The EMBO Journal vol.15 no.15 pp.4061–4068, 1996

SMC proteins constitute two subunits of the mammalian recombination complex RC-1

Rolf Jessberger[1], Brigitte Riwar, Heidi Baechtold and Alexandre T.Akhmedov

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland

[1]Corresponding author

Recombination protein complex RC-1, purified from calf thymus nuclear extracts, catalyzes cell-free DNA strand transfer and repair of gaps and deletions through DNA recombination. DNA polymerase ε, DNA ligase III and a DNA structure-specific endonuclease co-purify with the five polypeptide complex. Here we describe the identification of two hitherto unknown subunits of RC-1. N-terminal amino acid sequences of the 160 and 130 kDa polypeptides display up to 100% identity to proteins of the structural maintenance of chromosomes (SMC) subfamilies 1 and 2. SMC proteins are involved in mitotic chromosome segregation and condensation, as well as in certain DNA repair pathways in fission (*rad18* gene) and budding (*RHC18* gene) yeast. The assignment was substantiated by immunocross-reactivity of the RC-1 subunits with polyclonal antibodies specific for *Xenopus laevis* SMC proteins. These antibodies, and polyclonal antibodies directed against the bovine 160 and 130 kDa polypeptides, named BSMC1 and BSMC2 (bovine SMC), inhibited RC-1-mediated DNA transfer, indicating that the SMC proteins are necessary components of the reaction. Two independent assays revealed DNA reannealing activity of RC-1, which resides in its BSMC subunits, thereby demonstrating a novel function of these proteins. To our knowledge, this is the first evidence for the association of mammalian SMC proteins with a multiprotein complex harboring, among others, DNA recombination, DNA ligase and DNA polymerase activities.

Keywords: DNA renaturation/recombination/ recombination complex 1/SMC proteins

Introduction

The isolation, identification and characterization of enzymes and other proteins involved in DNA recombination reactions of the mammalian cell is still a considerable challenge in research on DNA metabolism (Gellert, 1992; West, 1992; Kowalczykowski and Eggleston, 1994; Jessberger *et al.*, 1996b).

Several strategies have been devised. They include an evolutionary approach, which uses sequence homologies between prokaryotic and lower eukaryotic, i.e. yeast, DNA repair and recombination genes in the search for corresponding mammalian homologs. This approach allowed cloning of a number of genes from mice to man, including those for human Rad51 (Shinohara *et al.*, 1993; Benson *et al.*, 1994), chicken and mouse Rad52 (Bezzubova *et al.*, 1993) and Rad54 (Bezzubova and Buerstedde, 1994). There remains, however, the need to prove recombination-specific roles *in vivo* and the respective biochemical functions. In another strategy, cell-free reactions are designed to specifically mimic individual steps of recombination reactions or complete multistep reactions. A recent example is the establishment of a cell-free assay for initiation of site-specific V(D)J recombination of the antigen receptor loci (van Gent *et al.*, 1995), and the identification of the RAG-1 and -2 proteins as necessary and sufficient components of this reaction (McBlane *et al.*, 1995).

We have described a different assay, the DNA transfer assay (DTA), in our studies on repair of gaps and deletions through homologous recombination (Jessberger and Berg, 1991). Stable transfer of DNA between two double-stranded, linear or circular DNA molecules, regarded as the essence of DNA recombination, can be measured quantitatively in the assay. The DTA has been used to reproduce, in a cell-free system, the recombination-deficient *in vivo* phenotype of the SCID (severe combined immunodeficiency) mouse mutant, and allowed complementation of the reaction deficiency *in vitro* (Jessberger *et al.*, 1995). In SCID cells, which are defective in DNA-dependent protein kinase activity (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995), DNA transfer activity is reduced, indicating that modulation of the phosphorylation status of recombination activities, or of the respective signaling molecules, is of great importance. The DTA proved useful also in monitoring changes in recombination activity during lymphocyte development (Jessberger *et al.*, 1995) and to study sequence-specific recombination reactions (Jessberger *et al.*, 1996b).

The assay lends itself especially to protein purification experiments and has thus been used to isolate several DNA recombination activities, among them recombination protein complex RC-1 (Jessberger *et al.*, 1993). The analysis of RC-1 revealed a preference for homologous DNA substrates in the recombination reaction and a number of individual enzymatic activities, which co-purify with the complex. A DNA polymerase present in RC-1 was identified by biochemical and immunological methods as DNA polymerase ε (Jessberger *et al.*, 1993, 1996a) and is most likely responsible for repair synthesis of gaps during the recombination reaction. As a second activity, a DNA ligase was observed and identified as DNA ligase III (Jessberger *et al.*, 1993, and unpublished results). The RC-1-associated DNA polymerase and DNA ligase can act in concert to repair a gapped DNA substrate. RC-1's DNA transfer activity can be stimulated by addition of replication protein A (RP-A), but not of proliferating cell nuclear antigen (PCNA; Jessberger *et al.*, 1993). Finally,

an endonuclease which specifically cleaves cruciform-structured DNA substrates has been observed in purified RC-1 preparations (Jessberger *et al.*, 1996a). Analysis of DNA recombination products showed that RC-1 can catalyze recombinational repair of gaps and deletions (Jessberger *et al.*, 1993). Of the initially reported five subunits of RC-1, the 230, 210, 150–160, 130–140 and 40 kDa polypeptides, the largest, 230 kDa polypeptide has not been obtained in all RC-1 preparations and does not seem to be necessary for basic activity. The 210 and 40 kDa subunits were identified as the two polypeptides of DNA polymerase ε. As has been observed in chromatography experiments, DNA ligase III, with its 100 kDa native and 85 kDa active proteolytic fragment polypeptides (for review, see Lindahl and Barnes, 1992), is more loosely associated with the complex (Jessberger *et al.*, 1993). The remaining two subunits, the 150–160 and 130–140 kDa proteins, were until now of unknown identity.

Based on amino acid data and immunoblotting experiments, we report here the identification of the 160 and 130 kDa polypeptides of RC-1. They both belong to the structural maintenance of chromosomes (SMC) protein family, represent the two subfamilies SMC1 and SMC2 and are named bovine SMC1 and bovine SMC2 (BSMC1, BSMC2). While most SMC proteins are known to be involved in chromosome segregation and condensation (for reviews, see Peterson, 1994; Gasser, 1995; Hirano, 1995; Hirano *et al.*, 1995; Saitoh *et al.*, 1995), one member recently identified in yeast has been shown to act in a DNA repair pathway, possibly post-replicative recombinational repair (Lehmann *et al.*, 1995). SMC antibody inhibition experiments indicate that the SMC polypeptides are necessary components of the DNA transfer reaction mediated by RC-1. Furthermore, DNA reannealing activity of RC-1 could be assigned to its BSMC subunits. Collectively, we present molecular evidence for the involvement of mammalian SMC proteins in DNA recombination as components of a multiprotein complex which harbors several other DNA metabolic activities.

Results

Amino acid sequences of the 160 and 130 kDa RC-1 polypeptides

Nuclear extract was prepared from 150 g fetal calf thymus, and RC-1 purified as described in Materials and methods. For N-terminal amino acid sequence determination, 0.15 mg of RC-1 was separated into the individual polypeptides by SDS–polyacrylamide gel electrophoresis, and the polypeptides transferred onto a PVDF membrane. Sequences were obtained by automated Edman degradation cycles in an Applied Biosystem 475 machine.

Twenty one amino acids of the 160 kDa and 20 of the 130 kDa polypeptides were identified unambiguously (Figure 1). N-terminal amino acid sequences from three independent RC-1 preparations were determined. The sequences were compared with the Swiss Protein and EMBL databank sequences using the programs FastA and Blast, which yielded identical information.

The 160 kDa polypeptide sequence displays homologies to subfamily I SMC proteins. The most striking result is a 100% identity over the whole stretch of 21 amino acids to the N-terminus of the human SB1.8 protein (Rocques

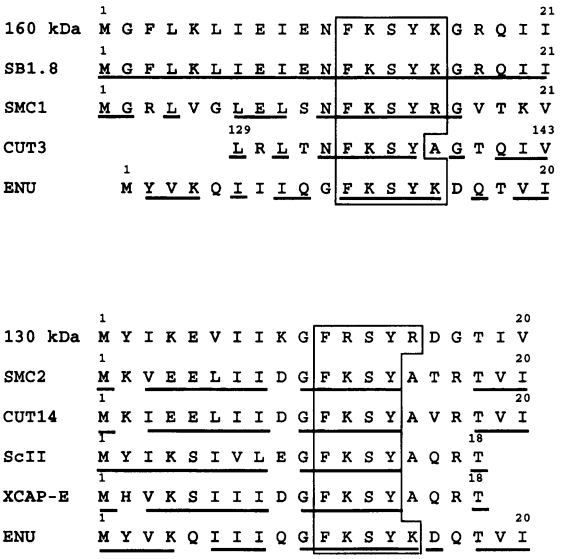

Fig. 1. N-terminal amino acid sequences of the 160 and 130 kDa polypeptides of RC-1. (Top) Sequence of the 160 kDa polypeptide compared with four members of the SMC1 subfamily. (Bottom) Sequence of the 130 kDa polypeptide compared with five members of the SMC2 subfamily. Identical and similar amino acids are underlined. The conserved FKSYK motif is boxed.

et al., 1995), a member of the SMC1 subfamily. Out of the 10 highest score pairs, seven are chromosome condensation or scaffold proteins belonging to the SMC1 family. Beside the SB1.8 protein, they include the *Saccharomyces cerevisiae* SMC1 protein (Strunnikov *et al.*, 1993) (48% homology, 62% identity), which also aligns at the N-terminus, the *Schizosaccharomyces pombe* cut3 protein (Saka *et al.*, 1994), an *Aspergillus nidulans* chromosome scaffold protein (Holt and May, 1996) and three sequences from *Caenorhabditis elegans* (Wilson *et al.*, 1994). One aligning protein is of unknown identity, and two are unrelated (not shown).

Comparing the amino acid sequence of the 130 kDa polypeptide revealed homologies to proteins of the SMC2 subfamily. The six highest score pairs include five members of this family, among them the group-defining *S.cerevisiae* SMC2 protein (Strunnikov *et al.*, 1995) (50% identity, 75% similarity), *Xenopus laevis* XCAP-E (Hirano and Mitchison, 1994), chicken ScII (Saitoh *et al.*, 1994), *S.pombe* Cut14 (Saka *et al.*, 1994) and *A.nidulans* chromosome scaffold protein (Holt and May, 1996). The sixth protein is represented by a hypothetical open reading frame (not shown). The 130 kDa subunit N-terminal sequence fits in every case to the N-termini of the six proteins.

In some preparations, we observed an ~110 kDa polypeptide, the N-terminal sequence of which was also determined and found to be identical to the N-terminal sequence of the 130 kDa subunit (not shown). The 110 kDa polypeptide, therefore, is regarded as a proteolytic degradation product of the 130 kDa polypeptide.

Immunoreactivity with anti-SMC protein antibodies

Further substantiation was sought for the assignment to the SMC protein family of the 130 kDa subunit of RC-1, which does not show the complete amino acid sequence

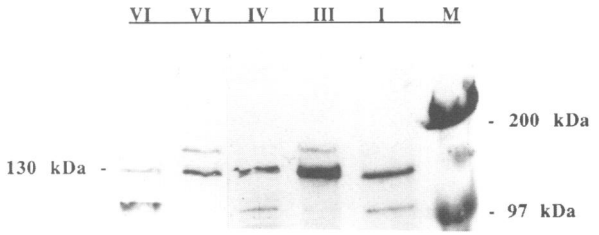

Fig. 2. Immunoblot analysis of RC-1 polypeptides. Anti-XCAP-E polyclonal antibodies (R5-3) were used to probe RC-1 fractions from different levels of purification (Fractions I–VI). See text for details.

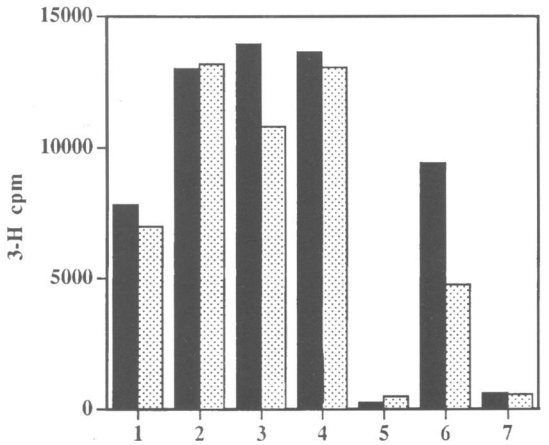

Fig. 3. DNA transfer inhibition assay. Two RC-1 preparations were used (black and stippled columns) in DNA transfer reactions with or without 2 µl of different 1:5 diluted sera. (1) No addition, (2) pre-immune sera, (3) M8-7, (4) M14-7, (5) R3-3, (6) R5-3, (7) RJ322.

identity with SMC proteins as does the 160 kDa polypeptide. Immunoblotting experiments were performed using polyclonal antibodies directed against *X.laevis* SMC protein XCAP-E (Hirano and Mitchison, 1994) (Figure 2). The antibodies react with a protein of the correct size in crude nuclear calf thymus extract (Fraction I), and recognize the 130 kDa polypeptide in the more purified RC-1 fractions (III and IV), up to the most purified one (VI). A weak cross-reactivity, probably due to the limited amino acid sequence homologies known to exist between the two SMC proteins (Hirano *et al.*, 1995), is detectable with the 160 kDa subunit. Also, the 110 kDa proteolytic fragment of the 130 kDa polypeptide (see above), present in some RC-1 preparations, is recognized by the antibodies.

Inhibition of cell-free DNA transfer

The question remained as to whether the SMC components of RC-1 are necessary for the DNA transfer reaction. Therefore, we undertook inhibition experiments aimed at functional blocking of SMC proteins in the DTA (Figure 3). We have used a panel of four different polyclonal anti-*X.laevis* SMC protein antibodies (anti-XCAP-C: M8-7, R3-3; anti-XCAP-E: M14-7, R5-3; Hirano and Mitchison, 1994), the antisera raised against RC-1 160 and 130 kDa proteins (RJ322; see Materials and methods; Figures 4 and 8) and, for control, a pre-immune sera. The amounts of sera added to the reaction were titrated to yield an optimal effect (not shown). Four different RC-1 preparations, Fraction VI or VII, were used, two of which are shown in Figure 3 (represented by black and stippled columns).

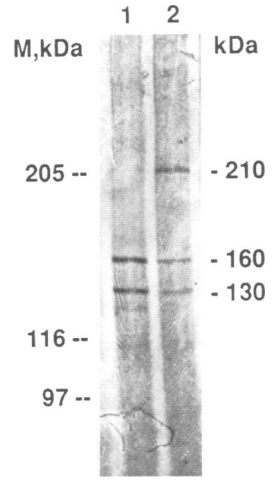

Fig. 4. RC-1-derived BSMC (3 ng; #1) and RC-1 (5 ng; #2) preparations. Proteins were separated in a 5% polyacrylamide–SDS gel and stained with silver. M = size marker positions.

Three sera and the pre-immune sera did not yield any inhibition, some even stimulated the reaction ~1.6-fold. This stimulation may be caused by non-specific protein–protein stabilization effects on the diluted RC-1 proteins. Similar effects were observed upon addition of other proteins like bovine serum albumin or ovalbumin to the DTA reaction. Two of the sera, R3-3 (column #5) and RJ322 (#7), drastically inhibited the reaction. For R3-3, the average activity is 5.1% of the untreated (#1) and 2.8% of the pre-immune (#2) control. For RJ322, the activity is 8.1% of the untreated and 4.6% of the pre-immune control. Compared with the pre-immune control, there is a weak effect of antibody R5-3 (#6) as well (54%), which is not apparent if compared with the untreated reaction. Thus, two of the six sera clearly inhibited the DNA transfer reaction.

DNA renaturation and ATPase activities

SMC proteins may exist as elongated heterodimers, possibly forming filamentous structures with chromosomes (Hirano and Mitchison, 1994). This, and the role which SMC proteins play in maintaining specific mitotic chromosome structures, being involved in segregation and condensation of chromosomes, suggests that they might support pairing or reannealing of homologous DNA strands. Such activities are known from almost all recombination proteins investigated in this respect (for review, see Kowalczykowski and Eggleston, 1994).

Renaturation of complementary DNA single strands can be measured by acquisition of resistance to single strand-specific nucleases like S1 through formation of a DNA duplex (Weinstock *et al.*, 1979). The use of [^{3}H]DNA strands allows the measurement of non-degraded, acid-precipitable material. Three different amounts (7.5–30 ng each) of RC-1 were tested alongside three different amounts of the purified BSMC heterodimer in this S1 nuclease protection assay (Figure 5). The purification of the two BSMC polypeptides was as described in Materials and methods (see Western blotting and Figure 4).

Both preparations display an activity that at least matches that of recA protein (lane 1, 200 ng). Assuming a 1:1 ratio of the RC-1 subunits (Jessberger *et al.*, 1993),

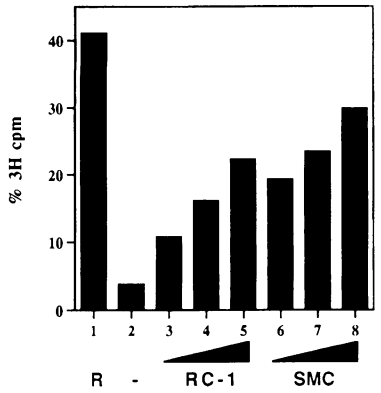

Fig. 5. S1 nuclease protection assay for DNA renaturation. Preparations of RC-1 (#3–5; 7.5, 15 and 30 ng) and its isolated SMC subunits (#6–8; 7.5, 15 and 30 ng) were assayed and compared with no protein (#2) or RecA protein (#1; 200 ng) controls. The activity is expressed as percent S1 nuclease-resistant, acid-precipitable ^{3}H-labeled material. 100% = 22 000 c.p.m.

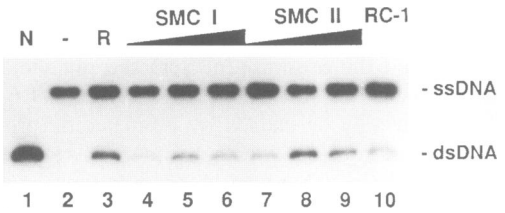

Fig. 6. DNA reannealing gel assay. Two preparations of RC-1-derived SMC protein (SMC I, 3, 6 and 12 ng; SMC II, 1.5, 3 and 6 ng) and RC-1 (6 ng) were assayed as described in Materials and methods for promotion of reannealing of two complementary DNA single strands. R, RecA protein (100 ng); –, no protein, denatured DNA; N, native, undenatured DNA. Substrates (ssDNA) and products (dsDNA) are indicated.

the relative amounts of BSMC in the reaction are about twice as large in reactions 6–8 compared with 3–5. Thus, the activity of the BSMC preparation is roughly the same as that of RC-1, indicating, that the BSMC subunits of RC-1 are responsible for the reannealing activity of the complex. With an input of 1.3 fmol (nucleotides) of DNA substrate (two single strands) and ~0.1 pmol of BSMC heterodimer, ~30% of the substrate DNA is protected from S1 nuclease digestion through renaturation. About 100 BSMC heterodimer molecules per two single-stranded DNA substrate molecules are needed for this conversion, equalling one BSMC heterodimer per 115 nucleotides of one substrate strand.

The reannealing activity of RC-1 was confirmed by a second independent assay which visualizes reannealing of complementary single-stranded DNA by gel electrophoresis of the products (Figure 6). RC-1 (lane 10) or two preparations of its two BSMC subunits (I and II, lanes 4–6 and 7–9) were incubated with the single-stranded substrate alongside the controls without protein (lane 2) and with RecA protein (lane 3). Both RC-1 and BSMC proteins promoted reannealing of the substrate, with increasing amounts of BSMC protein (3, 6 and 12 ng and 1.5, 3 and 6 ng respectively) generating more product. With the medium amounts (lanes 5 and 8), a plateau is reached under our reaction conditions. Product formation seems even to decrease with an increase in added protein, an effect already known from RecA protein (Bryant and Lehman, 1985). The signal generated with 6 ng of BSMC

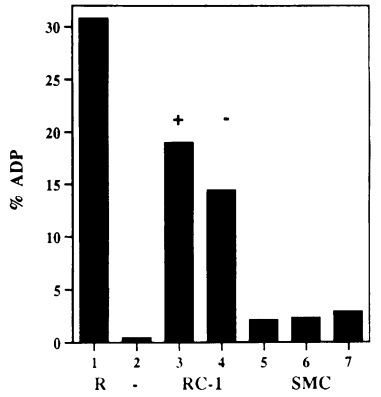

Fig. 7. ATPase assay. RC-1 (#3 and 4; 15 ng; with or without added DNA) and RC-1-derived SMC (#5–7; 15, 21 and 30 ng) preparations were assayed. No protein (#2) and RecA protein (#1; 120 ng) are controls. The activity is expressed as percent conversion of ATP to ADP. 100% = 22 000 c.p.m.; 73 pmol [^{3}H]ATP per reaction.

proteins is somewhat stronger than that obtained with 6 ng of RC-1, in accordance with the higher relative amounts of BSMC in these reactions (see above). No signal can be detected in the negative control (lane 2); the native, double-stranded DNA yields no single-stranded substrate signal (lane 1) and RecA protein efficiently promotes the reaction (lane 3). It should be noted that, in contrast to RecA, the BSMC-mediated renaturation reaction does not depend on the presence of ATP (not shown).

SMC proteins share two characteristic motifs, the P-loop motif and the DA box, which are possibly involved in ATP binding (Gasser, 1995; Hirano, 1995; Hirano *et al.*, 1995; Saitoh, 1995). It is assumed, that binding and hydrolysis of nucleotide triphosphates yield the energy required for dynamic changes of chromosomes, which are mediated by the SMC proteins. Finding two SMC proteins as components of RC-1, we asked whether there is an ATPase activity detectable in the complex (Figure 7). Incubation of [^{3}H]ATP with 15 ng of RC-1 in the presence of DNA under standard recombination buffer conditions revealed an ATPase activity (#3 19.1% ADP) ~30 times above background (#2; 0.6% ADP) and roughly comparable with *Escherichia coli* RecA protein (#1; 31% ADP, 120 ng). Omission of DNA from the reaction mixture renders RecA inactive (not shown) and RC-1 about a quarter less active (#4, 14.2% ADP). Purified BSMC proteins incubated with DNA, however, displayed a low activity of 1.9–2.8% ADP with 15–30 ng of protein per reaction (#5–7). Omission of DNA gave nearly identical results (not shown). This activity is not regarded as significant (see Discussion).

Discussion

After the first paper on budding yeast SMC1 appeared (Strunnikov *et al.*, 1993), a family of proteins involved in chromosome dynamics, the SMC family, was defined in 1994 (Gasser, 1995; Hirano, 1995; Hirano *et al.*, 1995; Saitoh *et al.*, 1995). These proteins are required for chromosome condensation and segregation during mitosis. One of the first proteins of this family to be isolated was the chicken ScII protein, which is a major component of the chromosome scaffold, co-localizing with topoisomerase II

(Saitoh *et al.*, 1994). At about the same time, Hirano and Mitchison (1994) described two proteins from *X.laevis* which co-sediment with *in vitro* assembled mitotic chromosomes. The two proteins, XCAP-C and -E, are essential for assembly and structural maintenance of mitotic chromosomes in this system (Hirano and Mitchison, 1994). Similar SMC proteins were reported from *S.pombe* (Saka *et al.*, 1994) and *C.elegans* (Chuang *et al.*, 1994). Coiled-coil domains in a head–rod–tail structure, together with DA box and P-loop motifs, are distinct features of SMC proteins.

Sequence homologies of SMC proteins to several known prokaryotic and lower eukaryotic repair and recombination enzymes suggested that certain members of the SMC family might be involved in these processes as well. Structural similarities and amino acid sequence motif homologies are shared with the *E.coli* RecN, RecF, SbcC, UvrA proteins and the *S.cerevisiae* Rad50 protein (Hirano *et al.*, 1995; Saitoh *et al.*, 1995). As the first evidence supporting this hypothesis, the *S.pombe rad18* gene, was reported recently by Lehmann *et al.* (1995) to belong to the SMC family. Like most *rad* mutants, the *rad18* mutant of *S.pombe* is highly sensitive to UV and γ irradiation (Nasim and Smith, 1975), indicating its involvement in a repair pathway, which might use recombinational repair mechanisms. Sequence data revealed that the *rad18* gene product is structurally related to the SMC family, with an ATP binding site, coiled-coil domains and nuclear localization signals, and might define a new SMC subfamily. A homolog from *S.cerevisiae*, *RHC18*, indicates evolutionary conservation of the gene (Lehmann *et al.*, 1995).

Our assignment of the 160 and 130 kDa polypeptides of RC-1 to the SMC1 and SMC2 families is based on their extensive N-terminal amino acid sequence homologies, and on immuno-cross-reactivity and inhibition with antibodies specific for a known SMC protein.

The 160 kDa polypeptide N-terminal sequence shows 100% identity to the N-terminal amino acid sequence of the human SB1.8 protein (Rocques *et al.*, 1995). The probability for such a match is 2.1×10^{-8}. There is also high identity (48%) and similarity (62%) to the N-terminal sequence of the *S.cerevisiae* SMC1 protein, which defines the SMC1 subfamily. In addition, the RC-1-derived polypeptide sequence shows significant homologies to two other members of the SMC1 family (Figure 1). From this, we conclude that the 160 kDa subunit of RC-1, named BSMC1, is a member of the SMC1 subfamily and possibly a bovine homolog of the human SB1.8 protein.

The human *SB1.8* gene was originally described as a genetic marker for the X chromosome region Xp11.1–p11.2, localizing to the vicinity of a specific chromosomal translocation found in synovial sarcomas. The protein was found to be homologous, with 30% overall identity, and to be very similar structurally with respect to motifs to the *S.cerevisiae* SMC1 protein (Rocques *et al.*, 1995). The *SB1.8* cDNA, however, was not sufficient to complement the *S.cerevisiae* SMC1 mutant. The SB1.8 protein does not share homologies to kinesin or other mechanochemical domains found in motor proteins. Therefore, Rocques *et al.* suggest a closer relationship of SB1.8 to a group of recombination and repair enzymes like the *S.cerevisiae* RAD50 protein. So far, however, no molecular function has been confirmed for the SB1.8 protein, which remained the only mammalian SMC protein reported so far.

Similarly, the second subunit of RC-1 examined in this study displayed high N-terminal homology to the *S.cerevisiae* SMC2 protein (50% identity, 75% similarity) and the *S.pombe* Cut14, chicken ScII and *X.laevis* XCAP-E proteins, which are all members of the SMC2 group (45–50% identity).

Our assignment of the 130 kDa RC-1 polypeptide as BSMC2 was supported further by immunoblotting experiments, where antibodies specific for the *X.laevis* XCAP-E protein recognized the polypeptide (Figure 2).

Furthermore, the sizes of the two RC-1 polypeptides fit to sizes reported for the corresponding SMC proteins. Gel electrophoresis of RC-1 in analytical polyacrylamide gels and staining with silver showed an ~1:1 stoichiometry of the two polypeptides to each other and to the two subunits of DNA polymerase ε (Figure 4 and Jessberger *et al.*, 1993). Not only do SMC1 and SMC2 proteins form 1:1 heterodimers (Hirano and Mitchison, 1994), but it was also observed, that these dimers sometimes are contained in higher order complexes (T.Hirano, personal communication).

Antibody inhibition studies, using antibodies either specific for known *X.laevis* SMC proteins or polyclonal antibodies raised against the two RC-1 polypeptides, again supported the assignment. With two of five different sera, a significant inhibition (>90%) of the DNA transfer reaction was achieved (Figure 3). These experiments also indicate that the SMC proteins, or at least one of them, are necessary for the RC-1-mediated cell-free DNA transfer reaction.

As a novel activity of SMC proteins, we demonstrate promotion of DNA reannealing by RC-1-derived BSMC proteins (Figures 5 and 6). DNA renaturation or reannealing are considered important steps early in homologous recombination reactions, where duplex-invading single strands are to pair with the complementary strands of the recombination partner. Two independent assays showed reannealing activities of RC-1 and its isolated BSMC subunits which, under our reaction conditions, are roughly comparable with, if not higher than, that of *E.coli* RecA protein. There is a long list of strand exchange and homologous pairing proteins from prokaryotic and eukaryotic organisms which also possess such an activity. The ATP-dependent proteins besides RecA include UvsX (phage T4) and Rec1 (*Ustilago maydis*), and the ATP-independent proteins are RecT (*E.coli*), protein β (phage λ), STPα (*S.cervevisiae*), Rrp1 (*Drosophila melanogaster*) and others (for review, see Kowalczykowski and Eggleston, 1994). Several other proteins, not involved in DNA recombination reactions, also stimulate DNA reannealing in the presence or absence of certain macromolecular crowding agents, which in some cases by themselves support renaturation as well. It should be noted that the reaction conditions used to test RC-1 and BSMC reannealing activities did not include any such agents or high salt, and thus are stringent. Also, two DNA substrates of different length were used: 5730 nt for the S1 protection assay and 422 nt for the gel analysis, both with high efficiencies. The amounts needed to obtain ~30% reannealing relate to a stoichiometry of approximately one BSMC heterodimer per stretch of 115 nucleotides of one single-

Table I. Summary of DNA metabolic activities tested for RC-1

Activity	Presence
DNA ligase	yes
DNA helicase	no
DNA polymerase	yes
dsDNA 5′-3′ exonuclease	weak[a]
dsDNA 3′-5′ exonuclease	weak[b]
dsDNA nicking activity	weak[b]
DNA transfer (4-strand reaction)	yes
ssDNA nuclease	weak[a]
Terminal transferase	no
Topoisomerase I	no
Topoisomerase II	no
Cruciform DNA structure-specific endonuclease	yes
ATPase	yes[c]
DNA renaturation	yes
RNA polymerase	no

[a]Almost undetectable.
[b]Probably a function of DNA polymerase ε.
[c]Partially DNA dependent, possibly two separate activities.

stranded substrate, which is lower than that of RecA, reported to be one monomer per 30 nucleotides (Weinstock *et al.*, 1979; Bryant and Lehman, 1985). The renaturation activity of RC-1 and BSMC proteins was not affected by omission of ATP in the reaction, while RecA is stimulated several fold by ATP (Bryant and Lehman, 1985). The double-stranded monomeric DNA remained the only product observed in gel analysis. No high molecular weight aggregated structures have been observed.

Although SMC proteins possess a P-loop nucleotide binding site and a DA box, which is frequently found in ATP hydrolyzing proteins (Gasser, 1995; Hirano, 1995; Hirano *et al.*, 1995; Saitoh *et al.*, 1995), an ATP hydrolyzing activity of SMC proteins has not yet been described. Earlier (Jessberger *et al.*, 1993), the RC-1 reaction was found to depend on the presence of ATP. Experiments presented here (Figure 7) revealed ATP hydrolysis to ADP by RC-1. The activity was found in at least six different, highly purified RC-1 preparations (not shown), is only partially dependent on DNA and may possibly represent two different ATPases, one of which is DNA dependent. One of the most frequently observed ATPase activities is that of DNA helicases, but no DNA helicase activity has been detected in RC-1 (Jessberger *et al.*, 1993; compare Table I). RC-1s ATPase activity, however, cannot be attributed to its BSMC components. The weak activity observed in BSMC preparations is not regarded as significant, but rather may be an unrelated contamination, for which this assay, in contrast to the renaturation assays, is very sensitive, since it requires catalytic amounts of protein only.

The precise function which the BSMC heterodimer might have in its association with RC-1 has to be elucidated if individual components become available. Finding a DNA reannealing activity suggests, however, that the BSMC subunits might be involved in the initial steps of the RC-1-mediated DNA transfer reaction. An SMC protein recently has been shown in *S.pombe* and *S.cerevisae* to be involved in an unusual repair pathway, which probably uses post-replicative recombinational mechanisms (Lehmann *et al.*, 1995). The BSMC subunits in concert with other enzymatic activities residing in RC-1 might act in a similar pathway in mammalian cells. The identification of recombination- and repair-related SMC proteins as components of RC-1 further supports our hypothesis of this protein complex being a biologically relevant DNA metabolic activity in higher eukaryotes. A summary of the individual enzymatic activities tested so far for RC-1 is presented in Table I.

Finally, the identification of two hitherto unknown subunits of RC-1 and the availability of the DNA polymerase and DNA ligase are important assets for reconstitution of the reaction from individual, cloned components. Future studies should then be possible to determine the mode of interaction of the SMC subunits with these other enzymes.

Materials and methods

Preparation of RC-1

Preparation of nuclear extracts from fetal calf thymus and purification of RC-1 were described in detail earlier (Jessberger *et al.*, 1993). From 1000 mg of nuclear extract protein, ~0.2 mg of RC-1 Fraction VII was obtained. In analytical SDS–PAGE and silver staining of the gel, Fraction VII displayed five prominent polypeptides of ~230, ~210, ~160, ~130 and 40–45 kDa. The intensities of two additional bands of 100 and 85 kDa, representing DNA ligase III (Jessberger *et al.*, 1993, and unpublished results), varied between preparations and was generally weak. The 230 kDa polypeptide was absent from some preparations without a significant effect on the overall DNA transfer activity of the complex. The 210 and 45 kDa polypeptides were identified as the subunits of DNA polymerase ε (Jessberger *et al.*, 1993, 1996a).

N-terminal amino acid sequencing and computer analysis

About 0.15 mg of RC-1, Fraction VII, was loaded on a preparative SDS–polyacrylamide gel, the polypeptides were separated from each other and transferred (transfer buffer 10 mM CAPS pH 11.0, 15% MeOH) onto a PVDF membrane. Proteins were stained with Coomassie blue G 250, destained, and individual bands excised. The proteins bound to the membrane pieces were then subjected to consecutive Edman degradation cycles in an Applied Biosystem 475 Protein Sequencer apparatus. Sequences were read by the computer and controlled manually.

N-terminal sequences of the 160 kDa (21 amino acids) and the 130 kDa (20 amino acids) polypeptides were compared with the EMBL and SwissProt databanks using the computer programs FastA and Blast.

Western blotting

Polyclonal antibodies directed against the *X.laevis* SMC proteins XCAP-C and -E were a gift from Dr T.Hirano, Cold Spring Harbor Laboratories (Hirano and Mitchison, 1994). Polyclonal antibodies against the bovine RC-1-derived BSMC1 and BSMC2 polypeptides were raised by injecting 1×30 μg and 3×20 μg of purified polypeptides into one rabbit on a weekly schedule. Blood was taken 1 month after the first immunization. The 160 and 130 kDa polypeptides were obtained by treatment of RC-1, Fraction V, with 0.5% Triton X-100, followed by gel filtration chromatography (Superdex 200, FPLC, Pharmacia, Sweden). This procedure disassembles and inactivates the DNA transfer activity of RC-1, and yields the 160 and 130 kDa polypeptides together in one fraction, presumably in their heterodimeric form (Figure 4). The sera, named RJ322, were found to react in Western blot experiments specifically with the two BSMC polypeptides of purified RC-1 preparations (Figure 8). Notably, they do not cross-react with the RC-1 polymerase or ligase polypeptides. In crude nuclear calf thymus extract preparations, a number of additional proteins are recognized by the antibodies (Figure 8)

For Western blot experiments, protein samples were resolved by 6% SDS–PAGE, and transfered onto a nitrocellulose membrane (Hybond N, Amersham Inc.) in a transfer buffer containing 25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol. The membrane was then blocked with 5% non-fat milk in phosphate-buffered saline (PBS)-Tween (50 mM Na phosphate pH 7.4, 150 mM NaCl, 0.1% Tween 20; 16 h at 4°C). The membrane was washed with PBS-Tween. The sera were diluted 1:500 in PBS-Tween and applied to the membrane for a minimum of 12 h at 4°C. Horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit Igs at a 1:5000 dilution were used as the secondary antibody.

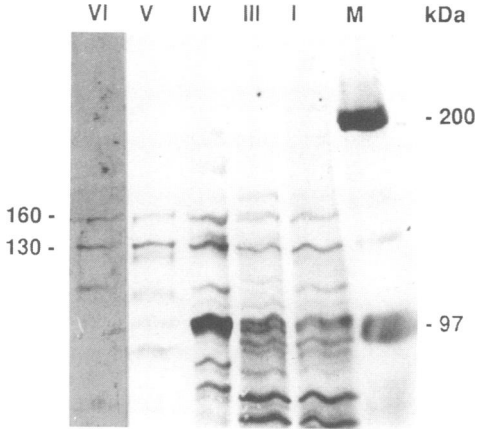

Fig. 8. Recognition of BSMC polypeptides of RC-1 by newly generated antisera. Crude nuclear calf thymus extract (20 μg protein, Fraction I) and consecutive protein fractions derived from RC-1 purification (Fractions III–VI) were loaded. The left lane (Fraction VI) is shown as a longer exposure (30 versus 10 s) of the same filter. The position of the 160 and 130 kDa polypeptides is indicated.

Visualization was achieved with the ECL system (Amersham Inc.), and a 5 s to 5 min exposure time.

DNA transfer assay and inhibition experiments

The DTA has been described extensively earlier (Jessberger and Berg, 1991; Jessberger *et al.*, 1993, 1995, 1996a) and is only briefly summarized here. The DNA substrates used in this study are double-stranded plasmid DNAs, a deletion-bearing recipient (pSV2neoΔ70) and a full-length, homologous donor plasmid (3H-pSV2neo), in their circular form. This four-strand DNA recombination assay monitors transfer of radioactively labeled donor DNA into recipient DNA. Reaction mixtures typically include 5 ng of RC-1 incubated with 0.1 μg of each of the two DNA substrates in a buffer containing 30 mM EPPS pH 7.4, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM ATP and 0.1 mM of the four dNTPs, for 20 min at 37°C. Input ^3H radioactivity is betwen 100 000 and 200 000 c.p.m. and is the same for each experimental series. The products go through a rigid extraction procedure including EDTA–SDS treatment and phenol–chloroform extraction and are stable in that the majority are heat resistant (20% reduction after 85°C for 20 min) and do not depend on the continuous presence of proteins or bivalent metal ions. The activity is expressed as input donor ^3H radioactivity transferred to the recipient (c.p.m.). Among the products are those expected for homologous recombination reactions: crossover and non-crossover products (Jessberger and Berg, 1991; Jessberger *et al.*, 1993).

For antibody inhibition experiments, equal volumes of sera, either before (pre-immune) or after immunization, in various dilutions made in pre-immune sera were added to a mixture of RC-1 in reaction buffer. The concentration of DTT was lowered in all reactions of these experiments to 0.1 mM. The mixture was incubated on ice for 15 min to allow binding of the antibodies, and the transfer reaction was started by addition of the two DNA substrates and incubation at 37°C. The reaction was then carried through as described above.

ATPase assay

This assay was performed as described by Weinstock *et al.* (1979). Reaction mixtures (30 μl) contained 20 mM Tris–HCl (pH 7.5), 5 (for RC-1) or 10 (for RecA) mM $MgCl_2$, 1 mM DTT, 2.4 μM [^3H]ATP (41 Ci/mmol; Amersham), 30 μM single-stranded φX174 DNA (as nucleotide) and protein fractions. After incubation at 37°C for 60 min, reactions were quenched by addition of EDTA to 25 mM. Aliquots (1 μl) were spotted onto PEI–cellulose plates, overspotted with ATP, ADP and AMP markers and developed in 1 M formic acid, 0.5 M LiCl. The spots containing ATP and ADP were identified with a UV lamp, excised, and the radioactivity determined by scintillation counting.

DNA renaturation assays

DNA renaturation was monitored by the S1 nuclease protection assay (Weinstock *et al.*, 1979). pSV2neo [^3H]DNA, uniformly labeled to a specific activity of 2.5×10^5 c.p.m./nmol total nucleotide, was digested with *Hin*dIII and heat denatured. Reaction mixtures (40 μl) contained 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 2.1 μM heat-denatured pSV2neo [^3H]DNA (as nucleotide) and protein fractions. After an incubation at 37°C for 30 min, reactions were stopped by the addition of 4 μl of 10% SDS. The mixtures were then diluted into 550 μl of S1 digestion buffer containing 150 mM NaCl, 50 mM sodium acetate (pH 4.6), 1 mM $ZnCl_2$; then 1 μl of heat-denatured calf thymus DNA (5 mg/ml) and 70 U of S1 nuclease (Boehringer-Mannheim) were added. Digestions were performed at 37°C for 30 min, and the reactions were terminated by the addition of 4 μl of calf thymus DNA (5 mg/ml) and 600 μl of 10% trichloroacetic acid. Acid-precipitable radioactivity was measured by liquid scintillation counting.

Complementary DNA strands used in the second DNA renaturation assay were obtained by heat denaturation of a ^{32}P-end-labeled dsDNA fragment, 422 bp in length, produced by *Ava*I restriction of M13mp18 RFI DNA. Reaction mixtures (20 μl) contained 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.13 μM (nucleotides) heat-denatured 422 bp [^{32}P]DNA fragment and various amounts of protein. After incubation at 37°C for 30 min, reactions were stopped by addition of 0.1 vol of 10% SDS and 100 mM EDTA and analysed by SDS–PAGE (10% polyacrylamide) and autoradiography.

Acknowledgements

The authors thank Drs T.Hirano, Cold Spring Harbor Laboratories and T.Mitchison, University of California, San Francisco, for the generous gift of anti-*Xenopus laevis* SMC antibodies, and for helpful discussions. We thank Drs K.Karjalainen and H.R.Rodewald, Basel Institute for Immunology, for critical reading of the manuscript, and D.Avila for amino acid sequencing. The Basel Institute for Immunology is founded and supported by Hoffmann-LaRoche Ltd, Basel, Switzerland.

References

Benson,F.E., Stasiak,A. and West,S.C. (1994) Purification and characterization of the human Rad51 protein, an analogue of *E.coli* RecA. *EMBO J.*, **13**, 5764–5771.

Bezzubova,O.Y. and Buerstedde,J.-M. (1994) Gene conversion in the chicken immunoglobulin locus: a paradigm of homologous recombination in higher eukaryotes. *Experientia*, **50**, 270–276.

Bezzubova,O., Schmidt,H., Ostermann,K., Heyer,W.D. and Buerstedde, J.-M. (1993) Identification of a chicken RAD52 homologue suggests conservation of the RAD52 recombination pathway throughout the evolution of higher eukaryotes. *Nucleic Acids Res.*, **21**, 5945–5949.

Blunt,T., Finnie,N.J., Taccioli,G.E., Smith,G.C.M., Demengeot,J., Gottlieb,T.M., Mizuta,R., Varghese,A.J., Alt,F.W., Jeggo,P.A. and Jackson,S.P. (1995) Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell*, **80**, 813–823.

Bryant,F.R. and Lehman,I.R. (1985) On the mechanism of renaturation of complementary DNA strands by the recA protein of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **82**, 297–301.

Chuang,P.T., Albertson,D.G. and Meyer,B.J. (1994) DPY-27: a chromosome condensation protein homolog that regulates *C.elegans* dosage compensation through association with the X chromosome. *Cell*, **79**, 459–474.

Gasser,S.M. (1995) Coiling up chromosomes. *Curr. Biol.*, **5**, 357–360.

Gellert,M. (1992) Molecular analysis of V(D)J recombination. *Annu. Rev. Genet.*, **22**, 425–446.

Hirano,T. (1995) Biochemical and genetic dissection of mitotic chromosome condensation. *Trends Biochem. Sci.*, **20**, 357–361.

Hirano,T. and Mitchison,T.J. (1994) A heterodimeric coiled-coil protein required for mitotic chromosome condensation *in vitro*. *Cell*, **79**, 449–458.

Hirano,T., Mitchison,T.J. and Swedlow,J.R. (1995) The SMC family: from chromosome condensation to dosage compensation. *Curr. Biol.*, **7**, 329–336.

Holt,C.L. and May,G.S. (1996) An extragenic suppressor of the mitosis-defective *bimD6* mutation of *Aspergillus nidulans* codes for a chromosome scaffold protein. *Genetics*, in press.

Jessberger,R. and Berg,P. (1991) Repair of deletions and double-strand gaps by homologous recombination in a mammalian *in vitro* system. *Mol. Cell. Biol.*, **11**, 445–457.

Jessberger,R., Podust,V., Hübscher,U. and Berg,P. (1993) A mammalian protein complex that repairs double-strand breaks and deletions by recombination. *J. Biol. Chem.*, **268**, 15070–15079.

Jessberger,R., Riwar,B., Rolink,A. and Rodewald,H.R. (1995) Stimulation of defective DNA transfer activity in recombination deficient SCID cell extracts by a 72-kDa protein from wild-type thymocytes. *J. Biol. Chem.*, **270**, 6788–6797.

Jessberger,R., Chui,G., Linn,S. and Kemper,B. (1996a) Analysis of the mammalian recombination protein complex RC-1. *Mutat. Res.*, **350**, 217–227.

Jessberger,R., Wabl,M. and Borggrefe,T. (1996b) Biochemical studies of class switch recombination. *Curr. Top. Microbiol. Immunol.*, **217**, in press.

Kirchgessner,C.U., Patil,C.K., Evans,J.W., Carter,T. and Brown,J.M. (1995) DNA dependent kinase (p350) is a candidate gene for murine scid defect. *Science*, **267**, 1178–1182.

Kowalczykowski,S.C. and Eggleston,A.K. (1994) Homologous pairing and DNA strand-exchange proteins. *Annu. Rev. Biochem.*, **63**, 991–1044.

Lehmann,A.R., Walicka,M., Griffiths,D.J.F., Murray,J.M., Watts,F.Z., McCready,S. and Carr,A.M. (1995) The *rad18* gene of *Schizosaccharomyces pombe* defines a new subgroup of the SMC superfamily involved in DNA repair. *Mol. Cell. Biol.*, **15**, 7067–7080.

Lindahl,T. and Barnes,D.E. (1992) Mammalian DNA ligases. *Annu. Rev. Biochem.*, **61**, 251–281.

McBlane,J.F., van Gent,D.C., Ramsden,D.A., Romeo,C., Cuomo,C.A., Gellert,M. and Oettinger,M.A. (1995) Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*, **83**, 387–395.

Nasim,A. and Smith,B.P. (1975) Genetic control of radiation sensitivity in *Schizosaccharomyces pombe*. *Genetics*, **79**, 573–582.

Peterson,C.L. (1994) The SMC family: novel motor proteins for chromosome condensation? *Cell*, **79**, 389–392.

Rocques,P.J., Clark,J., Ball,S., Crew,J., Gill,S., Christodoulo,Z., Borts,R., Louis,E.J., Davies,K.E. and Cooper,C.S. (1995) The human SB1.8 gene (DXS423E) encodes a putative chromosome segregation protein conserved in lower eukaryotes and prokaryotes. *Hum. Mol. Genet.*, **4**, 243–249.

Saka,Y., Sutani,T., Yamashita,Y., Saitoh,S., Takeuchi,M., Nakaseko,Y. and Yanagida,M. (1994) Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. *EMBO J.*, **13**, 4938–4952.

Saitoh,N., Goldberg,I.G., Wood,E.R. and Earnshaw,W.C. (1994) ScII: an abundant chromosome scaffold protein is a member of a family of putative ATPases with an unusual predicted tertiary structure. *J. Cell Biol.*, **127**, 303–318.

Saitoh,N., Goldberg,I.G. and Earnshaw,W.C. (1995) The SMC proteins and the coming of age of the chromosome scaffold hypothesis. *BioEssays*, **17**, 759–766.

Shinohara,A.H., Ogawa,H., Matsuda,Y., Ushio,N., Ikeo,K. and Ogawa,T. (1993) Cloning of human, mouse and fission yeast recombination genes homologues to *RAD51*. *Nature Genet.*, **4**, 239–243.

Strunnikov,A.V., Larionov,V.L. and Koshland,D. (1993) SMC1: an essential yeast gene encoding a putative head–rod–tail protein is required for nuclear division and defines a new ubiquitous protein family. *J. Cell Biol.*, **123**, 1635–1648.

Strunnikov,A.V., Hogan,E. and Koshland,D. (1995) SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family. *Genes Dev.*, **9**, 587–599.

van Gent,D.C., McBlane,J.F., Ramsden,D.A., Sadofsky,M.J., Hesse,J.E. and Gellert,M. (1995) Initiation of V(D)J recombination in a cell-free system. *Cell*, **81**, 1–20.

Weinstock,G.M., McEntee,K. and Lehman,I.R. (1979) ATP catalyzed renaturation of DNA catalyzed by the recA protein of *E.coli*. *Proc. Natl Acad. Sci. USA*, **76**, 126–130.

West,S.C. (1992) Enzymes and molecular mechanism of genetic recombination. *Annu. Rev. Biochem.*, **61**, 603–640.

Wilson,R. *et al.* (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C.elegans*. *Nature*, **368**, 32–38.

Received on February 5, 1996; revised on April 23, 1996