Molecular Cell, Vol. 6, 583–592, September, 2000, Copyright ©2000 by Cell Press

Rad54 Protein Is Targeted to Pairing Loci by the Rad51 Nucleoprotein Filament

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Summary

Rad51 and Rad54 proteins are important for the repair of double-stranded DNA (dsDNA) breaks by homologous recombination in eukaryotes. Rad51 assembles on single-stranded DNA (ssDNA) to form a helical nucleoprotein filament that performs homologous pairing with dsDNA; Rad54 stimulates this pairing substantially. Here, we demonstrate that Rad54 acts in concert with the mature Rad51-ssDNA filament. Enhancement of DNA pairing by Rad54 is greatest at an equimolar ratio relative to Rad51 within the filament. Reciprocally, the Rad51-ssDNA filament enhances both the dsDNA-dependent ATPase and the dsDNA unwinding activities of Rad54. We conclude that Rad54 participates in the DNA homology search as a component of the Rad51-nucleoprotein filament and that the filament delivers Rad54 to the dsDNA pairing locus, thereby linking the unwinding of potential target DNA with the homology search process.

Introduction

Proficient repair of DNA double-stranded breaks (DSBs) is important for the survival of all organisms. Homologous recombination plays an important role in the repair of potentially lethal DNA lesions in all domains of life (Kowalczykowski et al., 1994; Camerini-Otero and Hsieh, 1995; Baumann and West, 1998; Kuzminov, 1999; Kowalczykowski, 2000). In Prokarya, a central enzyme of the homologous recombination machinery is RecA protein; in Eukarya and Archaea the Rad51 and RadA proteins, respectively, play a similarly important role. These proteins polymerize on ssDNA, assembling into helical nucleoprotein filaments with strikingly similar structures (Ogawa et al., 1993; Benson et al., 1994; Sung, 1994; Bianco et al., 1998; Seitz et al., 1998). The nucleoprotein filaments share a unique enzymatic activity: they promote both a search for homologous sequences in dsDNA and an exchange of DNA strands between the ssDNA bound within the filament and homologous dsDNA. This process, called DNA strand exchange, lies at the core of homologous recombination (Radding, 1993; Kowalczykowski et al., 1994). DNA strand exchange is stimulated by the respective single-stranded DNA binding (SSB) proteins, either bacterial SSB protein or eukaryotic and archaeal RPA (replication protein A) (Cox et al., 1983; Sung, 1994; Sugiyama et al., 1997; Chédin et al., 1998; Mazin and Kowalczykowski, 1998).

In *Saccharomyces cerevisiae*, the best studied eukaryotic organism, many of the proteins required for efficient recombinational repair of chromosomal DSBs are defined by the *RAD52* epistasis group of genes, which includes *RAD51* as well as *RAD50, RAD52, RAD54, RAD55, RAD57, MRE11*, *XRS2, and RFA1* (Game, 1993; Hays et al., 1998). Based largely on two-hybrid analyses and immunoprecipitation, it was shown that the protein products of the *RAD52* epistasis group in yeast interact extensively with one another. Rad51 protein interacts with Rad52 (Shinohara et al., 1992; Donovan et al., 1994), Rad55 (Hays et al., 1995), and Rad54 proteins (Jiang et al., 1996; Clever et al., 1997). Rad55 and Rad57 proteins interact with each other to form a stable heterodimer (Sung, 1997a), and Rad52 protein interacts with RPA protein (Shinohara et al., 1998; Sugiyama et al., 1998). In addition, work in both yeast and human cells established that Rad50, Mre11, and Xrs2 proteins form a heterotrimer that possesses a nuclease activity (Trujillo et al., 1998; Usui et al., 1998; Paull and Gellert, 1999).

In vitro, the Rad52, Rad55, Rad57, and Rad54 proteins stimulate DNA strand exchange promoted by Rad51 protein. Rad52 protein stimulates DNA strand exchange both by mediating the exchange of Rad51 protein for RPA that is bound to ssDNA (Sung, 1997b; New et al., 1998; Shinohara and Ogawa, 1998) and, in an RPA-independent mode, by acting directly on Rad51 protein (J. New and S. C. K., unpublished data). The latter mode of stimulation mimics the behavior of the human Rad52 protein (Benson et al., 1998). The Rad55/Rad57 protein heterodimer acts by enhancing the ability of Rad51 protein to compete with RPA for ssDNA binding (Sung, 1997a). Finally, Rad54 protein stimulates DNA strand exchange by an as yet unknown mechanism (Petukhova et al., 1998, 1999).

The stimulation of DNA pairing activity of Rad51 protein by Rad54 protein is especially interesting because *rad54* mutations have a very strong effect on DSB repair (Game, 1993) and also because there is no known homolog in the well-defined prokaryotic system. Rad54 protein belongs to the Swi2/Snf2 class of DNA binding proteins that participate in the remodeling (assembly/disassembly) of multiprotein complexes associated with transcription, recombination, and DNA repair (Pazin and Kadonaga, 1997). Like most members of the Swi2/Snf2 class, Rad54 protein possesses dsDNA-dependent ATPase activity (Petukhova et al., 1998; Swagemakers et al., 1998). It was demonstrated that both human and yeast Rad54 protein use energy derived from the hydrolysis of ATP to topologically unwind dsDNA (Petukhova et al., 1999; Tan et al., 1999). Because mutations that abolish this activity are recombination deficient (Clever et al., 1999; Petukhova et al., 1999), it is believed that this DNA unwinding activity is responsible for stimulation of DNA strand exchange. The dsDNA unwinding activity of Rad54 protein could act either at the time of homologous pairing to make the dsDNA recipient more accessible for interaction with the ssDNA, or it could act after pairing to promote extension of the dsDNA heteroduplex product. In either case, since Rad51 protein is associated with ssDNA during the homology search and because

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Rad54 protein acts nonspecifically on any dsDNA sequence, the manner by which Rad54 protein is targeted to the unique dsDNA homologous site is an important issue.

We found that Rad54 protein exerts its stimulatory effect on the DNA pairing activity of Rad51 protein by binding to the assembled Rad51-ssDNA nucleoprotein filament rather than by binding to free Rad51 protein or to dsDNA. In conjunction with this enhanced DNA pairing, the dsDNA-dependent ATPase and dsDNA unwinding activities of Rad54 protein are also stimulated. Our results explain how Rad54 protein is targeted to a homologous locus, a formidable task when viewed in the context of a genomic search process.

Results

Rad54 Protein Enhances the DNA Pairing Activity of Rad51 Protein via a Stoichiometric and Species-Specific Interaction with Rad51 Protein

Previously, it was demonstrated that Rad54 protein stimulates the DNA pairing activity of Rad51 protein (Petukhova et al., 1998). Using ssDNA and supercoiled dsDNA as substrates, we confirmed that Rad54 protein increased the yield of joint molecules, which are the products of Rad51 protein-mediated invasion of supercoiled DNA (Figure 1A). By varying the Rad54 protein concentration, we observed that optimal joint molecule formation occurred when the Rad54 protein concentration was approximately equal to that of Rad51 protein (Figures 1A and 1B). This finding suggested that a stoichiometric complex of Rad54 and Rad51 nucleoprotein filament might be the active species in this reaction.

To establish that this optimum is defined with respect to the ratio of Rad54 protein to Rad51 protein, the concentration of the Rad51-ssDNA nucleoprotein filament was changed, decreasing or increasing it 2-fold, and titrations with Rad54 protein were repeated (Figure 1C). In this case, the concentration of Rad54 protein required for optimal DNA pairing activity also decreased or increased in direct proportion to the Rad51 protein concentration. Hence, the most efficient joint molecule formation requires equimolar amounts of Rad51 and Rad54 proteins.

To determine whether this interaction is specific to the cognate yeast proteins, we examined the stimulatory effect of Rad54 protein on joint molecule formation promoted by human Rad51 protein. No stimulation of joint molecules was observed in this case (compare Figures 2A and 2B). This lack of stimulation was not due to low activity of human Rad51 protein; it was fully active in DNA strand exchange with linear DNA substrates (data not shown), as described previously (Mazin et al., 2000). Also, Rad54 protein does not stimulate joint molecule formation promoted by *Esherichia coli* RecA protein; in fact, it inhibited this reaction (data not shown). Thus, a specific protein-protein interaction between Rad54 and Rad51 proteins is essential for the stimulatory function of Rad54 protein.

Rad54 Protein Is Delivered to the Site of DNA Pairing by Interaction with Rad51 Protein

Both the species-specific stimulation of joint molecule formation and the requirement for equimolar concentrations of Rad51 and Rad54 proteins suggested that these

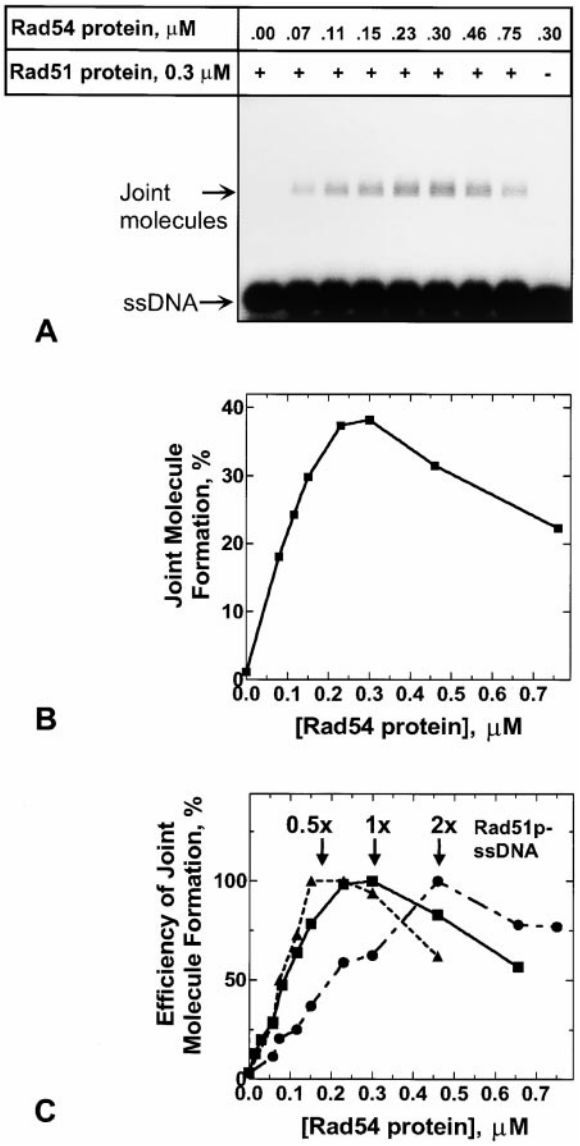

Figure 1. A Stoichiometric Ratio of Rad51 and Rad54 Proteins Is Required for Maximal Stimulation of Joint Molecule Formation

(A) Shows joint molecule formation promoted by Rad51 protein, under standard assay conditions, between supercoiled pUC19 dsDNA and ssDNA (#90, 90-mer) as a function of Rad54 protein concentration.

(B) Displays the results shown in (A) as a graph. Because the molar molecule concentration of the pUC19 DNA is limiting, the percentage of joint molecule formed is calculated based on the dsDNA concentration.

(C) Graphically shows the optimal Rad54 protein concentration for joint molecule formation at three different concentrations of the Rad51-ssDNA nucleoprotein filament. In these experiments, nucleoprotein filaments were formed at ssDNA (#5, 90-mer) and Rad51 protein concentrations twice (2×) as high as standard, 1.8 μM and 0.6 μM, respectively. Then the reaction mixture was diluted with binding buffer 2-fold (1×), 4-fold (0.5×), or left undiluted (2×). Rad54 protein was added to each concentration of the Rad51-ssDNA filament. Joint molecule formation was initiated by the addition of pUC19 plasmid DNA (9.0 μM); the maximal absolute amounts of joint molecule formation were 6%, 12.5%, and 22%, for the 0.5×, 1×, and 2× reactions, respectively. The yield of pairing product with ssDNA #5 is less than that obtained with ssDNA #90 because the former contains 36 nucleotides of nonhomology at the 3′-end of the oligonucleotide (Mazin et al., 2000). All panels represent joint molecule formation after 5 min of incubation.

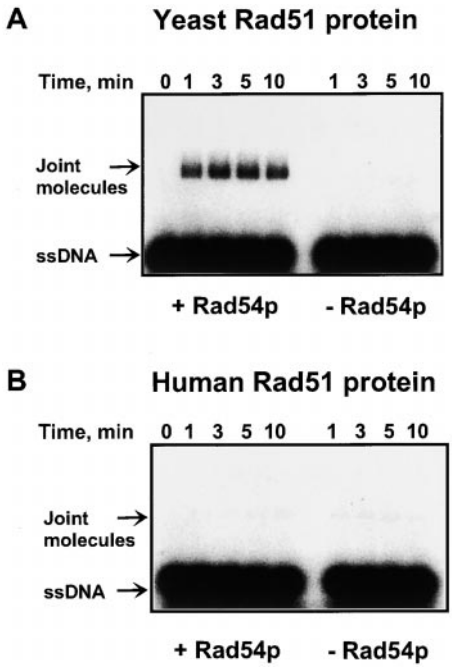

Figure 2. Rad54 Protein Stimulates Joint Molecule Formation via a Species-Specific Interaction with Rad51 Protein

(A and B) Show the effects of Rad54 protein on joint molecule formation between supercoiled pUC19 dsDNA and ssDNA (#90, 90-mer) promoted by the yeast and human Rad51 protein, respectively, under standard assay conditions.

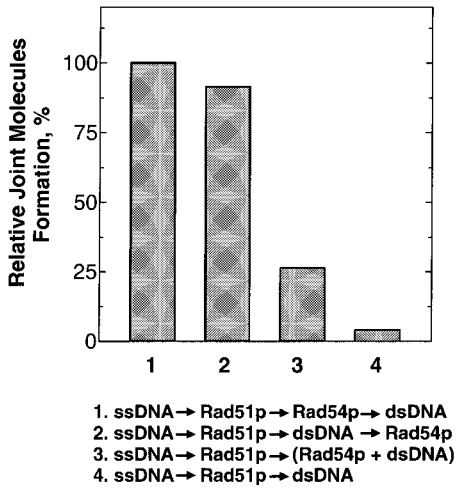

Figure 3. Preincubation of Rad54 Protein with dsDNA Diminishes Its Stimulatory Effect on Rad51 Protein-Dependent Joint Molecule Formation

The bar graph illustrates the relative yield of joint molecule formation between ssDNA (#5, 90-mer) (0.45 μM) and supercoiled dsDNA pUC19 (4.5 μM) promoted by Rad51 protein (0.15 μM). Rad54 protein (0.12 μM) was added either to the preformed Rad51-ssDNA nucleoprotein filaments 2 min before (column 1) or immediately after addition of plasmid DNA (column 2). Alternatively, Rad54 protein was preincubated with dsDNA in assay buffer at 37°C for 2 min and then the mixture was added to the Rad51-ssDNA filament (column 3). The control reaction omitted Rad54 protein (column 4). Joint molecule formation was carried out for 5 min. The yield of the most efficient reaction (column 1), which represents 15% joint molecules formation, was designated as 100%.

two proteins interacted physically during DNA strand exchange. However, these experiments did not delineate whether Rad54 protein interacted with the Rad51 protein, which then promoted pairing, or whether the Rad54 protein bound to the dsDNA and recruited the Rad51 nucleoprotein to itself in order to promote pairing. To distinguish between these two possibilities, Rad54 protein was preincubated with either the Rad51 nucleoprotein filament or with dsDNA, and the yield of joint molecules was measured.

We found that Rad54 protein strongly stimulated joint molecule formation when it was added to the nucleoprotein filament prior to dsDNA (Figure 3, column 1). In contrast, when Rad54 protein was bound to dsDNA prior to the introduction of the Rad51-ssDNA nucleoprotein filament, the stimulatory effect of Rad54 protein was reduced by 75% (Figure 3, column 3). This result agrees with independent observations made with the three-strand exchange reaction using ϕX174 DNA substrates in the presence of replication protein-A: the prebinding of Rad54 protein to dsDNA abolished its stimulatory effect (J. A. S., G. Lutz, S. C. K., and W.-D. H., unpublished data). When Rad54 protein was not preincubated with the dsDNA, but rather added after the addition of dsDNA, only a modest reduction was observed (Figure 3, column 2); note, however, that because joint molecule formation in the absence of Rad54 protein is poor ($\approx$ 3%, Figure 3, column 4), most of the Rad51 presynaptic filament remains free and fully capable of interacting with the added Rad54 protein. Thus, when not prebound to dsDNA, Rad54 protein could readily associate with the free Rad51 nucleoprotein filament to produce the observed stimulation in column 2. Collectively, these results argue that Rad54 protein exercises its stimulatory effect via an interaction with the Rad51 nucleoprotein filament directly rather than by first binding to the dsDNA.

Rad54 Protein Interacts after Formation of the Rad51 Nucleoprotein Presynaptic Filament

Though the preceding experiments demonstrate a synaptic function for Rad54 protein, they do not exclude a presynaptic role. Potentially, Rad54 protein could stimulate the presynaptic step by facilitating the polymerization of Rad51 protein onto ssDNA. To explore this possibility, we varied the order by which the Rad51 and Rad54 proteins were assembled on ssDNA. We found that Rad54 protein stimulated joint molecule formation best when it was added after the Rad51 protein-ssDNA complex had formed (Figure 4). When the two proteins were mixed together, or when Rad54 protein was preincubated with ssDNA prior to Rad51 protein, the efficiency of joint molecule formation decreased. This result indicates that Rad54 protein most efficiently stimulates DNA pairing activity when it interacts with the Rad51-ssDNA filament, rather than with either free Rad51 protein or ssDNA. Essentially the same conclusion emerges from similar order-of-addition experiments performed using the three-strand exchange reaction with ϕX174 DNA substrates (J. A. S., G. Lutz, S. C. K., and W.-D. H., unpublished data). These findings, in turn, suggest that Rad54 protein does not serve, as a major function, to promote Rad51 nucleoprotein filament formation on ssDNA.

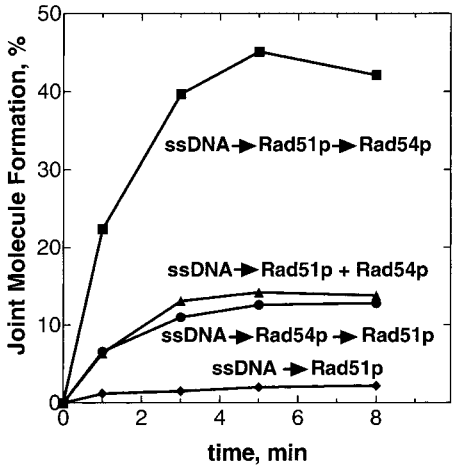

Figure 4. Rad54 Protein Stimulates DNA Pairing by Acting after Formation of the Rad51 Presynaptic Filament

Joint molecule formation was assayed after incubating ssDNA (#90, 90-mer) with either Rad51 protein for 10 min followed by addition of Rad54 protein (designated ssDNA→Rad51p→Rad54p); Rad51 protein for 10 min (designated ssDNA→Rad51p); a mixture of Rad51 protein and Rad54 protein for 10 min (designated ssDNA→ Rad51p+ Rad54p); or Rad54 protein for 1 min, followed by addition of Rad51 protein, and then by additional incubation for 10 min (designated ssDNA→Rad54p→Rad51p). Most of Rad51p was on ssDNA at the time when Rad54p was added. The Rad54 protein concentration was 0.24 μM in all cases, and joint molecule formation was initiated by the addition of pUC19 plasmid dsDNA; standard assay conditions were used. In a separate experiment, we ascertained that formation of the Rad51 nucleoprotein filament was completed within 10 min: increasing the time for presynaptic complex formation from 10 to 30 min did not increase product formation in DNA strand exchange reactions with short linear DNA substrates (data not shown).

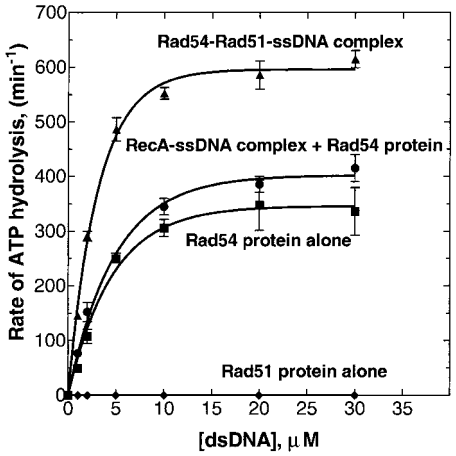

Figure 5. The Rad51-ssDNA Nucleoprotein Filament Enhances the dsDNA-Dependent ATPase Activity of Rad54 Protein

The ATPase activity of either free Rad54 protein (squares) or the complexes formed between Rad54 protein and the Rad51-ssDNA nucleoprotein (triangles) was measured as a function of dsDNA (#1 and #2, 63-mer) concentration. An otherwise identical set of experiments using RecA protein instead of Rad51 protein is shown (circles); the intrinsic ssDNA-dependent ATPase activity of RecA protein (10 min^{-1}) was subtracted from the graph. The concentration of Rad54 protein in the assays was 0.15 μM. The activity of Rad51 protein alone (0.17 μM) is also shown (diamonds). The rate of hydrolysis is given as moles of ATP hydrolyzed per minute per mole of Rad54 protein.

Interaction with the Rad51 Nucleoprotein Filament Enhances the ATPase Activity of Rad54 Protein in a Homology-Independent Manner

Rad54 protein possesses dsDNA-dependent ATPase activity (Petukhova et al., 1998; Swagemakers et al., 1998). This ATPase activity is essential for Rad54 protein function: a mutation (K341R) that strongly suppresses ATP hydrolysis confers a null phenotype (Clever et al., 1999; Petukhova et al., 1999). In vitro, in agreement with its phenotypic behavior, the Rad54 K341R mutant protein stimulates neither three-strand DNA strand exchange with long DNA substrates (Petukhova et al., 1999) nor D loop formation with supercoiled DNA (data not shown).

To better understand the role of Rad54 protein's ATPase activity in joint molecule formation, we examined the consequences of the interaction with the Rad51-ssDNA nucleoprotein complex on this activity. Namely, we wanted to ascertain whether either this interaction or DNA sequence homology affected the dsDNA-dependent activity of Rad54 protein. We found that the Rad51 nucleoprotein filament enhanced the ATPase activity of Rad54 protein: the dsDNA-dependent ATPase activity of Rad54 protein increased between 3.0- and 1.8-fold, depending on the dsDNA concentration (Figure 5). Although Rad51 protein possesses an ssDNA-dependent ATPase activity, several lines of evidence indicate that the observed ATP hydrolysis is entirely attributable to the activity of Rad54 protein. First, ATP turnover of Rad51 protein is negligible ($\leq$ 0.6 min^{-1}) compared to that of Rad54 protein ($>$ 300 min^{-1}). Second, in the absence of dsDNA, Rad54 protein has no effect on the ATPase activity of the Rad51-ssDNA filament (data not shown). Finally, the residual ATP turnover of the K341R mutant protein (6.4 $\pm$ 1.3 min^{-1}) remains low (8.3 $\pm$ 1.4 min^{-1}) in the presence of the Rad51 nucleoprotein filament (data not shown), even though this mutant is capable of interacting with Rad51 protein (Petukhova et al., 1999).

Table 1. The Rad51-ssDNA Nucleoprotein Filament Enhances the dsDNA-Dependent ATPase Activity of Rad54 Protein

dsDNA Substrate	Rad54 Protein ATPase Activity, k_{cat}, min^{-1}		Fold Increase
	Minus (−) Rad51 Protein-ssDNA Filament[a]	Plus (+) Rad51 Protein-ssDNA Filament[a]	
Supercoiled pUC19	555 ± 12	1106 ± 60	2.0
Linear pUC19	558 ± 29	1315 ± 95	2.4
Linear 63 bp fragment	346 ± 15	596 ± 10	1.7

[a]The Rad51 nucleoprotein filament was assembled on the 94-mer ssDNA (#71). ATP hydrolysis was measured as described in Experimental Procedures, using the following concentrations: Rad51 protein, 0.17 μM; ssDNA, 0.5 μM; and Rad54, protein 0.15 μM. The k_{cat} was obtained from a fit of the dsDNA concentration dependence of ATP hydrolysis, from experiments such as those shown in Figure 5.

Table 2. Stimulation of the dsDNA-Dependent ATPase Activity of Rad54 Protein by the Rad51-ssDNA Nucleoprotein Filament Does Not Require DNA Homology

dsDNA	ssDNA[a] within the filament	ATPase Activity, k_{cat}, min^{-1}
pUC19	Homologous	1320 ± 20
	Heterologous	1310 ± 120
Linear 63 bp fragment	Homologous	610 ± 20
	Heterologous	550 ± 16

[a] Rad51 nucleoprotein filaments were assembled on ssDNA that was either homologous (#90, 90-mer) or heterologous (#71, 94-mer) either to supercoiled pUC19 dsDNA or to linear dsDNA fragments (#1 and #2, 63-mer) that were used as the substrates for the ATPase activity of Rad54 protein. The concentration of Rad54 protein was either 0.15 or 0.09 μM in the reaction with supercoiled or linear dsDNA, respectively. The k_{cat} was determined as described in the legend to Table 1.

This enhancement of ATPase activity requires a specific Rad51-Rad54 protein interaction, since the RecA nucleoprotein filament has no effect on the ATP hydrolysis by Rad54 protein (Figure 5), and the human Rad51 nucleoprotein filament enhances it by only ~20% (data not shown). Although the length of the dsDNA substrates affected the observed ATPase activity of Rad54 protein, the stimulatory effect of the Rad51 nucleoprotein filament remained almost unchanged (Table 1). Importantly, enhancement of ATPase activity was independent of homology between dsDNA and the ssDNA bound within the Rad54-Rad51-ssDNA (Table 2), demonstrating that the stimulation is intrinsic to the Rad54-Rad51 protein interaction and is not dependent on DNA pairing.

Thus, interaction with the Rad51-ssDNA nucleoprotein complex not only preserves the ability of Rad54 protein to interact with dsDNA, but this interaction enhances its dsDNA-dependent ATPase activity.

Interaction with the Rad51 Nucleoprotein Filament Enhances the DNA Unwinding Activity of Rad54 Protein

Previously, it was demonstrated that Rad54 protein topologically unwinds dsDNA, introducing negative supercoils into relaxed circular dsDNA (Petukhova et al., 1999; Tan et al., 1999). The DNA unwinding activity requires ATP hydrolysis and, since the ATPase-deficient Rad54 mutant protein (K341R) also lacks this activity, it was suggested that DNA unwinding is important for stimulation of DNA strand exchange promoted by Rad51 protein (Tan et al., 1999). Using *E. coli* DNA topoisomerase I, we tested the effect of the Rad51 nucleoprotein filament on this dsDNA unwinding activity. *E. coli* topoisomerase I relaxes negatively supercoiled DNA, and it also relaxes positively supercoiled DNA if that DNA contains a region of ssDNA (Kirkegaard and Wang, 1985). We found that topoisomerase I can act on the complex of Rad54 protein and covalently closed circular DNA (Figure 6, lane 4; C. J. B. and S. C. K., unpublished data). As seen in Figure 6, the DNA unwinding activity of Rad54 protein is enhanced by the Rad51 nucleoprotein filament in a concentration-dependent manner (compare lane 4, which shows the basal level of topological unwinding induced by Rad54 protein alone, with lanes 5 to 12, which represent unwinding in the presence of the increasing amounts of Rad51 nucleoprotein filament). We quantified the amount of substrate DNA remaining, rather than DNA products, because the various supercoiled products bind different amounts of ethidium bromide. The result reveals that an optimal stimulation (≈ 8-fold) of DNA unwinding occurs at a stoichiometric ratio of Rad51 and Rad54 proteins (lanes 7 and 11). The unwinding observed in lanes 5–12 is due entirely to Rad54 protein, because the Rad51 nucleoprotein filament alone does not induce DNA topological unwinding under these conditions (lane 3). As with ATPase activity, the stimulation of the Rad54 protein unwinding activity by the Rad51 nucleoprotein filament did not require DNA homology: the Rad51-ssDNA filaments that were assembled on either homologous or heterologous ssDNA enhanced the DNA topological unwinding activity of Rad54 protein similarly (compare lanes 5–8 and lanes 9–12). Finally, as for stimulation of both the DNA pairing and ATPase activity, the RecA nucleoprotein filament did not affect DNA unwinding by Rad54 protein (data not shown). Thus, we conclude that a specific interaction between the Rad54 protein and the Rad51-ssDNA nucleoprotein filament enhances DNA unwinding capacity of Rad54 protein.

Discussion

RAD54 is an important member of the *RAD52* epistasis group of genes, which are responsible for the recombinational repair of chromosomal DNA. Mutations in the *RAD54* gene strongly impair homologous recombination and DNA repair in both yeast and mammals (Game, 1993; Bezzubova et al., 1997). Earlier, it was discovered that Rad54 protein stimulated DNA strand exchange promoted by Rad51 protein in vitro (Petukhova et al.,

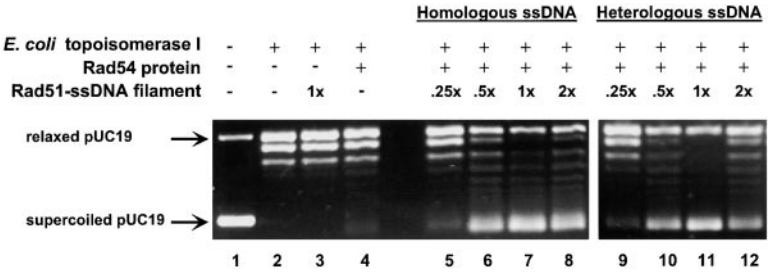

Figure 6. The Rad51-ssDNA Nucleoprotein Filament Enhances the DNA Unwinding Activity of Rad54 Protein

Lane 1 contains supercoiled pUC19 DNA prior to relaxation, as a marker. Lanes 2–12 contain relaxed pUC19 dsDNA and *E. coli* topoisomerase I. Lane 3 contains Rad51 nucleoprotein filament assembled on homologous ssDNA (#90, 90-mer), at a concentration of one nucleoprotein filament per molecule of relaxed pUC19 DNA (0.084 μM Rad51 protein and 0.28 μM ssDNA; this concentration was designated as 1×), but no Rad54 protein. Lanes 4–12 contain Rad54 protein (0.084 μM), and the following concentrations of Rad51 nucleoprotein filament: lane 4, zero; lanes 5 and 8, 0.25×; lanes 6 and 9, 0.5×; lanes 7 and 10, 1×; and lanes 8 and 12. Lanes 5–7 contain homologous ssDNA (#90, 90-mer), whereas lanes 8–10 contain heterologous ssDNA (#71, 94-mer).

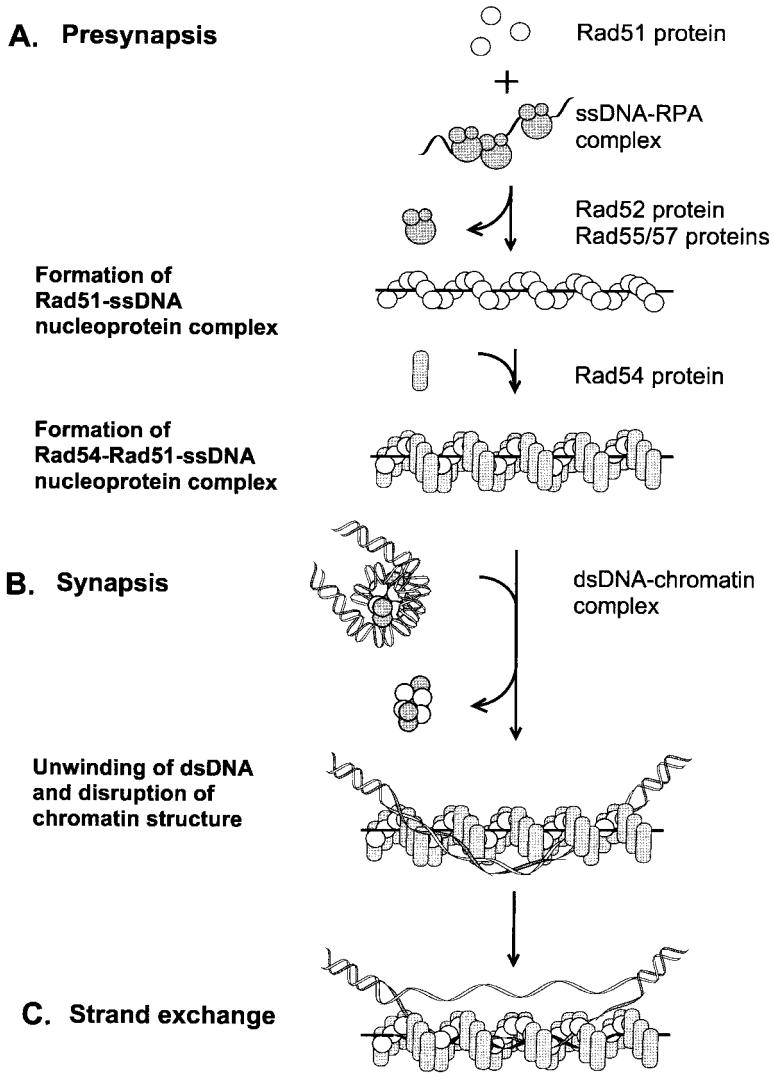

Figure 7. Scheme Illustrating the Stimulatory Role of Rad54 Protein in DNA Strand Exchange

In the presynaptic step (A), RPA binds to ssDNA produced by nucleolytic processing; replacement of the RPA by Rad51 protein results in formation of the Rad51-ssDNA filament. Rad54 protein binds to the Rad51 nucleoprotein filament forming a tertiary Rad54-Rad51-ssDNA nucleoprotein complex. In the synaptic step (B), Rad54 protein, as a part of the nucleoprotein complex, interacts with dsDNA and unwinds it, thereby making it more accessible for homologous recognition. Unwinding of dsDNA also facilitates the displacement of chromosomal proteins from dsDNA, thereby removing an additional constraint to DNA strand exchange (step C).

1998, 1999). Here, we studied the mechanism of this stimulation. Our data demonstrate that the key component of this stimulation resides in the interaction of Rad54 protein with the Rad51-ssDNA nucleoprotein filament, a central structure of the homologous recombination machinery. This interaction recruits Rad54 protein to the locus for homologous pairing, where Rad54 protein exerts its stimulatory effect on DNA strand exchange. In contrast, prebinding of Rad54 protein to dsDNA or to free Rad51 protein significantly decreases its stimulatory effect on DNA strand exchange. Since yeast Rad54 protein does not stimulate the joint molecule formation activity of either RecA protein or human Rad51 protein, this stimulation occurs, most likely, through a specific protein-protein interaction between Rad54 protein and Rad51 protein subunits within the nucleoprotein filament.

To understand the biochemical consequences of this stimulation, we examined the effects of the Rad51 nucleoprotein filament on Rad54 protein activities. Rad54 protein, like many other members of Swi2/Snf2 family of proteins, possesses a dsDNA-dependent ATPase activity that is important for both in vivo and in vitro functions (Petukhova et al., 1998, 1999; Clever et al., 1999). Recently, it was discovered that both yeast and human Rad54 proteins interact with dsDNA and topologically unwind it in an ATP-hydrolysis-dependent manner (Petukhova et al., 1999; Tan et al., 1999). Here, we found that upon interaction with the Rad51 nucleoprotein filament, Rad54 protein not only retains these activities but, in fact, displays significantly enhanced activities. Like the stimulation of the DNA pairing activity of Rad51 protein, this enhancement of both ATPase and dsDNA unwinding activities depends on a species-specific interaction between yeast Rad54 and Rad51 proteins.

Rad54 protein can exert its stimulatory effect on DNA strand exchange at concentrations that are significantly lower than that of Rad51 protein (Petukhova et al., 1998, 1999). Here, we also show that at a concentration of 0.014 μM, which is 21-fold lower than the Rad51 protein concentration, Rad54 protein stimulates joint molecule formation by 5-fold (Figure 1C). This result is comparable to the level of stimulation that was observed previously by Sung and coworkers using short linear DNA substrates, where they found that at a concentration of 0.1 μM (62-fold lower than the Rad51 protein concentration) Rad54 protein stimulated DNA strand exchange by 2.2–3.5-fold (Figure 1B, left panel, in Petukhova et al. [1999]).

However, we find that equimolar amounts of these two proteins elicit optimal DNA pairing activity and a resultant stimulation of 40-fold. Further substantiating our conclusion that a cocomplex of these two proteins comprises the functional form, the maximal DNA unwinding activity of Rad54 protein requires similar stoichiometric amounts of Rad54 and Rad51 proteins. This mutual stimulation of biochemical activities emphasizes the functional importance of Rad54 protein interaction with the Rad51-ssDNA nucleoprotein filament.

In vivo, Rad54 protein is significantly more abundant than was previously appreciated (Jiang et al., 1996). In *S. cerevisiae*, there are 3500 and 7000 molecules of Rad54 protein in exponentially growing haploid and diploid cells, respectively (Clever et al., 1999). The amount of Rad54 protein can be further induced about 10-fold by treatment of cells with methyl methane sulfonate (MMS) (Clever et al., 1999). Moreover, in exponentially growing cells, Rad54 protein was found to be as abundant as Rad51 protein (J. A. S. and W.-D. H., unpublished data). These recent observations are consistent with the idea that a stochiometric complex of Rad54 and Rad51 proteins, which is required for the highest DNA pairing activity in our biochemical experiments, is the physiologically active species in homologous recombination.

Based on our results, we suggest the following generalized role for Rad54 protein in DNA strand exchange promoted by Rad51 protein. In Figure 7, we illustrate this role in the generalized context of the early steps of homologous recombination. After ssDNA is produced as a result of DNA damage and/or the action of specific nucleases, it is initially bound by RPA (Gasior et al., 1998). Rad51 protein can assemble into a filament, only if the RPA can be removed from that ssDNA (Figure 7, step A). Both the Rad52 protein and the Rad55/Rad57 heterodimer facilitate formation of the Rad51 nucleoprotein complex by displacing RPA and mediating its replacement by Rad51 protein (Sung, 1997a, 1997b; New et al., 1998; Shinohara and Ogawa, 1998). We demonstrate here that Rad54 protein acts after presynaptic complex formation, by interacting with the Rad51-ssDNA nucleoprotein complex (Figure 7, step A). This result is consistent with the more recent cytological studies showing that Rad51 protein foci, which represent inferred intermediates of recombination process, both form independently of Rad54 protein and turn over slower in the absence of Rad54 protein (Shinohara et al., 1997; Takata et al., 2000). As a component of the presynaptic complex, Rad54 protein retains the ability to interact with dsDNA, either homologous or heterologous. Since the stimulation of both the ATPase and DNA unwinding activities of Rad54 protein is independent of DNA homology, we believe that Rad54 protein acts prior to formation of stable heteroduplex DNA. In fact, the interaction of Rad54 protein with dsDNA suggests that Rad54 protein plays an important role during the homology search process itself (Figure 7, step B). Indeed, Rad54 protein unwinds dsDNA, potentially making the normally stable base pairs more available for interaction with the ssDNA within the Rad51 nucleoprotein filament; this would facilitate both the search for homology and the DNA strand exchange event (Figure 7, step C). The fact that maximal DNA unwinding by Rad54 protein requires the same stoichiometric complex of Rad51 and Rad54 proteins as does optimal DNA pairing by Rad51 protein further emphasizes the importance of dsDNA unwinding by Rad54 protein. This dsDNA unwinding activity of the combined Rad54-Rad51 presynaptic filament parallels an activity of RecA protein that itself possesses an intrinsic ability to topologically unwind dsDNA by binding to it (Cunningham et al., 1979; Stasiak et al., 1981; Conley and West, 1990). This DNA unwinding function of RecA protein facilitates homologous pairing (Rould et al., 1992) by "activating" dsDNA (either homologous or nonhomologous) for interaction with ssDNA that is either inside or outside of the RecA nucleoprotein filament (Mazin and Kowalczykowski, 1999). The ability of Rad51 protein to unwind heterologous dsDNA during homology search has not yet been studied; it is possible that this activity of Rad51 protein is intrinsically lower than that of RecA protein and, therefore, Rad54 protein is needed to provide this function.

In eukaryotes, the unwinding of dsDNA during the homology search may have an additional important role. Eukaryotic chromosomal DNA is associated with histones in the form of chromatin; this chromatin structure is an impediment to DNA strand exchange (Ramdas et al., 1991). Despite the chromatin structure, genomic DNA obviously is available for DSB repair. We imagine that after an initial weak interaction with the dsDNA that is accessible within the chromatin structure (Ramdas and Muniyappa, 1995), the histones are displaced by the Rad54-Rad51 nucleoprotein filament. The unwinding of dsDNA by Rad54 protein at these sites of nascent homologous pairing can weaken the interaction between chromatin-associated proteins and DNA and, we speculate, facilitate their removal (Figure 7, step B). This property of Rad54 protein is in accord with the structural characteristics of Swi2/Snf2 family of proteins, whose function in broad terms is to remodel protein-DNA complexes including chromatin. Thus, targeting of Rad54 protein to the locus of homologous pairing by the Rad51 nucleoprotein may provide an economic way of effecting the removal of the chromatin proteins from the site of homologous pairing, without disrupting the structure of the entire chromosome.

Experimental Procedures

Proteins and DNA

E. coli RecA protein, *S. cerevisiae* Rad51 protein, and RPA were purified as described (LeBowitz, 1985; Sugiyama et al., 1997; New et al., 1998). Human Rad51 protein was a generous gift of Patrick Sung (The University of Texas Health Science Center at San Antonio). Rad54 protein was purified from yeast as a fusion with GST sequence as described (J. A. S. et al., unpublished data). The GST-Rad54 protein we used in this paper is fully active both in vitro and in vivo. The ATPase activity measured at 37°C (1100–1300 min^{-1}) is in accord with the published activity (1270 min^{-1}) (Petukhova et al., 1998). Also, the GST-Rad54 protein fully complements the recombination and DNA repair defects of *rad54*Δ cells (J. A. S. et al., unpublished data).

The oligonucleotides used in this study were #1, 63-mer, ACA GCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGA CCTCTTATCAAAAGGA; #2, 63-mer, TCCTTTTGATAAGAGGTCATT TTTGCGGATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGT; #5, 90-mer, CGCGTGTCGGGGGTGGCTTAACTATGCGGCATCAGAG CAGATTGTACTGAGAGTTAGAAGCGTTATTCTCTGGGCGAA TAGAGTGCTATC; #71, 94-mer, CTTTAGCTGCATATTTACAACATG TTGACCTACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCG CAAAAATGACCTCTTATCAAAAGGA; and #90, 90-mer CGGGTGT CGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGA GAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT. Oligonucleotides were prepared as described (Mazin and Kowalczykowski, 1996). Covalently closed circular supercoiled pUC19 dsDNA

was purified using alkaline lysis, followed by two rounds of CsCl-ethidium bromide equilibrium centrifugation (Sambrook et al., 1989). The pUC19 dsDNA was linearized by digestion with SmaI restriction endonuclease (Boehringer Mannheim). All DNA concentrations are provided as molar in nucleotide concentration.

Joint Molecule Formation

To form Rad51 nucleoprotein filaments, Rad51 protein (0.3 μM) was incubated with ssDNA (0.9 μM) in buffer containing 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 2 mM ATP, 3 mM phosphoenolpyruvate, and pyruvate kinase (20 U/ml), bovine serum albumin (100 μg/ml) at 37°C for 15 min. Where indicated, Rad54 protein was added to the reaction at the indicated concentrations. In all cases, pairing reactions were initiated by addition of pUC19 supercoiled DNA (9 μM) to the nucleoprotein filaments; in terms of molecule concentrations, the supercoiled DNA is limiting. When Rad54 protein was present, dsDNA was added immediately after Rad54 protein. Aliquots were withdrawn from the reaction mixture, deproteinized by addition of EDTA to 50 mM, SDS to 1% and proteinase K to 500 μg/ml with incubation for 5 min at 37°C. Samples were mixed with a $^1/_{10}$ volume of loading buffer (20% ficoll and 0.1% bromophenol blue) and analyzed by gel electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate [pH 8.0] and 2 mM EDTA). The yield of joint molecules was determined using a Storm 840 PhosphorImager (Molecular Dynamics).

ATPase Assay

The ATPase assays were carried out as described earlier (Kowalczykowski and Krupp, 1987). Standard reaction buffer contained 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 2 mM ATP, 3 mM phosphoenolpyruvate, pyruvate kinase (20 U/ml), lactate dehydrogenase (20 U/ml), and NADH (200 μg/ml). The oxidation of NADH, coupled to ADP phosphorylation, resulted in a decrease in absorbance at 340 nm, which was continuously monitored by a Hewlett-Packard 8452A diode array spectrophotometer using UV-Visible ChemStation software. The rate of ATP hydrolysis was calculated from the rate of change in absorbance using the following formula: rate of A_{340} decrease (s^{-1}) × 9880 = rate of ATP hydrolysis (μM/min). Kinetic parameters of these reactions were determined using GraphPad Prism software version 3.0. When Rad51 nucleoprotein filaments were present in the reaction, they were formed by incubation of Rad51 protein (0.17 μM) with ssDNA (0.5 μM) in standard reaction buffer at 37°C for 15 min. RecA nucleoprotein filaments were assembled by incubation of RecA protein (0.17 μM) with ssDNA (0.5 μM) at 37°C for 10 min in the same buffer.

Topological DNA Unwinding Assay

To measure DNA unwinding activity of Rad54 protein alone or in the presence of the Rad51 nucleoprotein filament, we used a topological assay described earlier (Wold et al., 1987; Harmon et al., 1999). The Rad51 nucleoprotein filaments were assembled by incubating ssDNA (90-mer, #90, or 94-mer, #71) at concentrations of either 0.56, 0.28, 0.14, or 0.07 μM with Rad51 protein at concentrations 0.168, 0.084, 0.042, or 0.021 μM, respectively. The concentrations of these Rad51 nucleoprotein filaments were designated as 2×, 1×, 0.5×, and 0.25×, respectively. Each filament assembly reaction was carried out for 20 min at 37°C in 20 μl of topoisomerase buffer, containing 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, 2 mM ATP, bovine serum albumin (100 μg/ml), 2 mM phosphoenolpyruvate, and pyruvate kinase (20 U/ml) and then cooled to 24°C for 1 min and mixed with Rad54 protein (0.084 μM). DNA unwinding assays were then immediately initiated by addition of 2 μl of the freshly prepared relaxed pUC19 DNA-topoisomerase I mix; this DNA was prepared by treating supercoiled pUC19 DNA (165 μM) with 1 μg of *E. coli* topoisomerase I in topoisomerase buffer for 30 min at 37°C. The DNA unwinding reactions were carried out for 20 min at 30°C. In control DNA unwinding assays, either Rad54 protein or the Rad51 nucleoprotein filaments was omitted. The reactions were stopped by the addition of SDS to 0.5% and proteinase K to 600 μg/ml and were further incubated for 20 min at 30°C. Gel loading buffer (3 μl) containing 0.1% bromophenol blue, 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 50% glycerol was added prior to electrophoresis through a 1% agarose gel in TBE buffer (90 mM Tris-borate [pH 8.3] and 1 mM EDTA) at 3 V/cm for 6 hr. DNA bands were visualized by staining with ethidium bromide (0.5 μg/ml) for 1 hr, destaining for 30 min in water, and photographing the gel on a UV light transilluminator (Fotodyne Inc.). DNA bands were quantitated using ImageQuant software. Since different species of supercoiled DNA bind ethidium bromide with different efficiency, we quantitated the amount of relaxed circular DNA that remained at the end of each reaction.

Acknowledgments

We are grateful to Piero Bianco, Jason Churchill, Frank Harmon, Cynthia Haseltine, Noriko Kantake, Konstantin Kiianitsa, Julie Kleiman, James New, Erica Seitz, and Tomohiko Sugiyama for their comments on the manuscript. We are especially grateful to Patrick Sung (The University of Texas Health Science Center at San Antonio) for providing human Rad51 protein. We thank Shunichi Takeda for communication of data prior to publication. A. M. is grateful to the Institute of Cytology and Genetics, Russian Academy of Science, Novosibirsk, for granting him a leave of absence. This work was supported by the following grants: National Institutes of Health (NIH) (AI-18987) and Human Frontiers Science Program (RG63) to S. C. K.; NIH Postdoctoral Fellowship (GM-20057) to C. J. B.; and Swiss National Science Foundation (31-4556495), NIH (GM-58015), and Human Frontier Science Program (RG63) to W.-D. H.

Received May 12, 2000; revised July 10, 2000.

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