeast cells, aliquots from log phase

cultures were examined without any prior damaging treatment. Control experiments by using a *rad51* null mutant indicated that the majority of foci detected in the *RAD51*⁺ strains used in this experiment did contain Rad51. An average of about 1.2 ± 1.7 SD foci/nucleus were observed in untreated WT cells and 3.8 ± 4.5 SD foci/nucleus in *rad52*. We interpret these data under the assumption that the same number of “Rad51-provoking” lesions occur in WT and *rad52*. That the number of Rad51 foci is greater in *rad52* than in WT is likely to indicate that the lifespan of the foci observed is longer in the mutant. Other observations indicate that disappearance of Rad51 foci is coupled with completion of recombination (23, 29, 30). Thus, the great number of foci observed in the mutant could indicate Rad52 is required either for assembly of functional Rad51 complexes (as discussed above), or that it is required for recombination at a postassembly stage. If the Rad51 foci seen in the *rad52* mutant reflect the number of lesions normally repaired by Rad51, yeast cells suffer an average of at least four such lesions. For related observations on Rad52 localization, see ref. 95.

Meiotic vs. Mitotic Assembly of Rad51. The role of Rad52 and Rad55/57 in assembly of Rad51 in mitotic cells after irradiation differs considerably from their role in meiosis. The two proteins appear to be able to substitute for each other in promoting the assembly of Rad51 after irradiation of mitotic cells. Again, this result is consistent with the biochemical finding that both Rad55/57 alone and Rad52 alone have mediator function in purified systems. In contrast to the requirements after mitotic DNA damage, both Rad55 and Rad52 are required for detectable assembly of Rad51 in meiosis. One possibility is that the enhanced mediator requirement in meiosis results from the fact that meiotic DSBs form in a highly regulated context. Several genes are required for the formation of meiotic DSBs (96), and the continued association of one or more of the corresponding proteins could create an assembly barrier that only the combined functions of Rad52 and Rad55/57 can overcome. However, a preliminary experiment indicated that radiation induction of Rad51 foci in meiosis depends strongly Rad52. This suggests that meiotic breaks need not form in the normal context to display strict mediator requirements (S.G. and D.K.B., unpublished work). If this result proves to be reproducible, it is likely that some difference in the expression of a factor controlling Rad51 assembly in meiosis as compared with mitosis is responsible for the observed difference in mediator requirement.

Do Mediators Promote Rad51 Assembly Before RPA Assembly in Meiosis? As mentioned previously, Ashley and colleagues found that Rad51 foci appeared at an earlier stage in meiosis than RPA foci (33). They proposed that Rad51 assembles in an inactive form before DSBs occur, and that RPA activates these complexes at the DSB stage. However, recent results indicate that formation of Rad51 foci depends on DSBs in the mouse (97) as it does in yeast (29). Thus, in mouse, Rad51 foci are likely to assemble at the sites of DSBs without prior assembly of visible quantities of RPA. Determining whether RPA or Rad51 associates first in yeast was complicated by the presence of replication-associated RPA foci at the times when Rad51 foci first appeared (29). However, we found that the subset of DSB-dependent RPA foci appeared at the same time as Rad51 foci. We also found that RPA foci appeared with normal kinetics in *rad51* mutants and in *rad55*, *rad57*, and *rad52* mutants. These results, along with biochemical data on Rad52 and Rad55/57 function, prompted us to propose that Rad51 assembles at the sites of prior RPA assembly during yeast meiosis, as we have shown here for Rad51 assembly after mitotic DNA damage. However, the available meiotic data leave open the possibility that the mediator-promoted Rad51 assembly occurs before RPA assembly in both yeast and mouse. Furthermore, some of the

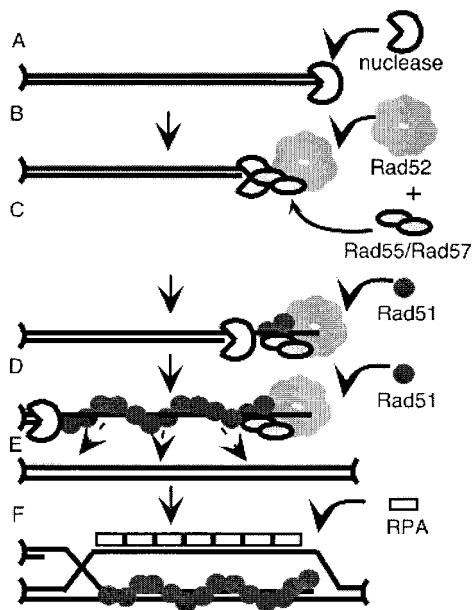


Fig. 4. Model for mediator-dependent coupling of ssDNA strand resection and recombinase assembly at DSBs during meiosis. (A) Strand-specific nuclease loads at DSB sites. (B) Mediators are loaded on DNA ends before nuclease proceeds a distance far enough to allow cytologically detectable amounts of RPA. (C) Rad51 is recruited to ends via mediator interactions, again before nuclease has proceeded far from the end. (D) Nucleolytic resection of DNA occurs in concert with elongation of the Rad51 filament. (E) Rad51 carries out homology search. (F) Strand exchange occurs with RPA loading on the outgoing ssDNA strand.

Rad51 foci observed in the radiation experiments described above did not colocalize with RPA, making it possible that a subset of damage-induced Rad51 foci might also assemble at sites not bound by RPA.

A model designed to account for assembly of Rad51 before RPA during meiotic recombination is shown in Fig. 4. In order for Rad51 to outcompete RPA for binding sites on ssDNA, the model proposes that initiation of Rad51 assembly be mechanistically coupled to the nucleolytic processing of DNA ends. (Under the nuclease-recombinase coupling model, the observation that RPA foci form in recombinase and mediator mutants is explained as a default pathway.) Rad52 binds to ssDNA ends and, with the help of Rad55/57, initiates a Rad51 filament before nucleolytic activity allows an inhibitory RPA filament to form. Although this model remains to be tested, coupling of recombinase assembly to nucleolytic processing of ends has precedent. As discussed above, RecBCD-dependent stimulation of RecA assembly at χ sites involves coupling of ssDNA formation and recombinase assembly. In addition, the T4-encoded nuclease gp46/47 associates strongly with UvsY, the bacteriophage T4 mediator, raising the possibility that recombinase assembly can be coupled to formation of ssDNA tracts in that system as well (35). This observation could be relevant to eukaryotes, given that gp46/47 nuclease complex is structurally related to the yeast Mre11/Rad50/Xrs2 complex (98), which controls both formation and nucleolytic processing of meiotic DSBs (99).

An alternative way to explain assembly of Rad51 before RPA in meiosis would be an alteration in the binding properties of RPA and/or Rad51 that enhanced Rad51's ability to compete with RPA for binding sites on ssDNA. This possibility seems unlikely to us, given that the requirement for mediators is stricter in meiosis than in mitosis. On the other hand, posttranslational modification of both Rad51 and RPA has been observed (as

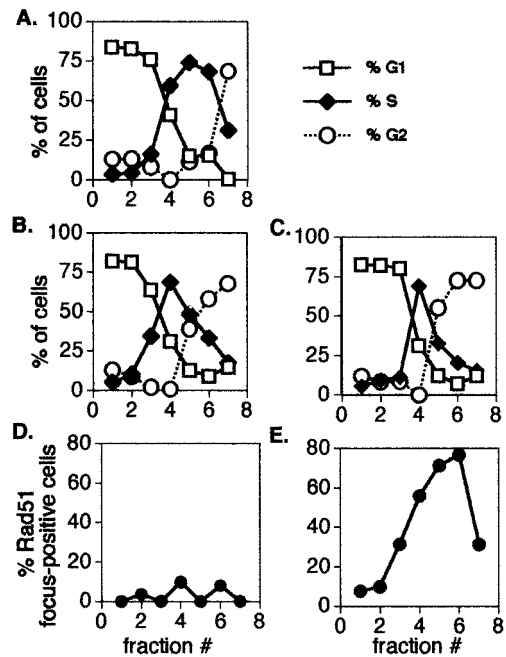


Fig. 5. Radiation induction of Rad51 foci is cell cycle stage dependent. Subconfluent CHO cells were fractionated by centrifugal elutriation and aliquots of fractions assayed for DNA content via conventional methods (propidium iodide staining) on a Becton Dickinson FACSscan. Raw data were analyzed to determine the percentage of the population in G₁, S, and G₂ by using CELLQUEST software (Becton Dickinson). The remaining cells in each fraction were split into two fractions; one fraction was irradiated to a dose of 0.9 krad (9 Gy) x-rays with a Maxitron generator (General Electric), and the other served as an unirradiated control. All fractions were incubated in culture medium for 3 h, after which all fractions were analyzed a second time by FACS and also by immunostaining for Rad51 foci by using a previously published method (60). (A) Percentage of cells in G₁, S, and G₂ as estimated by FACS analysis of each of seven fractions obtained by elutriation. (B) FACS analysis of untreated fractions after 3-h incubation. (C) FACS analysis of irradiated fractions after 3-h incubation. (D) Percentage of Rad51-focus positive cells in each untreated fraction after a 3-h incubation in culture medium. (E) Percentage of Rad51-focus positive cells in each irradiated fraction after a 3-h incubation in culture medium.

discussed further below), and such modifications could influence the mechanism of assembly during meiosis and/or mitosis.

Other Factors Controlling Rad51 Assembly. Mammalian cells in growing cultures are heterogeneous with respect to their ability to form Rad51 foci in response to radiation (27, 28, 61, 100). This heterogeneity does not appear to be accounted for by a random distribution of foci among cells; instead, there appear to be two populations of cells, those that can be induced to form many foci and those that fail to respond. Studies of steady-state mRNA and protein levels have shown that expression of the *RAD51* gene is low in serum-starved (G₀) cells (101, 102). *RAD51* mRNA levels are reported to be highest at the G₁/S boundary and protein levels highest in G₂. However, although the level of transcript was low in G₀ cells, the observed changes in expression during cell cycle progression were quite modest (less than 3-fold). Nonetheless, the observation that the steady-state level of Rad51 is not altered by radiation (61), together with the data on cell cycle control of Rad51 expression, suggested that it is cells in G₁ that are incapable of responding to radiation by forming Rad51 foci. To test the idea directly, we fractionated cycling CHO cells by centrifugal elutriation and tested each fraction's ability to form Rad51 foci in response to a dose of x-rays. The results are shown in Fig. 5. The results clearly show that fractions containing predominantly G₁ cells failed to form foci in response to

radiation, whereas those in S phase responded efficiently. A single fraction (no. 7) contained predominantly G₂ cells and this fraction showed less focus induction than did the previous fraction that contained mostly S-phase cells at the time of radiation treatment. However, this result could be misleading because the number of cells recovered in fraction no. 7 was quite low, reducing the reliability of FACS analysis. Furthermore, in other experiments, G₂-enriched fractions showed efficient induction of Rad51 foci (data not shown). In contrast to the variable result with G₂-enriched fractions, we consistently see failure of G₁ cells to induce Rad51 foci and efficient induction of foci in S-phase cells. Induction of recombinational repair capability in S phase makes biological sense, in that most of the spontaneous DNA damage repaired by the recombinational mechanism occurs as a result of detects in replication (see ref. 103 and references therein).

Although changes in Rad51 expression level alone could account for the failure of G₁ (and possibly G₂) cells to form Rad51 foci in response to radiation, other regulatory processes may influence this response as well. Several lines of evidence from mammalian cells and yeast indicate that RPA, Rad51, and Rad55 are all phosphorylated in response to radiation (104–108). Phosphorylation of these proteins is mediated by members of the ATM family of protein kinases in mammals and yeast. The activity of these kinases is induced in response to damage. ATM-dependent phosphorylation of Rad51 is mediated indirectly through ATM-dependent activation of the nonreceptor tyrosine kinase c-Abl (107, 108). One study found that phosphorylation of Rad51 by c-Abl increased Rad51-Rad52 interaction (108), a change that would be expected to enhance assembly of Rad51 in response to damage. However, another study found that phosphorylated Rad51 had reduced ability to bind ssDNA and was inactive in strand exchange reactions (107). Perhaps two pools of Rad51 are required for an efficient repair after damage, a phosphorylated pool to promote filament initiation via Rad52 interaction and an unphosphorylated pool critical for filament elongation and strand exchange. Another possibility is that

phosphorylation of Rad51 contributes to cell-cycle regulation of Rad51 activity. Further studies are required to determine the role of posttranslational modification in assembly of recombination complexes *in vivo* and *in vitro*.

Future Directions for the Study of Recombinase Assembly. Substantial progress has been made in the last few years on the mechanisms that promote assembly of RecA-like recombinases. Important questions remain to be addressed. What are the structural intermediates involved in recombinase assembly? How is mediator activity regulated to ensure that recombinase assembles only at sites of DNA damage and not on the tracts of ssDNA that form as normal intermediates in DNA replication? Does mediator binding at DNA ends play an important part in recombinase assembly? Are the requirements for assembly at ends different from the requirements for assembly at daughter strand gaps? Why are mediator requirements stricter in meiosis than in mitosis? Does down-regulation of Rad51 protein alone account for the inability of G₁ cells to form Rad51 foci in response to damage, or are other regulatory mechanisms in place? What role does damage-dependent phosphorylation play in recombinase assembly?

Bacteriophages have often provided paradigms of broad relevance to biological systems. This is certainly true in the case of recombinase assembly where the lessons learned from phage have guided experiments in bacteria, yeast, and higher eukaryotes. On the other hand, demonstration of different mediator requirements in mitosis and meiosis contributes to evidence indicating that some of the mechanisms regulating assembly of recombination complexes are unique to eukaryotes.

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