Conditions for strand-exchange reaction. We first incubated Rad52 or RPA protein with φX174 viral DNA (final concentration, 20 μM in nucleotide) for 5 min at 37 °C in 9 μl buffer (20 mM HEPES, pH 6.5, 1 mM DTT, 6.6 mM $MgCl_2$, 3 mM ATP, 20 mM creatine phosphate, 0.1 mg ml^{-1} creatine kinase, 50 μg ml^{-1} BSA), then Rad51 protein (0.5 μl of 123 μM) was added. After 5 min of further incubation, 0.1 μg of *Pst*I-linearized φX174 dsDNA in 1 μl and 100 mM $MgCl_2$ in 1 μl were added to start the reaction. After incubation at 37 °C for the indicated times, SDS and proteinase K were added to final concentrations of 0.5% and 0.5 mg ml^{-1}, respectively, followed by a 20-min incubation. Samples were mixed with 1 μl dye solution (0.01% of bromophenol blue, 20% glycerol) and analysed on a 0.9% agarose gel prepared in 1 × TAE buffer (40 mM Tris-acetate, pH 7.5, 0.5 mM EDTA). Electrophoresis was for 15 h at 1.2 V cm^{-1} at 4 °C. The gel was stained with 0.1 μg ml^{-1} of Syber green (Molecular Probe). The image of the gel was recorded with a CCD camera (Epi-UV FA1100, Aisin Cosmos); densitometric analysis was carried out using Quantity One software (PDI). Images were processed using Photoshop (Adobe). In the case of the RecA reaction, Rad51 was simply substituted by RecA (6 μM).

Assay for ssDNA-dependent ATP hydrolysis. ATP hydrolysis was analysed in 10 μl of buffer containing 20 mM HEPES, pH 6.5, 1 mM DTT, 5 mM $MgCl_2$, 0.2 mM ^{32}P-α-ATP, 50 μg ml^{-1} BSA, 20 μM ssDNA at 37 °C. The reaction was started by addition of the indicated amounts of Rad51 protein. At the indicated time, 1-μl aliquots were removed and directly spotted on a PEI paper. The PEI papers were developed in 0.85 M potassium phosphate (pH 2.8). Radioactive ATP and ADP spots were analysed by using the phsophorimager BAS2,000 (Fuji Film).

Gel shift assay of Rad52-p(dT)$_{60}$ complexes. 20 μM (in nucleotide) of p(dT)$_{60}$ oligonucleotide whose 5′ end was labelled with ^{32}P was incubated with Rad52 or Rad52–327 proteins in 10 μl buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 50 μg ml^{-1} BSA) at 37 °C for 10 min. Samples were analysed on a 1.0% of agarose gel in 0.1 × TAE. Electrophoresis was done at 10 V cm^{-1} for 90 min.

Received 1 September; accepted 4 December 1997.

1. Resnick, M. A. in *Meiosis* (ed. Moens, P. B.) 157–210 (Academic, New York, 1987).
2. Petes, T. D., Malone, R. E. & Symington, L. S. in *The Molecular and Cellular Biology of the Yeast* Saccharomyces. *Genome Dynamics, Protein Synthesis and Energetics.* (eds Broach, J. R., Pringle, J. R. & Jones, E. W.) 407–521 (Cold Spring Harbor Laboratory Press, New York, 1991).
3. Shinohara, A. & Ogawa, T. Homologous recombination and the roles of double-strand breaks. *Trends Biochem. Sci.* **20,** 587–591 (1995).
4. Shinohara, A., Ogawa, H. & Ogawa, T. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell* **69,** 457–470 (1992).
5. Aboussekhra, A., Chanet, R., Adjiri, A. & Fabre, F. Semidominant suppressors of Srs2 helicase mutations of *Saccharomyces cerevisiae* map in the *RAD51* gene, whose sequence predicts a protein with similarities to prokaryotic RecA proteins. *Mol. Cell. Biol.* **12,** 3224–3234 (1992).
6. Ogawa, T., Yu, X., Shinohara, A. & Egelman, E. H. Similarity of the yeast RAD51 filament ot the bacterial Rec filament. *Science* **259,** 1896–1899 (1993).
7. Ogawa, T. *et al.* RecA-like recombination proteins in eukaryotes: Functions and structures of *RAD51* gene. *Cold Spring Harb. Symp. Quant. Biol.* **58,** 567–576 (1994).
8. Sung, P. Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science* **265,** 1241–1243 (1994).
9. Sung, P. & Robberson, D. L. DNA strand exchange mediated by a RAD51–ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell* **82,** 453–461 (1995).
10. Sugiyama, T., Zaitseva, E. M. & Kowalczykowski, S. C. A single-stranded DNA binding protein is needed for efficient presynaptic complex formation by the *Saccharomyces cerevisiae* Rad51 proteins. *J. Biol. Chem.* **272,** 7940–7945 (1997).
11. Namsaraev, E. & Berg, P. Characterization of strand exchange activity of yeast Rad51 protein. *Mol. Cell. Biol.* **17,** 5359–5368 (1997).
12. Adzuma, K., Ogawa, T. & Ogawa, H. Primary structure of the *RAD52* gene in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4,** 2735–2744 (1984).
13. Milne, G. T. & Weaver, D. T. Dominant negative alleles of *RAD52* reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes Dev.* **7,** 1755–1765 (1993).
14. Shinohara, A. *et al.* Cloning of human, mouse and fission yeast recombination genes homologous to *RAD51* and *recA*. *Nature Genet.* **4,** 239–243 (1993).
15. Murris, D. F. R. *et al.* Cloning of human and mouse genes homologous to *RAD52*, a yeast gene involved in DNA repair and recombination. *Mutat. Res. DNA Repair* **315,** 295–305 (1994).
16. Ogawa, T., Shinohara, A. & Ikeya, T. A species-specific interaction of Rad51 and Rad52 proteins in eukaryotes. *Adv. Biophys.* **31,** 93–100 (1995).
17. Mortensen, U. H., Bendixen, C., Sunjevaric, I. & Rothstein, R. DNA strand annealing is promoted by the yeast Rad52 protein. *Proc. Natl Acad. Sci. USA* **93,** 10729–10734 (1996).
18. Sung, P. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev.* **11,** 1111–1121 (1997).
19. Kowalczykowski, S. C. & Eggleston, A. K. Homologous pairing and DNA strand exchange proteins. *Annu. Rev. Biochem.* **63,** 991–1043 (1994).
20. West, S. C. Enzymes and molecular mechanisms of genetic recombination. *Annu. Rev. Biochem.* **61,** 603–640 (1992).
21. Boundy-Mills, K. L. & Livingston, D. M. A *Saccharomyces cerevisiae RAD52* allele expressing a C-terminal truncation protein: Activities and intragenic complementation of missense mutations. *Genetics* **133,** 39–49 (1993).
22. Baumann, P., Benson, F. E. & West, S. C. Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. *Cell* **87,** 757–766 (1996).
23. Gupta, R. C., Bazemore, L. R., Golub, E. I. & Radding, C. M. Activities of human recombination protein Rad51. *Proc. Natl Acad. Sci. USA* **94,** 463–468 (1997).
24. Shen, Z., Cloud, K. G., Chen, D. J. & Park, M. S. Specific interactions between the human *RAD51* and *RAD52* proteins. *J. Biol. Chem.* **271,** 148–152 (1996).
25. Shinohara, A., Shinohara, M., Ohta, T., Matsuda, S. & Ogawa, T. Cooperation of Rad52 with a single-stranded DNA binding protein, RPA, in homologous recombination. *Genes-to-Cells* (submitted).
26. Alani, E., Thresher, R., Griffith, J. D. & Kolodner, R. D. Characterization of DNA-binding and strand-exchange stimulation properties of y-RPA, a yeast single-strand-DNA-binding protein. *J. Mol. Biol.* **227,** 54–71 (1992).
27. Benson, F. E., Baumann, P. & West, S. C. Synergistic actions of Rad51 and Rad52 in recombination and DNA repair. *Nature* **391,** 401–404 (1998).
28. New, J. H., Sugiyama, T., Zaitseva, E. & Kowalczykowski, S. C. Rad52 protein stimulates DNA strand exchange by Rad51 protein and replication protein A. *Nature* **391,** 407–410 (1998).
29. Sung, P. Functions of yeast Rad52 protein as a mediator between Replication protein A and the rad51 recombinase. *J. Biol. Chem.* **272,** 28194–28197 (1997).

Acknowledgements. We thank J. Tomizawa and H. Ogawa for encouragement and support; N. Kleckner, D. Bishop, E. Egelman, L. S. Symington and S. Gaisor for critically reading the manuscript; A. Sugino for yeast RPA protein; R. Kolodner for the overproducer strain of RPA; and D. Bishop and S. West for communicating unpublished results. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and the Human Frontier Science Organization to A.S. and T.O., and a grant from National Institute of Genetics to A.S.

Correspondence and requests for materials should be addressed to A.S. (e-mail: ashino@bio.sci.osaka-u.ac.jp) or T.O. (e-mail: tomogawa@lab.nig.jp).

Rad52 protein stimulates DNA strand exchange by Rad51 and replication protein A

James H. New, Tomohiko Sugiyama, Elena Zaitseva & Stephen C. Kowalczykowski*

Sections of Microbiology and of Molecular and Cellular Biology, University of California at Davis, Davis, California 95616-8665, USA

The generation of a double-strand break in the *Saccharomyces cerevisiae* genome is a potentially catastrophic event that can induce cell-cycle arrest or ultimately result in loss of cell viability. The repair of such lesions is strongly dependent on proteins encoded by the *RAD52* epistasis group of genes (*RAD50-55*, *RAD57*, *MRE11*, *XRS2*)[1,2], as well as the *RFA1*[3,4] and *RAD59* genes[5]. *rad52* mutants exhibit the most severe phenotypic defects in double-strand break repair[2], but almost nothing is known about the biochemical role of Rad52 protein. Rad51 protein promotes DNA strand exchange[6–8] and acts similarly to RecA protein[9]. Yeast Rad52 protein interacts with Rad51 protein[10,11], binds single-stranded DNA and stimulates annealing of complementary single-stranded DNA[12]. We find that Rad52 protein stimulates DNA strand exchange by targeting Rad51 protein to a complex of replication protein A (RPA) with single-stranded DNA. Rad52 protein affects an early step in the reaction, presynaptic filament formation, by overcoming the inhibitory effects of the competitor, RPA. Furthermore, stimulation is dependent on the concerted action of both Rad51 protein and RPA, implying that specific protein–protein interactions between Rad52 protein, Rad51 protein and RPA are required.

As with the RecA protein[13], assembly of a presynaptic filament composed of single-stranded (ss)DNA coated with Rad51 protein is a first step in DNA strand exchange. Secondary structure in ssDNA imposes a barrier to nucleoprotein filament continuity which RPA can overcome through its ability to destabilize duplex DNA[14,15]. Although RPA is required for optimal DNA strand exchange and ATP hydrolysis by Rad51 protein, because of their shared ability to bind ssDNA they are potential competitors: when RPA binds ssDNA first, a long delay in ATP hydrolysis (T.S., unpublished observation) and a substantial decrease in the amount of DNA strand exchange results[16]. Thus, displacement of RPA is necessary to form an active presynaptic complex[15]. Double-stranded DNA (dsDNA) is a substrate in DNA strand exchange but, as it is readily bound by Rad51 protein, it can also be a competitor of presynaptic filament formation[7]. Because both RPA and dsDNA are present *in vivo*

during Rad51 protein–ssDNA assembly, some mechanism presumably exists to allow filament formation in the presence of these competitors.

To examine the impact of Rad52 protein on Rad51 protein-promoted DNA strand exchange, Rad52 protein was purified from *Escherichia coli*. Figure 1 shows DNA strand exchange reactions in which ssDNA was incubated with RPA for one minute before addition of Rad51 and Rad52 proteins. DNA strand exchange was initiated by addition of dsDNA at 1, 5 or 15 min after the addition of these proteins. Product formation is stimulated significantly by Rad52 protein (Fig. 1a, lanes 3, 5 and 7); at longer times (30 min), stimulation occurs, but the relative magnitude decreases, because Rad51 protein can slowly displace RPA (data not shown). Stimulation is not due to Rad52 protein-mediated DNA strand exchange because the reaction is completely dependent on Rad51 protein (Fig. 1a, lane 1). The Rad52 protein concentration is optimal for these reactions: halving its concentration reduces the yield by approximately half, whereas increasing it by 50% increases it negligibly (data not shown). These results parallel other recent results[17]. Figure 2 shows the reaction time course. Rad52 protein increases the yield of paired products, but it has little impact on the progress of the reaction. These results indicate that Rad52 protein acts by increasing the amount of an essential DNA strand exchange participant, and that it does so by antagonizing the inhibitory effect of prebound RPA on the presynaptic phase of DNA strand exchange: we presume that Rad52 protein either increases dissociation of RPA and/or assembly of Rad51 protein.

To determine whether Rad52 protein moderates sequestration of Rad51 protein by dsDNA, Rad51 protein was incubated with dsDNA either in the simultaneous or subsequent presence of Rad52 protein; these DNA pairing reactions were completely blocked (data not shown), demonstrating that Rad52 protein does not reverse the inhibitory effect of Rad51 protein binding to dsDNA. Taken together, these results suggest that Rad52 protein facilitates the loading of Rad51 protein onto RPA–ssDNA.

The need for interaction between Rad52 protein and RPA, as previously suggested[4,18], was evaluated by substituting a ssDNA-binding protein for RPA. *E. coli* SSB protein, when added after Rad51 protein, stimulates its activity[15]; but when SSB protein is allowed to bind ssDNA before Rad51 protein is added, no product forms (Fig. 3). However, Rad52 protein cannot overcome this inhibition, even after 15 min of co-incubation, (Fig. 3, lanes 2 and 3) suggesting that a specific interaction between Rad52 protein and RPA is necessary for stimulation.

If Rad51 protein simply occupies a ssDNA site that is vacated by RPA, then RecA protein might act similarly; in contrast, if a Rad52–Rad51 protein interaction were essential, then RecA protein should not be stimulated by Rad52 protein. When RecA protein is substituted for Rad51 protein (Fig. 4), DNA strand exchange is efficient, even when RPA is allowed to prebind the ssDNA (Fig. 4, lane 2). However, when Rad52 protein is added (Fig. 4, lane 3), product formation is inhibited. Additionally, Rad52 protein completely blocks DNA strand exchange in an otherwise identical RecA protein reaction that used SSB protein instead of RPA (data not shown). From these experiments we conclude that the Rad52 protein–RPA interaction is not sufficient to stimulate RecA protein, and that the Rad51–Rad52 protein interaction is necessary for stimulation by Rad52 protein.

The potential of ssDNA-binding proteins to inhibit as well as to stimulate the activity of DNA strand exchange proteins underscores the need for proper coordination of the recombination machinery for efficient function. In this regard, the Rad51–Rad52–RPA system is like that of the bacteriophage T4 UvsX–UvsY–gene 32 protein recombination proteins, in which UvsY will stimulate DNA strand

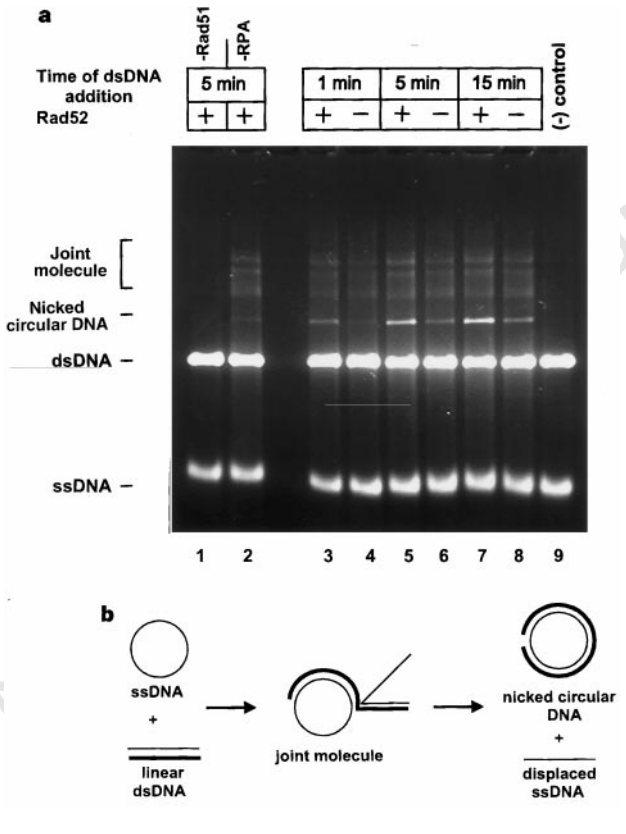

Figure 1 Stimulation of DNA strand exchange by Rad52 protein. **a**, Ethidium bromide stained DNA gel. Specific proteins were omitted from the reaction as indicated: lanes 3, 5 and 7 contained Rad52 protein (4.8 μM) and lanes 4, 6 and 8 contained storage buffer; dsDNA was added at the times indicated after protein addition. The negative (–) control reaction (lane 9) was terminated before addition of dsDNA. In the absence of RPA and Rad52 protein, negligible amounts of paired species are formed[6,15]. **b**, Diagram of the reaction substrates and products. **c**, Purity of Rad52 protein (1.1 μg in lane 1), as assessed by 8% SDS–PAGE and staining with Coomassie blue.

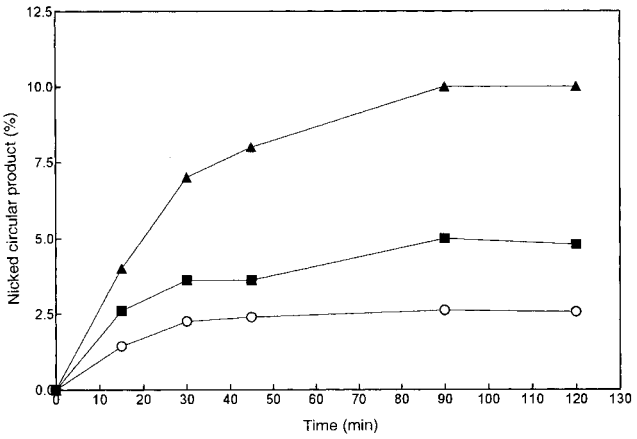

Figure 2 Time course for DNA strand exchange initiated at various times after addition of Rad52 protein. RPA was allowed to pre-bind ssDNA for 5 min before addition of Rad51 and Rad52 proteins, and DNA strand exchange was initiated by addition of dsDNA 1, 5 or 15 min after protein addition: Circles, 1 min; squares, 5 min; triangles, 15 min.

exchange by UvsX using a preformed gene 32 protein–ssDNA complex[19]. Like Rad51 protein, UvsX binds both dsDNA and ssDNA[9], and, like Rad52 protein, UvsY interacts with the strand exchange protein UvsX and the ssDNA-binding protein gene 32 protein[20]. Although the activities of Rad52 protein described here are important in double-strand DNA break repair and homologous recombination, they cannot completely account for all of the roles of *RAD52* in these processes. Other studies have suggested additional *RAD51*-independent functions for *RAD52* (refs 21, 22): whether or not these are dependent on the intrinsic ability of Rad52 protein to renature complementary ssDNA, on other as-yet undefined biochemical activities, or on additional protein factors remains to be investigated. □

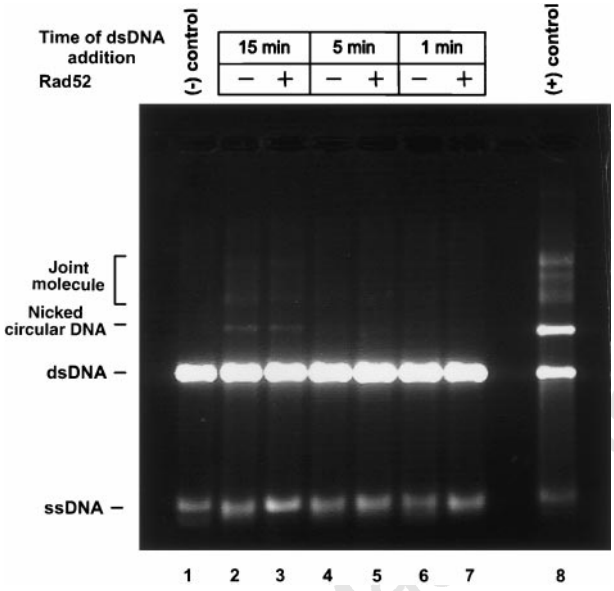

Figure 3 Inhibition of DNA strand exchange by SSB protein is not overcome by Rad52 protein. Standard reactions were performed, except that RPA was replaced by SSB protein (18 μM). Rad52 protein was added to reactions as indicated (lanes 3, 5 and 7), and dsDNA was added after Rad52 protein or buffer at indicated times. The negative (–) control reaction (lane 1) was the same as for Fig. 1. In the positive (+) control reaction (lane 8), the reaction was staged so that Rad51 protein was assembled on ssDNA first and, after a 15-min incubation at 37 °C, SSB protein was added; 15 min later, dsDNA was added to initiate the reaction.

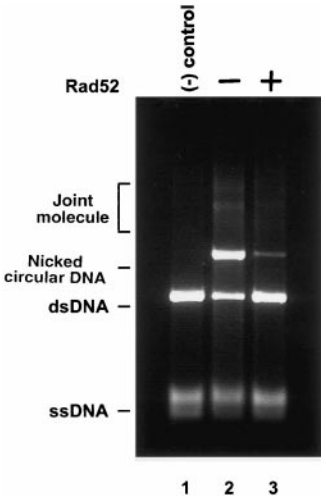

Figure 4 Rad52 protein does not stimulate RecA protein-promoted DNA strand exchange. Standard reactions containing RPA were supplemented with an ATP regeneration system, and Rad51 protein was replaced by RecA protein (10 μM). Rad52 protein or buffer was added (lanes 3 and 2, respectively) in the standard manner.

Methods

Strains and plasmids. BL21(DE3) Δ*recA* was constructed by E. Zaitsev of this laboratory, and BLR containing pLysS was from Novagen. PEZRad51 (Rad51 protein overexpression) and pEZRad52/3 (Rad52 protein overexpression) were constructed by cloning of the *RAD51* and *RAD52* (from the third ATG codon) ORFs into pET3a and pET21a (Novagen), respectively. Constructs were confirmed by sequence analysis.

Rad52 protein purification. A 12-litre culture of BL21(DE3) Δ*recA* containing pEZRad52/3 was grown at 30 °C in LB broth (Ap; 100 g ml^{-1}) and induced at $A_{600} = 1$ (IPTG, 0.1 mM final concentration) for 1 h. Cells were resuspended in 5 volumes of lysis buffer (450 mM NaCl in M_{50}DEG buffer (50 mM MES (pH 6.5), 1 mM DTT, 1 mM EDTA, 10% glycerol)); lysed by passage through a French press at 4 °C; and PMSF (1 mM), benzamidine (10 mM), and pepstatin A (1 μg ml^{-1}) added. After a low-speed spin (6,000 r.p.m., 10 min), the supernatant was loaded onto Q-Sepharose (35 ml). Ammonium sulphate (0.29 g ml^{-1}) was added to the flow-through fraction and, following dialysis of the pellet against lysis buffer, the solution was diluted 3-fold with M_{20}DEG buffer and applied to a P-11 phosphocellulose column (35 ml). Peak fractions (eluting at 400 mM NaCl in M_{20}DEG buffer) were diluted 2-fold with M_{20}DEG buffer and loaded onto ssDNA–cellulose (50 ml) which was washed with steps of 0.3 M and 0.6 M NaCl in M_{20}DEG buffer. The last wash was applied to a hydroxyapatite column (4 ml) and fractionated with a 10–200 mM potassium phosphate gradient in 0.2 M NaCl in M_{20}DG buffer. Rad52 protein eluted at 90 mM potassium phosphate, was made 1 M in NaCl, dialysed against 1 M NaCl in T_{20}DEG buffer (20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 10% glycerol) and loaded onto Superose-12 (25 ml). Rad52 protein, which eluted in the void volume, was dialysed against storage buffer (175 mM NaCl in T_{20}DEG buffer) and stored at −80 °C. Protein concentration was determined using an extinction coefficient of 2.43×10^4 at 280 nm. Approximately 1–2 mg purified protein was recovered from 10 g of cells. The identity of the protein was confirmed by immunoblotting.

Rad51 protein and RPA. Rad51 protein was purified as described[6], but with addition of a Cibacron-blue chromatography step (Rad51 protein eluted at 1 M KCl) and omission of gel filtration. RPA was purified as described[15].

DNA strand exchange. Reactions (12.5 μl) contained 42 mM MOPS (pH 7.4), 3 mM magnesium acetate, 1 mM DTT, 25 μg ml^{-1} BSA, 2.5 mM ATP, and 33 μM (nucleotides) ΦX174 ssDNA (New England Biolabs). RPA (1 μM) or SSB protein (18 μM) was added and incubated at 37 °C for 1 min, at which time Rad51 (13 μM) and Rad52 (3.2 μM) proteins were added. After further incubation for the indicated times, *Pst*I-linearized ΦX174 dsDNA (33 μM nucleotides) and spermidine acetate (4 mM) were added. Reactions were terminated after 90 min, or as indicated otherwise, and analysed[15].

RecA protein reactions were similar, except that the ATP concentration was 1 mM, an ATP regeneration system was included[23], dsDNA was added 1 min after addition of the last protein, and spermidine acetate was omitted.

Received 14 October; accepted 8 December 1997.

1. Game, J. C. DNA double-strand breaks and the *RAD50–RAD57* genes in *Saccharomyces*. *Semin. Cancer Biol.* **4,** 73–83 (1993).
2. Petes, T. D., Malone, R. E. & Symington, L. S. in *The Molecular and Cellular Biology of the Yeast* Saccharomyces*: Genome Dynamics, Protein Synthesis, and Energetics* (eds Broach, J. R., Jones, E. & Pringle, J.) 407 (Cold Spring Harbor Laboratory, New York, 1991).
3. Smith, J. & Rothstein, R. A mutation in the gene encoding the *Saccharomyces cerevisiae* single-stranded DNA-binding protein Rfa1 stimulates a RAD52-independent pathway for direct-repeat recombination. *Mol. Cell. Biol.* **15,** 1632–1641 (1995).
4. Firmenich, A. A., Elias-Arnanz, M. & Berg, P. A novel allele of *Saccharomyces cerevisiae* RFA1 that is deficient in recombination and repair and suppressible by RAD52. *Mol. Cell. Biol.* **15,** 1620–1631 (1995).
5. Bai, Y. & Symington, L. S. A Rad52 homolog is required for RAD51-independent mitotic recombination in *Saccharomyces cerevisiae*. *Genes Dev.* **10,** 2025–2037 (1996).
6. Sung, P. Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science* **265,** 1241–1243 (1994).
7. Sung, P. & Robberson, D. L. DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell* **82,** 453–461 (1995).
8. Sung, P. & Stratton, S. A. Yeast Rad51 recombinase mediates polar DNA strand exchange in the absence of ATP hydrolysis. *J. Biol. Chem.* **271,** 27983–27986 (1996).
9. Kowalczykowski, S. C. & Eggleston, A. K. Homologous pairing and DNA strand-exchange proteins. *Annu. Rev. Biochem.* **63,** 991–1043 (1994).
10. Shinohara, A., Ogawa, H. & Ogawa, T. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell* **69,** 457–470 (1992). [Erratum, *Cell* **71,** 180; 1992].

11. Milne, G. T. & Weaver, D. T. Dominant negative alleles of RAD52 reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes Dev.* **7,** 1755–1765 (1993).
12. Mortensen, U. H., Bendixen, C., Sunjevaric, I. & Rothstein, R. DNA strand annealing is promoted by the yeast Rad52 protein. *Proc. Natl Acad. Sci. USA* **93,** 10729–10734 (1996).
13. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. & Rehrauer, W. M. Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58,** 401–465 (1994).
14. Wold, M. S. Replication protein A: a heterotrimeric, single-stranded DNA-bindign protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem.* **66,** 61–92 (1997).
15. Sugiyama, T., Zaitseva, E. M. & Kowalczykowski, S. C. A single-stranded DNA-binding protein is needed for efficient presynaptic complex formation by the *Saccharomyces cerevisiae* Rad51 protein. *J. Biol. Chem.* **272,** 7940–7945 (1997).
16. Sung, P. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev.* **11,** 1111–1121 (1997).
17. Sung, P. Function of yeast Rad52 protein as mediator between replication protein A and the Rad51 recombinase. *J. Biol. Chem.* **272,** 28194–28197 (1997).
18. Park, M. S., Ludwig, D. L., Stigger, E. & Lee, S. H. Physical interaction between human RAD52 and RPA is required for homologous recombination in mammalian cells. *J. Biol. Chem.* **271,** 18996–19000 (1996).
19. Yonesaki, T. & Minagawa, T. Synergistic action of three recombination gene products of bacteriophage T4, uvsX, uvsY, and gene 32 proteins. *J. Biol. Chem.* **264,** 7814–7820 (1989).
20. Jiang, H., Giedroc, D. & Kodadek, T. The role of protein–protein interactions in the assembly of the presynaptic filament for T4 homologous recombination. *J. Biol. Chem.* **268,** 7904–7911 (1993).
21. Sugawara, N. *et al*. DNA structure-dependent requirements for yeast RAD genes in gene conversion. *Nature* **373,** 84–86 (1995).
22. Rattray, A. J. & Symington, L. S. Use of a chromosomal inverted repeat to demonstrate that the RAD51 and RAD52 genes of *Saccharomyces cerevisiae* have different roles in mitotic recombination. *Genetics* **138,** 587–595 (1994).
23. Kowalczykowski, S. C. & Krupp, R. A. Effects of the *Escherichia coli* SSB protein on the single-stranded DNA-dependent ATPase activity of *Escherichia coli* RecA protein: Evidence that SSB protein facilitates the binding of RecA protein to regions of secondary structure within single-stranded DNA. *J. Mol. Biol.* **193,** 97–113 (1987).

Acknowledgements. We thank T. Ogawa for antibodies against Rad52 and Rad51 proteins, K. Adzuma for advice on the purification of Rad52 protein, E. Zaitsev for strains, and T. Ogawa, A. Shinohara, P. Sung and S. West for communication of unpublished results. This work was supported by grants from the NIH and from the Human Frontier Science Program (to S.C.K.).

Correspondence and requests for materials should be addressed to S.C.K. (e-mail: sckowalczykowski@ucdavis.edu).

Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA

Frances E. Chen*, De-Bin Huang†, Yong-Qing Chen† & Gourisankar Ghosh†

*Departments of *Biology, †Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0359, USA*

The NF-κB p50/p65 heterodimer is the classical member of the Rel family of transcription factors which regulate diverse cellular functions such as immune response, cell growth, and development[1–3]. Other mammalian Rel family members, including the proteins p52, proto-oncoprotein c-Rel, and RelB, all have amino-terminal Rel-homology regions (RHRs)[4–7]. The RHR is responsible for the dimerization, DNA binding and cytosolic localization of these proteins by virtue of complex formation with inhibitor κB proteins[8]. Signal-induced removal of κB inhibitors allows translocation of dimers to the cell nucleus and transcriptional regulation of κB DNA-containing genes[9]. NF-κB specifically recognizes κB DNA elements[1,10,11] with a consensus sequence of 5′-GGGRNYYYCC-3′ (R is an unspecified purine; Y is an unspecified pyrimidine; and N is any nucleotide). Here we report the crystal structure at 2.9 Å resolution of the p50/p65 heterodimer bound to the κB DNA of the intronic enhancer of the immunoglobulin light-chain gene. Our structure reveals a 5-base-pair 5′ subsite for p50, and a 4-base-pair 3′ subsite for p65. This structure indicates why the p50/p65 heterodimer interface is stronger than that of either homodimer. A comparison of this structure with those of other Rel dimers reveals that both subunits adopt variable conformations in a DNA-sequence-dependent manner. Our results explain the different behaviour of the p50/p65 heterodimer with heterologous promoters.

The overall structure of the p50/p65 heterodimer is consistent with that of other Rel family proteins (Fig. 1a)[12,13,31]. Each subunit consists of two immunoglobulin-like domains connected by a 10-amino-acid flexible linker. Dimers form through a β-sheet sandwich of the carboxy-terminal dimerization domains. Unlike most DNA-binding proteins, which use α-helices for base-pair recognition, Rel family dimers use loops from the edges of the N- and C-terminal domains to mediate DNA contacts (Fig. 2a). Secondary structures of the subunits are equivalent, apart from a 32-amino-acid insert in the N-terminal domain of p50 that adds a second α-helix (Fig. 1b) (to simplify description, p50 residues will be written in normal type and p65 residues will be written in italics).

Dimerization in the heterodimer is localized to the C-terminal domains and consists of a hydrophobic core stapled by various polar interactions (Fig. 2b). The backbones of the dimerization domains are highly similar and superimpose with a root-mean-square deviation (r.m.s.d.) of 1.10 Å. The buried surface area upon dimerization is 1,442 Å^2. Of particular interest is the hydrogen bond between homologous residues Asp 254 of p50 and *Asn 200* of p65, which is unique in the heterodimer. In the homodimers, an interaction between homologous residues is energetically unfavourable because this juxtaposes two like charges. Also of interest are nine polar contacts, most of which occur between the backbones of hydrophobic core residues and the side chains of hydrogen-bonding residues. These results are consistent with the observation that the affinity between homodimers of p50 and p65 is weaker than for the heterodimers[14].

The co-crystallized DNA consists of 11 base pairs with a complementary overhanging base on each end (Fig. 1c). The 3′ ten base pairs of the target DNA define the immunoglobulin(Ig) κB element. Analysis of the central 11 base pairs with the program CURVES[15] revealed a 14-degree bend. The bases T_0 and G_{-1} are also twisted out of plane owing to this DNA bending. This DNA bending agrees with biochemical experiments indicating that the p50/p65 heterodimer induces a 17-degree bend in H2-κB DNA[16].

Previous experiments have indicated that the p50 subunit preferentially occupies the 5′ end of an Ig-κB target DNA[2,10,14]. The heterodimer crystal structure not only confirms this orientation, but also demonstrates the exact positioning (Fig. 3a). The observed base-specific contacts support the idea that the Ig-κB site consists of a 5-base-pair 5′-GGGAC-3′ subsite contacted by p50, and a 4-base-pair 5′-TTCC-3′ subsite contacted by p65. Upon binding to DNA, the heterodimer buries 3,754 Å^2 of the total solvent-accessible surface area, of which 58% is derived from interaction with p50. Overall, the DNA contacts mediated by p50 and p65 in the heterodimer structure are similar to those in the homodimer structures[12,13].

Base-specific binding by p50 to the subsite occurs through the residues Arg 54, Arg 56, Tyr 57, Glu 60, His 64 and Lys 241 (Fig. 3b, top). The strict conservation of the first three guanines in the p50 subsite is determined by the hydrogen bonds made by His 64 with the N7 group of G_{-5}, and by Arg 56 and Arg 54 with both the N7 and O6 groups of G_{-4} and G_{-3}. This interaction is strengthened by Glu 60, which hydrogen-bonds with these arginines and makes base-specific contacts to the N4 groups of C_{-5} and C_{-4}. Tyr 57 makes van der Waals contacts with C_{-3} and T_{-2} and is itself strongly

Table 1 Data collection and structure refinement statistics

Data collection

Resolution	Total reflections	Unique data	R_{merge}	I/sigma (last shell)	Completeness (last shell)
2.9 Å	88,449	26,430	0.082	15.8 (3.1)	97.2 (88.6)

Refinement

Resolution (Å)	Reflections ($F_o > 2\sigma$)	Atoms protein/DNA	*R* factor (%)* R_{work}	R_{free}	R.m.s.d. Bonds (Å)	Angles (°)
8.0–2.9	19562	4629/486	21.0	32.1	0.014	1.97

* $R_{work} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. $R_{free} = \Sigma_T||F_o| - |F_c||/\Sigma_T|F_o|$, where *T* is a test set containing a randomly selected 5% of the observations omitted from the refinement.