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Functional interaction of reverse gyrase with single-strand binding protein of the archaeon *Sulfolobus*

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ABSTRACT

Reverse gyrase is a unique hyperthermophile-specific DNA topoisomerase that induces positive supercoiling. It is a modular enzyme composed of a topoisomerase IA and a helicase domain, which cooperate in the ATP-dependent positive supercoiling reaction. Although its physiological function has not been determined, it can be hypothesized that, like the topoisomerase–helicase complexes found in every organism, reverse gyrase might participate in different DNA transactions mediated by multiprotein complexes. Here, we show that reverse gyrase activity is stimulated by the single-strand binding protein (SSB) from the archaeon *Sulfolobus solfataricus*. Using a combination of *in vitro* assays we analysed each step of the complex reverse gyrase reaction. SSB stimulates all the steps of the reaction: binding to DNA, DNA cleavage, strand passage and ligation. By co-immunoprecipitation of cell extracts we show that reverse gyrase and SSB assemble a complex in the presence of DNA, but do not make stable protein–protein interactions. In addition, SSB stimulates reverse gyrase positive supercoiling activity on DNA templates associated with the chromatin protein Sul7d. Furthermore, SSB enhances binding and cleavage of UV-irradiated substrates by reverse gyrase. The results shown here suggest that these functional interactions may have biological relevance and that the interplay of different DNA binding proteins might modulate reverse gyrase activity in DNA metabolic pathways.

INTRODUCTION

The generation of correct DNA topology and its regulation throughout the cell cycle is a complex process, not completely understood, that has implication in all DNA activities (replication, chromosome segregation, transcription, recombination and repair). All cells contain diverse topoisomerases, which co-operate with a variety of proteins and multiprotein complexes to maintain the homeostatic balance of DNA topology (1,2).

Reverse gyrase is a peculiar topoisomerase that positively supercoils DNA [reviewed in (3,4)]. The gene coding for this enzyme is the only one found in all and also only in hyperthermophilic organisms, *Bacteria* and *Archaea* living above 80°C (5). The DNA of these organisms is, in general, more positively supercoiled than that of mesophiles, a feature associated with the increased stability of DNA to thermal denaturation. For these reasons, reverse gyrase has been suggested to be essential for life at high temperature. This assumption has been challenged by the finding that inactivation of the reverse gyrase gene in *Thermococcus kodakaraensis* did not result in a lethal phenotype; however, growth of the mutant strain was significantly retarded specifically at high temperature (6), thus confirming that the enzyme plays a role in the adaptation of the cell to high temperature. Recently, reverse gyrase was reported to have DNA chaperone activity *in vitro*, preventing heat-induced breakage and aggregation of the double strand (7). Moreover, we have previously shown that reverse gyrase is recruited to DNA *in vivo* after UV irradiation in the hyperthermophilic archaeon *Sulfolobus solfataricus* and its activity is inhibited by UV-induced lesions *in vitro*, suggesting that it, directly or indirectly, plays a role in the response to UV-induced DNA damage (8).

Structural and biochemical analyses indicate that reverse gyrase comprises two distinct modules, a C-terminal type IA topoisomerase domain and an N-terminal region containing

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conserved ATP-binding domains that are common to helicases (9–11). The enzyme as a whole (or the two domains expressed separately and mixed) performs strand passage unidirectionally towards increasing linking number, and this reaction requires ATP hydrolysis. The mechanism of the positive supercoiling reaction and the roles and relationships of the two domains are not completely understood. The C-terminal domain alone is able to perform an ATP-independent DNA relaxation reaction typical of type IA enzymes, although with low efficiency. In contrast, neither the N-terminal domain nor the whole enzyme shows helicase activity (10).

Reverse gyrase is able to unwind DNA in the absence of ATP and without DNA cleavage (10,12). The enzyme binds both double-stranded and single-stranded DNA (ssDNA), but requires a single-stranded region in order to bind DNA with high affinity. The temperature dependence of reverse gyrase activity might reflect the need for single-stranded regions caused by melting (13). It has been proposed that the N-terminal domain unwinds DNA locally, providing a single-stranded region that acts as a substrate for the topoisomerase domain.

Although reverse gyrase is unique in its positive supercoiling activity, there are multiple examples of interaction between helicase and topoisomerase in every organism (14). Such complexes are involved in different aspects of DNA metabolism. In particular, type IA Topo III enzymes associate physically and functionally with helicases of the RecQ family, forming an evolutionary conserved complex that has essential functions in the cell response to DNA damage (15–18). Genetic defects in human homologues of the RecQ helicase are associated with the Bloom and Werner syndromes, characterized by premature aging, high incidence of chromosomal rearrangements and cancer [reviewed in (19)]. Several reports have shown that helicases and helicase–topoisomerase complexes interact with other cellular proteins which influence their activity. Among these, single-strand binding proteins (SSBs) play an important role. These proteins bind and protect ssDNA transiently exposed during most DNA interactions.

S.solfataricus encodes an SSB which resembles the *Escherichia coli* SSB, because it holds a single oligonucleotide binding (OB) fold; however, the OB fold domain of the *S.solfataricus* protein is more similar to that of the eukaryotic SSB, RPA (20–22). *S.solfataricus* SSB interacts with RNA polymerase stimulating transcription *in vitro*, induces melting of A–T rich promoter regions and overcomes repression by the general transcription repressor Alba (23). Moreover, SSB has been reported to stimulate the helicase activity of the replicative MCM (mini-chromosome maintenance) helicase of *S.solfataricus* (24).

Here, we show that SSB from *S.solfataricus* stimulates activity of reverse gyrase purified from the phylogenetically close strain *Sulfolobus shibatae*. Our data show that SSB affects all the steps of the reverse gyrase reaction and are consistent with a model in which stimulation is mainly due to SSB-induced enhancement of reverse gyrase binding to DNA. By co-immunoprecipitation of cell extracts we show that reverse gyrase and SSB can associate through DNA, but they do not form stable interactions in the absence of DNA. In addition, SSB stimulates reverse gyrase positive supercoiling activity on DNA templates associated with the chromatin protein Sul7d, binding of reverse gyrase and cleavage of UV-irradiated substrates. The possible implications of these interactions are discussed.

MATERIALS AND METHODS

DNA manipulations

Reactions were performed according to standard techniques.

Proteins

Reverse gyrase was purified from *S.shibatae* B12 by four chromatographic steps (including hydrophobic interaction and affinity chromatography on heparin) as described previously (25). Recombinant *S.solfataricus* SSB was purified from *E.coli* transformed with plasmid pET28c-SSB (provided by M. F. White, St Andrews University, UK) using a two-step procedure described previously (20), consisting of thermoprecipitation of *E.coli* proteins followed by chromatography on an SP-Sepharose high performance column. Sul7d was purified from *S.solfataricus* MT4 as described previously (26). Recombinant His-tagged *S.solfataricus* Smj12 was purified from *E.coli* by affinity chromatography on nickel nitrilotriacetic acid (27). All proteins were diluted in the following buffer: 20 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, 150 mM NaCl, 0.1% Triton X-100.

Cell growth and extract preparation

S.solfataricus P2 cultures were grown and soluble cell extracts were prepared as described previously (8). The protein concentration was determined using a BioRad protein assay kit.

Western blot

Total and fractionated extracts were analysed using the Amersham ECL-Plus kit and a ChemiDoc apparatus (BioRad). Polyclonal antibodies against *Sulfolobus acidocaldarius* reverse gyrase, which recognize all the reverse gyrase tested from thermophilic organisms (9,28) and *S.solfataricus* Sul7d (27), were raised in rabbits, and against *S.solfataricus* SSB [gift from M. F. White, St Andrews University, UK; (23)] and *S.solfataricus* Smj12 (27) were raised in goats. Samples were run on 4–12% gradient gels in MES buffer (BioRad). The QuantityOne software (BioRad) was used for quantitations.

Reverse gyrase assays

Positive supercoiling assays were performed at 70°C [as reported in (29)] using either *S.solfataricus* P2 cell extracts or reverse gyrase purified from *S.shibatae* B12 as described previously (9). Standard assays were performed at 70°C with plasmid pGEM3 (Promega) for the indicated time spans. Relaxation assays were performed in the same way but ATP was omitted. Controls (plasmid alone and plasmid with reverse gyrase but without SSB) were included in every experiment and, to minimize variations within each experiment, a single mix with all components, except for SSB, was set up. Samples were analysed by 2D agarose gel electrophoresis with ethidium bromide (0.01 μg/ml), in the second dimension. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml), analysed and quantified

under UV light with a ChemiDoc apparatus and the QuantityOne software. Quantitations were performed as reported previously (30). Briefly, the intensity of each band was experimentally determined and used to calculate the amount of total DNA in each reaction (given by the sum of the amount of unprocessed substrate and all products), and the fraction of total products and unprocessed substrate. The relative abundance of each topoisomer in a reaction was expressed as a fraction of the total amount of DNA in that reaction. The specific linking difference (σ) was determined using the equation $\sigma = \Delta Lk/Lk_0$. σ values of the topoisomers whose intensity was >30% of the intensity of the most intense topoisomer were considered for the calculation of the mean σ value. Each assay was performed at least four times.

Electrophoretic mobility shift assays

The RGA oligonucleotide (8): 5′-GGCTGTCGACGAAGATAGAGGACTTAATCGATATC (top strand) 5′-GGGATATCGATTAAGTCCTCTATCTTCGTCGACAG (bottom strand) was labelled at the 3′ end with ^{32}P-αdCTP (3000 Ci/mmol) and Klenow enzyme, after annealing of complementary strands. The standard reaction mixture (10 μl) contained: 20 mM Tris–HCl (pH 8.0), 10% glycerol, 50 mM KCl, 0.1 mM DTT, 4×10^4 c.p.m. of labelled DNA probe (final concentration of 0.06 μg/ml, 2.4 nM). Samples were preincubated for 10 min at 70°C, then the appropriate proteins were added and incubated for 10 min at 37°C. Samples were immediately loaded on non-denaturing 5% polyacrylamide gels in 0.5× TBE buffer and run at 100 V for 1 h at room temperature. Each assay was performed five times. Radioactivity was determined by autoradiography with a Storm PhosphoImager and quantified with the IQ-Mac software (Molecular Dynamics).

Covalent complex assays (CCA)

Assays were performed as described previously (8). Purified reverse gyrase and SSB were preincubated for 10 min at 70°C; the end-labelled RGA oligonucleotide was added and incubation continued for 10 min at 70°C. Reactions were blocked by quickly chilling on ice and adding SDS–PAGE sample buffer. Samples were denatured and loaded on 6% SDS–polyacrylamide gels. Radioactivity was determined with a Storm PhosphoImager. The assay was performed five times.

Helicase assays

The following single-stranded oligonucleotides were used: 3-tailed (5′-GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTGCTGAGATCTCCTAGGGGCCCA) and 5-tailed (5′-CCCAAAAGGGTCAGTGCTGCAAGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGT). They were complementary to M13 with a 3′ or 5′ non-complementary tail, respectively (underlined sequence). Oligonucleotides were 5′ end labelled with T4 polynucleotide kinase and ^{32}P-γ−ATP and annealed to single-strand M13 as reported previously (24). About 5×10^4 c.p.m. (3.2 nM) of the oligonucleotides were incubated with reverse gyrase and SSB, as needed, for 10 min at different temperatures in a final volume of 20 μl. Reactions were stopped by adding 2% SDS. Products were analysed on non-denaturing 8% polyacrylamide gels.

Immunoprecipitation

About 10 mg of soluble *S.solfataricus* extracts was prepared as described previously (8) and incubated with 15 μl of a polyclonal antibody raised in goats against *S.solfataricus* SSB (23) or *S.solfataricus* Smj12 (27) in dilution buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.2 μg/ml Aprotinin, 1.2 μg/ml Leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride; final volume, 1.5 ml) for 3 h at 4°C with shaking. About 150 μl of Protein G-agarose (Roche Applied Science) was added and the incubation continued overnight at 4°C with shaking. This solution was centrifuged at 12 000 *g* for 30 s and the beads were washed three times for 20 min at 4°C with 1 ml of wash buffer 1 (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40) and once with 1 ml of wash buffer 2 (25 mM Tris–HCl, pH 7.5). The beads were resuspended in 100 μl of SDS–PAGE loading buffer and heated at 100°C for 5 min. The beads were removed by centrifugation at 12 000 *g* for 30 s and one-third of the supernatant was analysed by western blot. If needed, ethidium bromide (50 μg/ml) was added to the extract before the first incubation. In the sample with DNaseI, the soluble extract was incubated for 1 h at 37°C with 0.5 U/μl of DNaseI before the first incubation. The experiment was repeated three times.

RESULTS

SSB stimulates positive supercoiling activity of reverse gyrase

Incubation of reverse gyrase purified from *S.shibatae* with a negatively supercoiled plasmid DNA produces relaxed/positively supercoiled topoisomers, which can be separated using 2D agarose gel electrophoresis (Figure 1A). Figure 1B shows the typical pattern of products obtained using increasing reverse gyrase concentration: the ratio of products/substrate gradually increased until all substrate was consumed; further increase of enzyme concentration determined a shift in the distribution of products towards more positive linking numbers (ΔLk; Figure 1B). These data, in agreement with earlier observations [(13,31) and references therein], showed that: (i) the enzyme displays higher affinity for negatively supercoiled substrates and (ii) products with high positive superhelical density can be obtained using high enzyme concentrations, i.e. when multiple enzyme molecules bind and process a single DNA molecule simultaneously (13).

We then tested the effect of the *S.solfataricus* SSB on reverse gyrase activity. Because reverse gyrase is prone to degradation, the specific activity of the enzyme in each batch can vary from time to time, thus appropriate controls were included in each experiment (see Materials and Methods). Addition of SSB stimulated reverse gyrase positive supercoiling activity with respect to the activity observed in the absence of SSB under identical conditions (Figure 1D). The extent of reverse gyrase stimulation correlated with SSB concentration. SSB saturated the substrate at 2.75 μM in our assay (data not shown), as expected assuming a binding site of 5 nt (20). The stimulation of the positive supercoiling activity of reverse gyrase was observed in a wide range of SSB concentrations from sub-saturating amounts (0.3 μM, corresponding to 0.1 protein molecules/binding site) up to 8.2 μM,

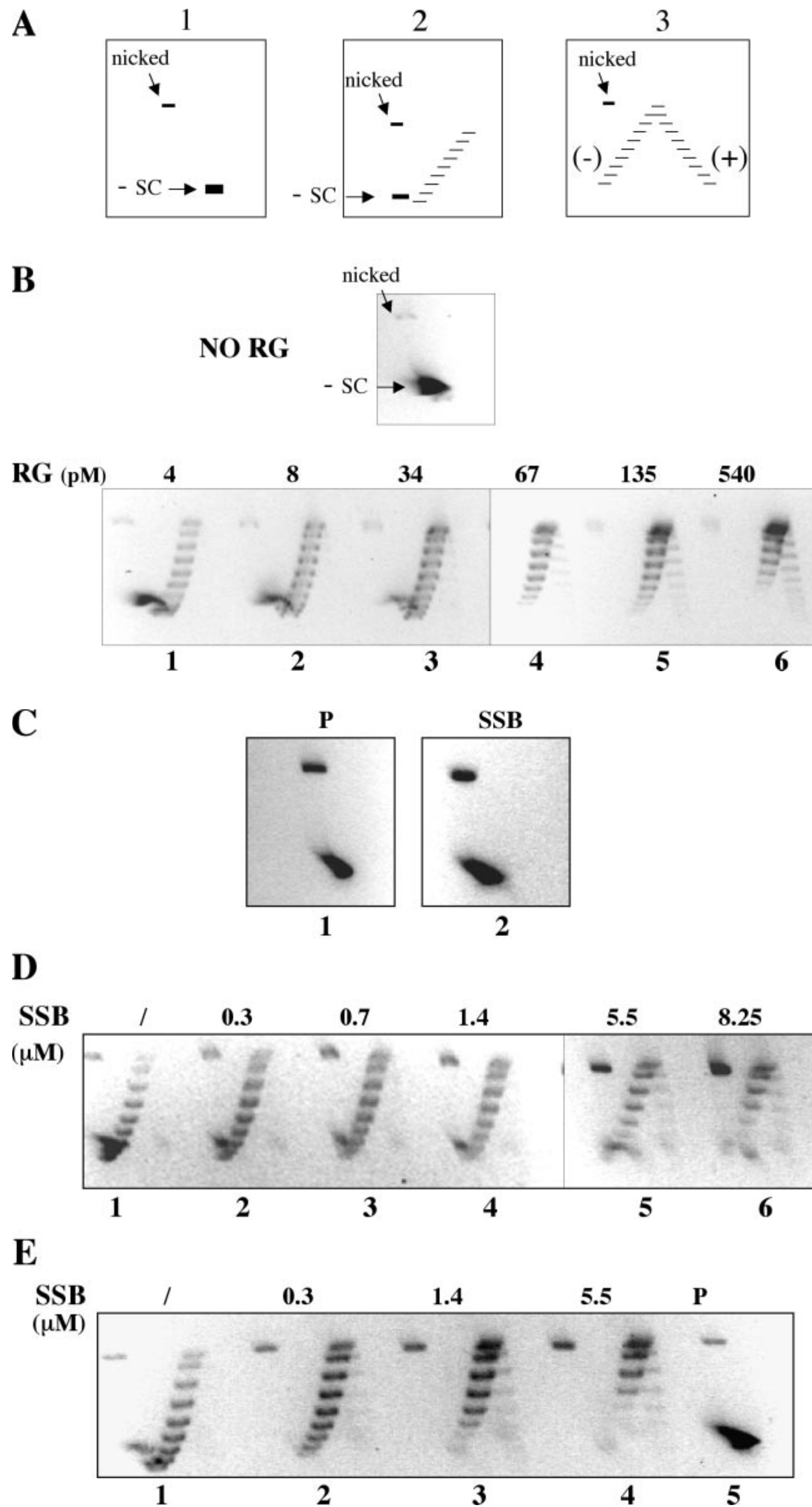

Figure 1. (**A**) Schematic representation of 2D gel electrophoresis of a negatively supercoiled plasmid (panel 1), of negative topoisomers (panel 2) and positive and negative topoisomers (panel 3). −SC, negatively supercoiled plasmid. (**B**) Positive supercoiling assay: 200 ng (5 nM) of pGEM3 plasmid DNA ($\Delta Lk > -12$) were incubated with reverse gyrase purified from *S.shibatae* (9) for 10 min at 70°C in a final volume of 20 μl, and subjected to 2D agarose gel electrophoresis. The amounts of reverse gyrase used were: panels 1–6, 0.012, 0.024, 0.1, 0.2, 0.4 and 1.6 ng, respectively (4, 8, 34, 67, 135 and 540 pM). (**C**) SSB has no effect on plasmid supercoiling. Assays were performed as in (B) but reverse gyrase was omitted. Panel 1, plasmid alone; panel 2, plasmid incubated with 3 μg of SSB. (**D**) Effect of SSB on reverse gyrase positive supercoiling activity. Positive supercoiling assays were performed as in (B). Each reaction contained 0.1 ng of reverse gyrase (34 pM) and the following amounts of SSB: panel 1, no SSB; panels 2–6, 0.1, 0.25, 0.5, 2 and 3 μg of SSB (0.3, 0.7, 1.4, 5.5 and 8.25 μM). (**E**) Positive supercoiling assay as in (B), but each reaction contained 0.4 ng of reverse gyrase (135 pM) and the following amounts of SSB: panel 1, no SSB; panels 2–4, 0.1, 0.5 and 2 μg of SSB (0.3, 1.4 and 5.5 μM); panel 5, plasmid alone. P, Plasmid.

corresponding to ∼3-fold molar excess of SSB with respect to its binding site. The concentrations used in this assay correspond to 1 reverse gyrase molecule/148 plasmid DNA molecules. The same result was obtained in assays performed at higher reverse gyrase concentrations (1 reverse gyrase molecule/37 DNA molecules; Figure 1E).

Table 1. Quantitation of reverse gyrase activity in the presence of SSB

	No SSB (%)	0.3 μM SSB (%)	0.7 μM SSB (%)	1.4 μM SSB (%)	5.5 μM SSB (%)	8.25 μM SSB (%)
RG (34 pM)	58.7 ± 1	94.0 ± 1	96.2 ± 2	97.0 ± 2	97.2 ± 2	97.7 ± 2
RG (135 pM)	92.7 ± 1	98.7 ± 2	99.2 ± 2	99.3 ± 2	99.9 ± 2	99.9 ± 2

RG, reverse gyrase.
Values report the relative amount of total products (given by the sum of all topoisomers) versus the total amount of DNA in each reaction.

Quantitation of positive supercoiling activity should take into account both the amount and ΔLk of products. We used two methods for quantitative comparison of the effect of SSB on reverse gyrase activity. First, we calculated the relative amount of total products in each sample (Table 1); at the lowest reverse gyrase concentration used (34 pM), addition of SSB at 0.3 μM increased total products from 58 to 94% of the input DNA. Thus, this method was not suitable to quantify the SSB effect at higher reverse gyrase or SSB concentrations. Therefore, we calculated the variations of the mean specific linking number (mean σ), obtained by considering the σ values of major topoisomers produced in each reaction (Figure 2A). At both reverse gyrase concentrations used (34 and 135 pM), the addition of increasing amounts of SSB resulted in a parallel increase of the mean σ, which reflected the overall products' ΔLk. This was also clear from the analysis of the distribution of topoisomers in each reaction at a given reverse gyrase

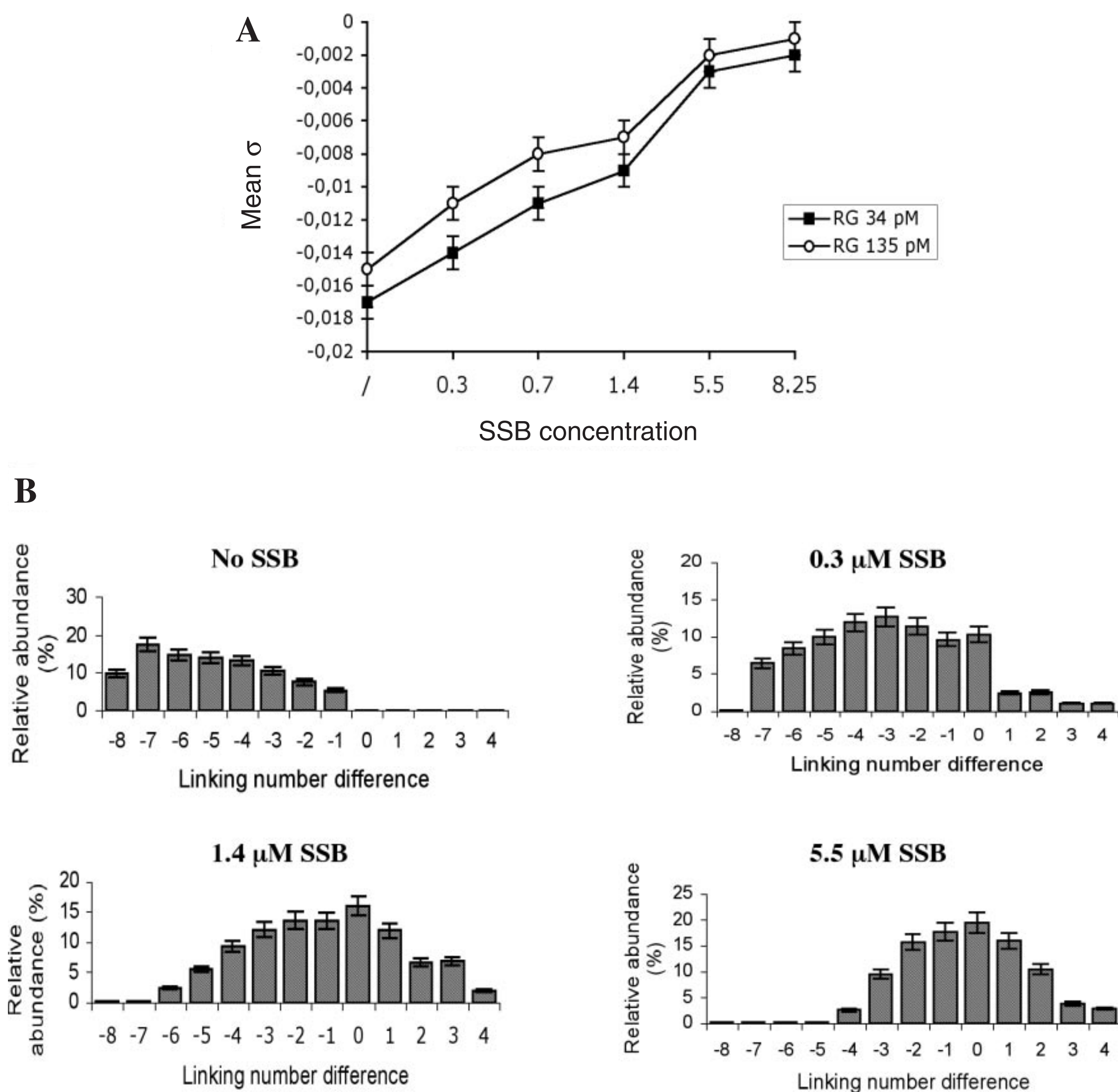

Figure 2. (**A**) Quantification of reverse gyrase activity; the mean specific linking difference (mean σ) was calculated as described in Materials and Methods. Reactions were performed with the indicated reverse gyrase (pM) and SSB (μM) concentrations. Values are the mean of three independent experiments. (**B**) Quantitation of the topoisomer distribution obtained in Figure 1E. The amount of DNA of each topoisomer is expressed as a fraction of the total amount of DNA in each reaction.

concentration, which showed that the topoisomers generated became more positive with increasing concentrations of SSB (Figure 2B). Thus, the effect of increasing the SSB concentration was similar to that observed on increasing the concentration of reverse gyrase (Figure 1B). However, it should be noted that neither of these methods provide a measure of the enzyme processivity, thus it is not clear whether reverse gyrase, in the presence of SSB, performs multiple isomerization cycles following a single association event with the substrate or after multiple association–dissociation events.

Interestingly, we noticed an SSB concentration-dependent accumulation of the nicked form of the substrate, which became very evident at higher protein concentrations (Figure 1D and E). This plasmid form might result from a block of the reverse gyrase reaction at an intermediate step, after cleavage and before ligation, and suggests an imbalance between the rates of these two steps (see below).

Reverse gyrase is not active on highly positively supercoiled substrates, probably because they do not expose single-strand regions. SSB was not able to stimulate reverse gyrase activity on plasmids with ΔLk ranging from 0 to +4 (data not shown).

SSB stimulates DNA binding activity of reverse gyrase

The catalytic cycle of reverse gyrase can be dissected into four steps: DNA binding, DNA cleavage, strand passage and religation of the DNA ends [reviewed in (1)]. We sought to determine which step of the reaction was affected by the presence of SSB. It has been shown previously that reverse gyrase binds initially to a region of ssDNA, but it is also in contact with flanking double-strand regions (13). These contact points allow the enzyme to unwind the DNA locally (10,12). We analysed the effect of SSB on binding of reverse gyrase to DNA in polyacrylamide gel shift experiments using a 35 bp double-strand oligonucleotide (RGA), which contains a high affinity reverse gyrase binding site (8). Reverse gyrase alone produced a sharp complex, although very faint (Figure 3A, lane 1). Under the same conditions, SSB alone

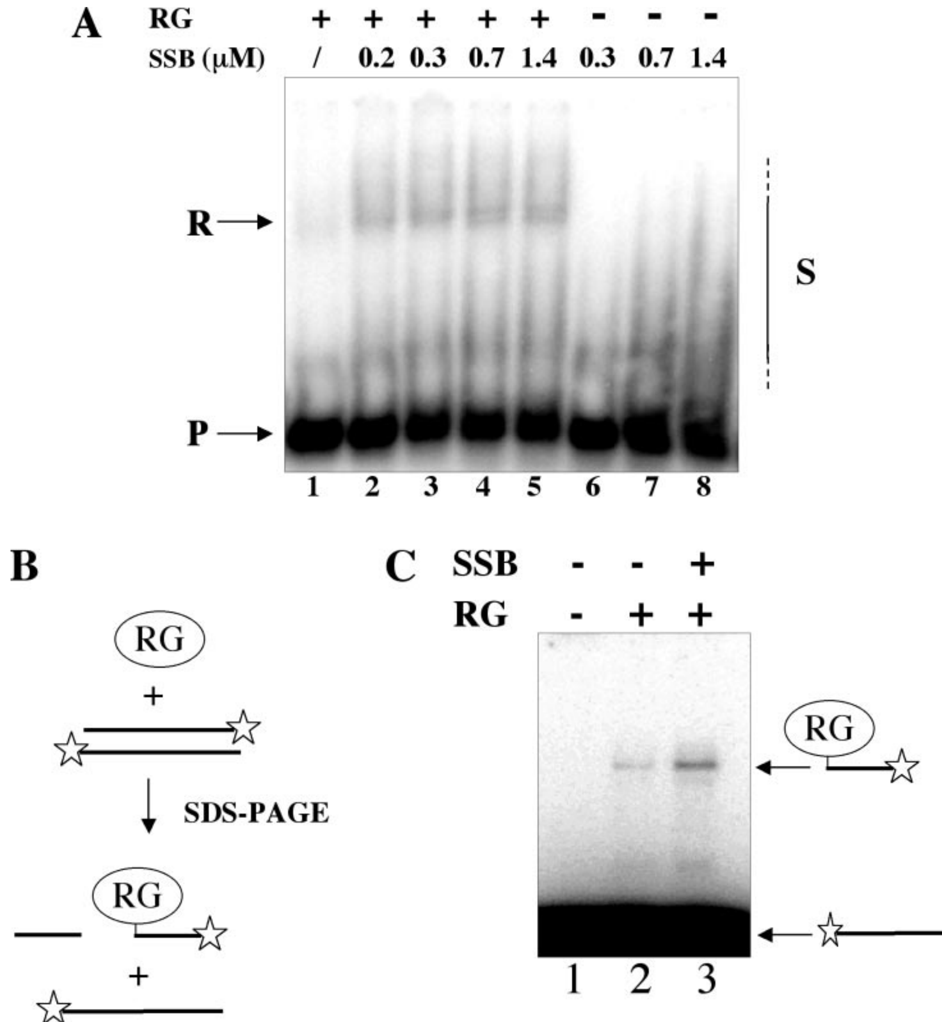

Figure 3. (**A**) Effect of SSB on reverse gyrase binding activity. The 3′ end-labelled RGA oligonucleotide (2.4 nM, 4×10^4 c.p.m./lane) was incubated for 10 min at 37°C with purified reverse gyrase (1 ng, 350 pM) in lanes 1–5 and the following amounts of purified SSB: lane 1, no SSB; lane 2–5, 30, 60, 125 and 250 ng (0.2, 0.3, 0.7 and 1.4 μM); lanes 6–8, 60, 125 and 250 ng of SSB (0.3, 0.7 and 1.4 μM). R, reverse gyrase–DNA complexes; S, SSB–DNA complexes; P, free probe. (**B**) Principle of the CCA (8). (**C**) Effect of SSB on reverse gyrase cleavage complexes formation. The 3′ end-labelled RGA oligonucleotide was incubated and assayed by CCA with: lane 1, no protein; lane 2, purified reverse gyrase (1 ng, 350 pM); lane 3, as lane 2 but with 500 ng of SSB (1.4 μM). RG, reverse gyrase. The arrow indicates the oligonucleotide–protein covalent complex.

Table 2. Quantitation of reverse gyrase binding to the RGA oligonucleotide, either normal or UV-irradiated (UV-RGA)

	No SSB (%)	0.2 μM SSB (%)	0.3 μM SSB (%)	0.7 μM SSB (%)	1.4 μM SSB (%)
RGA	0.04 ± 0.01	3.2 ± 0.5	6.5 ± 1	6.8 ± 1	5.82 ± 1
UV-RGA	0.05 ± 0.02	NT	NT	5.2 ± 1	5.1 ± 1

NT, not tested.
Reverse gyrase concentration was 350 pM.

formed unstable complexes (20), which were detectable as smeared bands (Figure 3A, lanes 6–8). When SSB and reverse gyrase were incubated with DNA, the formation of the reverse gyrase–DNA complexes was enhanced; the enzyme binding to DNA was stimulated with the same efficiency in a wide range of SSB concentrations, from 6 to 50 protein molecules/binding site (Figure 3A, lanes 2–5; Table 2). Similar results were obtained at different SSB/reverse gyrase ratios and in the presence of 1 mM ATP (data not shown). Sikder *et al*. (32) reported that the *E.coli* SSB stimulates binding of the cognate topoisomerase I (a type IA enzyme) to DNA; like for reverse gyrase, stimulation was independent of the concentration of SSB. Such all-or-nothing behaviour might be explained if binding of both enzymes to DNA is cooperative, as suggested for reverse gyrase (7).

SSB stimulates DNA cleavage activity of reverse gyrase

Like all type IA topoisomerases, reverse gyrase forms a covalent intermediate containing a phosphodiester bond between a DNA 5′ end and the catalytic tyrosine. It has been previously shown that covalent complexes between DNA and purified reverse gyrase can be trapped if reactions are performed in the absence of ATP and quickly blocked with detergents (32,33). We have previously identified a strong reverse gyrase cleavage site in the RGA oligonucleotide (8). We evaluated the efficiency of covalent complex formation by CCA in which reverse gyrase was incubated with end-labelled oligonucleotides and covalent complexes were visualized as radioactive protein bands in SDS–PAGE (Figure 3B). The efficiency of covalent complex formation can be used as a measure of the cleavage activity of reverse gyrase (8,31). To determine whether SSB was acting at a step prior to cleavage, we assessed the formation of the covalent RG–DNA complex in the presence of SSB. Addition of SSB increased the efficiency of covalent complex formation by reverse gyrase when used at concentration of 1.4 μM, corresponding to 50 protein molecules/DNA binding site (Figure 3C). Similar results were obtained at different SSB/reverse gyrase ratios (data not shown). Although careful quantitation in CCA is difficult for the lack of any possible internal control and the transient nature of the intermediate DNA–protein complex, this experiment was highly reproducible and showed that SSB stimulates reverse gyrase cleavage activity.

SSB does not activate reverse gyrase helicase activity

Although reverse gyrase contains an N-terminal helicase domain including an ATP binding motif and, like helicases, is able to hydrolyse ATP, neither reverse gyrase nor its N-terminal domain showed helicase activity *in vitro*. However, it induces a certain degree of unwinding upon binding to DNA (10,12). It is a common property of SSBs from the three domains of life to be able to stimulate helicases. The *Sulfolobus* SSB has been shown to stimulate the MCM helicase activity (24); SSB from *E.coli* stimulates the RecQ helicase (18); human replication protein A (hRPA) stimulates the RecQ1 and WRN helicases (34–37). It has been suggested that SSBs stimulate helicase-mediated unwinding by trapping the separated ssDNA strands and preventing the formation of non-productive enzyme–ssDNA complexes (38).

Reverse gyrase on its own is only able to induce, upon binding, unwinding of very short helical segments, which is reverted when the enzyme is released from DNA. We reasoned that the presence of SSB might stabilize and extend these single-strand regions, and the combination of these two activities might result in a ‘helicase’ activity. To test this hypothesis we designed two 60 base oligonucleotides containing 40 bases complementary to M13 DNA and a 20 base non-complementary extension at either the 3′ or the 5′ end. The oligonucleotides were annealed to M13 and oligonucleotide displacement assays were performed. We failed to detect any helicase activity with either of the oligonucleotides using different protein concentrations, protein–DNA ratios ranging from 0.5 to 2 protein molecules/binding site and temperatures ranging from 37 to 70°C (Figure 4A and data not shown). We therefore conclude that reverse gyrase, in combination with SSB, does not show detectable helicase activity under the conditions used.

We tested the effect of SSB on reverse gyrase ssDNA-stimulated ATPase activity. SSB failed to stimulate reverse gyrase ATPase activity either in the presence or in the absence of DNA (data not shown). This result is consistent with that obtained for the human RecQ1 helicase, which is stimulated by RPA without stimulation of its ATPase activity (35).

Effect of SSB on reverse gyrase DNA relaxation activity

The *E.coli* SSB and human RPA stimulated both DNA strand passage activity by the *E.coli* Topo III–RecQ complex and relaxation of the negatively supercoiled DNA by Topo III alone (18). The presence of ATP is absolutely essential for reverse gyrase to complete its reaction cycle. In the absence of ATP, the enzyme is able to bind and cleave DNA but cannot actively introduce positive supercoils; instead it performs a low efficiency DNA relaxation reaction typical of type IA enzymes (10). We investigated the effect of SSB on the DNA relaxation activity of reverse gyrase (Figure 4B). Under the conditions used the enzyme barely showed relaxation activity, which was weakly enhanced in the presence of SSB. Stimulation increased with increasing SSB concentration with maximum stimulation observed when SSB was used at 1.4 μM (corresponding to 0.5 protein molecules/binding site). Interestingly, SSB at 5.5 μM (corresponding to two protein molecules/binding site) resulted in inhibition of relaxation. This finding is in contrast to what we have observed for the positive supercoiling reaction, which is still stimulated by SSB at ~3-fold higher concentration, and for binding to DNA and cleavage activity, which were enhanced by SSB at ten-fold higher concentration (see Discussion).

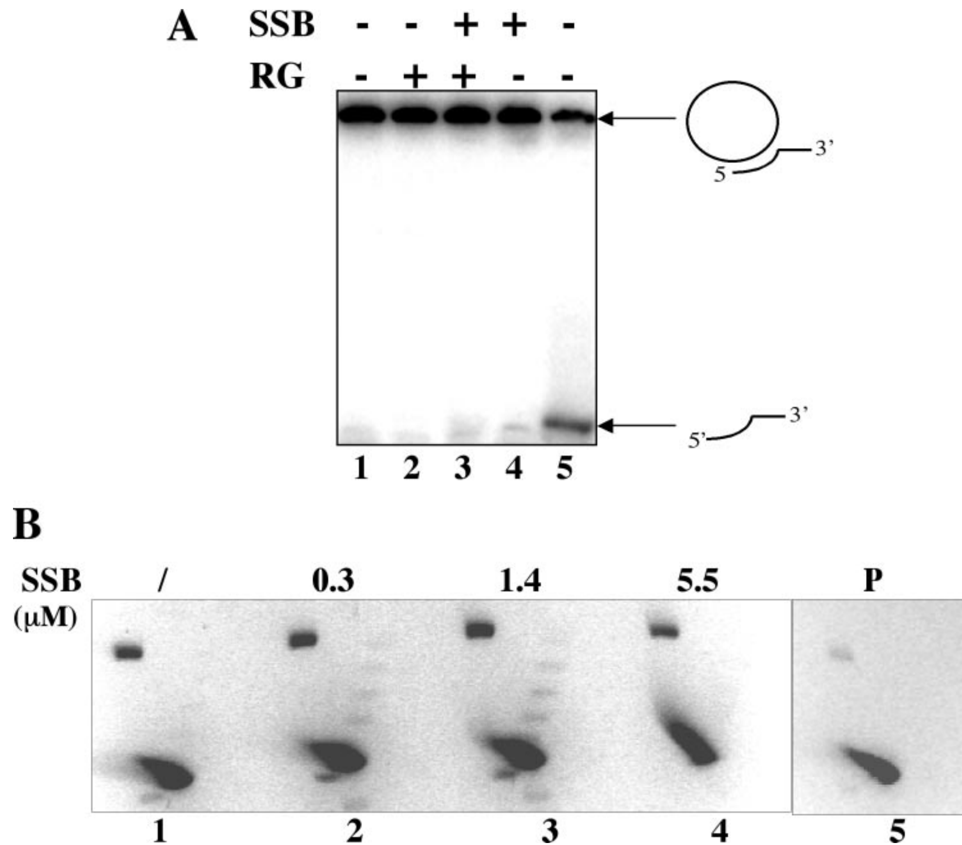

Figure 4. (**A**) Helicase assay. The 3-tailed oligonucleotide annealed to M13 DNA was incubated for 10 min at 70°C with: lane 1, no protein; lane 2, 1.6 ng of reverse gyrase (540 pM); lane 3, as lane 2 but with 250 ng of SSB (1.4 μM, corresponding to 0.5 protein molecules/binding site); lane 4, 250 ng of SSB. In lane 5 the substrate was partially denatured. Arrows indicate the annealed product and the displaced oligonucleotide. (**B**) Effect of SSB on reverse gyrase relaxation activity. Assays were performed as in Figure 1 but omitting ATP. Reverse gyrase 0.4 ng (135 pM) and the following amounts of SSB were used: panel 1, no SSB; panels 2–4, 100 ng, 500 ng and 2 μg (0.3, 1.4 and 5.5 μM), panel 5, plasmid alone.

SSB stimulates reverse gyrase activity in the presence of chromatin components

Unlike organisms belonging to the *Euryarchaea*, the crenarchaeon *S.solfataricus* does not hold eukaryal-type histones but contains 'architectural' DNA binding proteins called Sul7d and Alba (39). Sul7d (formerly known as Sso7d) is one of the most abundant chromatin components of *S.solfataricus* and has been reported to stabilize DNA by increasing its melting point (40), to promote the annealing of complementary single strands at high temperature (41) and to compact DNA (29). We have previously shown that reverse gyrase is inhibited by Sul7 (29). Although the mechanism of this inhibition is not clear, possible explanations are that Sul7d limits DNA accessibility to the enzyme either by steric hindrance or stabilizes the double helix to the temperature-induced unwinding.

We tested the effect of increasing concentrations of SSB on reverse gyrase activity in the presence of Sul7d. As reported previously, Sul7d inhibited reverse gyrase activity (Figure 5A); interestingly, addition of SSB could overcome the inhibitory effect of Sul7d. This compensatory effect was observed using different Sul7d:SSB:DNA ratios; the resulting shift of the mean σ reflected the protein composition of each reaction (Figure 5B).

When DNA was preincubated with SSB and then Sul7d and reverse gyrase were added, the efficiency of reaction was similar to that observed if the proteins were added simultaneously (Figure 5C, panel 2). When DNA was preincubated with Sul7d and then SSB and reverse gyrase were added, the reaction was less efficient than when the two proteins were added at the same time, showing that SSB could antagonize Sul7d less efficiently if the latter is prebound to DNA (Figure 5C, panel 3). Quantitation of these data is shown in Figure 5E. Moreover, in the presence of Sul7d, we also noted the SSB-dependent accumulation of the nicked plasmid. These results suggest that SSB might stimulate reverse gyrase activity in chromatin and raise the possibility that the interplay among reverse gyrase, SSB and Sul7d might have biological relevance.

We also tested the effect of Smj12 on reverse gyrase activity. This is another non-specific DNA binding protein that stabilizes the double strand and induces conformational changes that, in association with a DNA topoisomerase, result in positive supercoiling (27). Although its function is unknown, it has been suggested that Smj12 is not a structural component of chromatin. Smj12 when used at different concentrations did not show any effect on reverse gyrase activity (Figure 5D). Thus the inhibition of reverse gyrase activity by Sul7d is a specific effect.

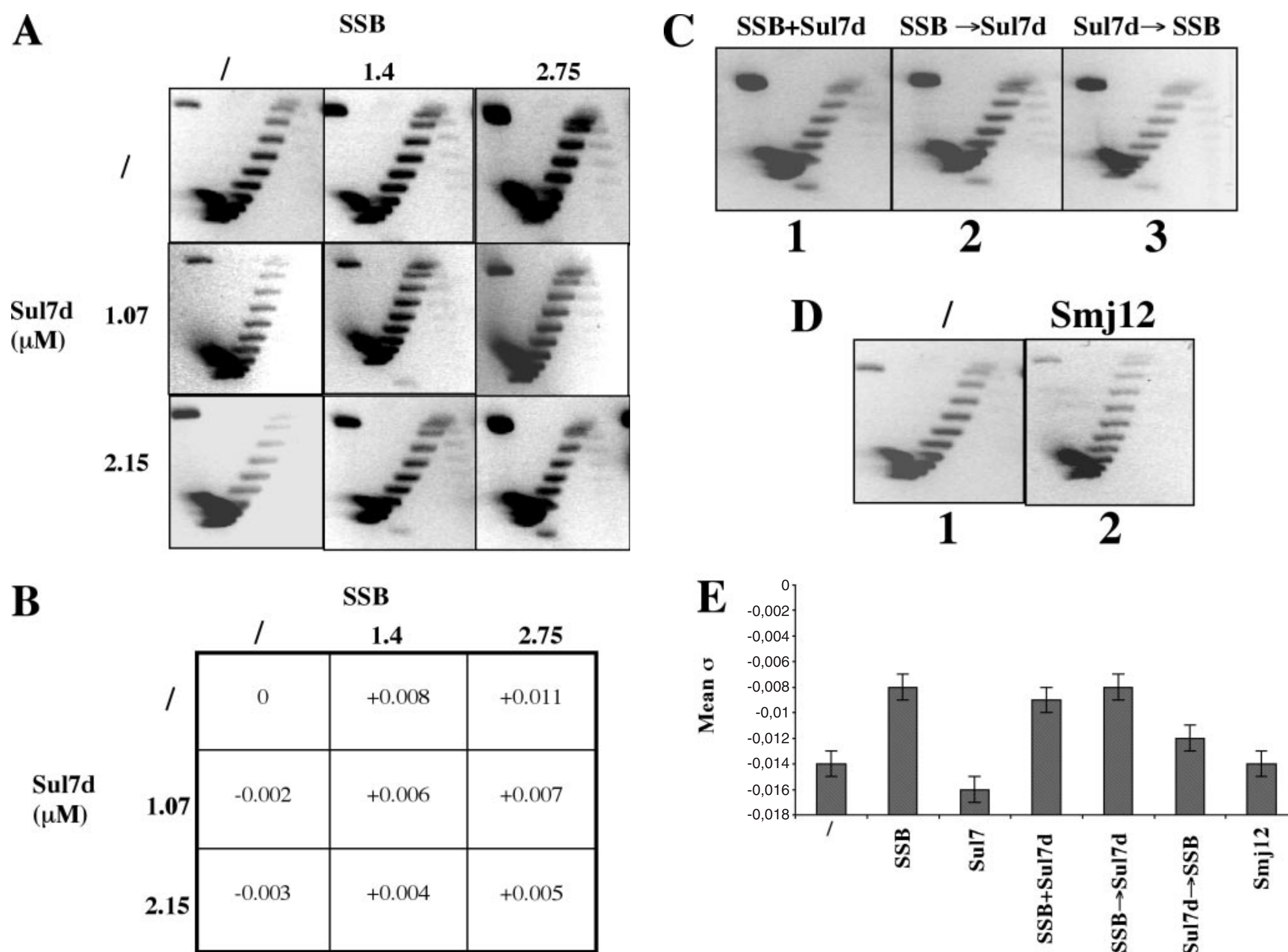

Figure 5. Effect of DNA binding proteins on reverse gyrase activity. (**A**) Positive supercoiling assays were performed as in Figure 1, but with 0.4 ng of reverse gyrase (135 pM) and the indicated amounts of SSB and Sul7d. Considering a binding site of 6 bp for Sul7d, 2.15 μM is a saturating concentration. Thus, at the highest concentrations used the ratio SSB:Sul7d:DNA is 1:1:1. (**B**) Quantification of reverse gyrase activity shown in (A); numbers represent the difference of the mean σ obtained in each reaction with respect to that obtained with reverse gyrase alone (which was assigned a value 0). (**C**) Assays were as in Figure 1, but each panel contained 0.4 ng of reverse gyrase (135 pM), 1 μg of SSB and 0.3 μg of Sul7d; in panel 1 all proteins were added together; in panel 2 DNA was preincubated with SSB for 10 min at 70°C, then reverse gyrase and Sul7d were added; in panel 3 DNA was preincubated with Sul7d, then reverse gyrase and SSB were added. (**D**) Effect of Smj12 on reverse gyrase activity. Assays were as in Figure 1, but 0.4 ng of reverse gyrase (135 nM) was incubated with: panel 1, no other protein; panel 2, 0.3 μg of Smj12 (1.25 μM). (**E**) Quantitation of reverse gyrase activity shown in (A) and (B); the mean specific linking difference (mean σ) was calculated as described in Materials and Methods.

Reverse gyrase colocalizes with SSB in the presence of DNA

The functional interaction between reverse gyrase and SSB prompted us to test whether the two proteins interact physically. We carried out co-immunoprecipitation experiments on *S.solfataricus* soluble extracts using a polyclonal antibody directed against SSB (23). The antibody immunoprecipitated both SSB and reverse gyrase; however, if extracts were previously incubated with ethidium bromide, which disrupts most protein–DNA interactions, reverse gyrase was no longer co-immunoprecipitated with SSB (Figure 6A). The same result was obtained if extracts were previously treated with DNaseI (data not shown), suggesting that the interaction of two proteins is mediated by DNA. The extracts used in these experiments are enriched for soluble proteins and devoid of the bulk of high-molecular weight DNA, but do contain limited amount of fragmented DNA [(8), and data not shown]. To test the specificity of the observed co-immunoprecipitation, we used several controls. Neither Smj12 nor Sul7d (which is very abundant) were co-immunoprecipitated with SSB; moreover, reverse gyrase was not immunoprecipitated using an antibody directed against Smj12 in the absence of either ethidium bromide or DNaseI (Figure 6B). Thus, under the conditions used we did not observe unspecific co-immunoprecipitation of DNA binding proteins. Although we cannot rule out the possibility that binding to DNA triggers conformational changes in one or both proteins required for their interaction, the most likely explanation of our data is that SSB and reverse gyrase do not make direct stable interactions but they may colocalize to the same DNA sites. Functional interaction without direct protein–protein interactions has been observed between the *E.coli* SSB and topoisomerase I (32).

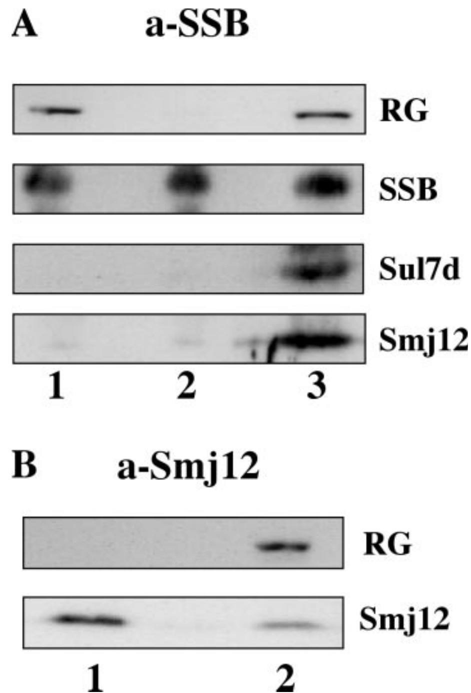

Figure 6. Co-immunoprecipitation of SSB and reverse gyrase. (**A**) Polyclonal antibody against SSB (23) was used to immunoprecipitate *S.solfataricus* soluble extracts. Blots were probed with polyclonal antibodies against the indicated proteins. Soluble extracts were immunoprecipitated without (lane 1) or with 50 μg/ml ethidium bromide (lane 2). Lane 3 contains 200 μg of soluble extracts (**B**) Polyclonal antibody against Smj12 (27) was used to immunoprecipitate *S.solfataricus* soluble extracts. Blots were probed with polyclonal antibodies against the indicated *S.solfataricus* proteins. Lane 1, immunoprecipitation and lane 2, soluble extracts (200 μg).

SSB stimulates reverse gyrase binding and cleavage of UV-irradiated substrates

We have previously reported that UV light irradiation of *S.solfataricus* cultures induces relocalization of reverse gyrase from the cytoplasmic compartment to chromatin *in vivo* (8). The chromatin-bound reverse gyrase is active, suggesting a role for the enzyme in the repair mechanism. On the other hand, SSB is equally distributed among cytoplasmic and chromatin fractions and its localization is not affected by UV irradiation. Although the mechanism of this recruitment has not been determined, it seems likely that UV-induced lesions trap reverse gyrase onto DNA. Indeed the presence of UV-induced lesions in plasmid substrates inhibits positive supercoiling activity of the enzyme *in vitro* (8). We, therefore, tested the effect of SSB on reverse gyrase activity on damaged DNA. When positive supercoiling activity on irradiated DNA plasmids was assayed, reverse gyrase was highly inefficient and produced a weak smear due to the aberrant migration of damaged topoisomers [Figure 7A; (8)]. Addition of increasing concentrations of SSB did not significantly change the efficiency of the smear formation but resulted in the increase of the nicked DNA intermediate. When DNA oligonucleotides which were previously exposed to UV light were used, SSB stimulated reverse gyrase binding in electrophoretic mobility shift assays (Figure 7B and Table 2) and covalent complex formation in CCA (data not shown). The efficiency of binding was similar to that obtained with the control oligonucleotide (Table 2). The affinity of SSB for irradiated substrates (both linear and supercoiled) was similar to that for control substrates (data not shown). Taken together, these results suggest that, as with undamaged substrates, SSB stimulates reverse gyrase binding to irradiated DNA, but the enzyme can not complete the reaction because of the presence of lesions, resulting in the accumulation of the nicked plasmid forms.

DISCUSSION

Among DNA topoisomerases, reverse gyrase is peculiar in its ability to actively introduce positive supercoiling, using the energy of ATP hydrolysis. Although detailed functional and structural studies have been performed with the enzyme purified from different hyperthermophiles, its physiological function *in vivo* has not been established. It is likely that its main role is maintaining the correct DNA topology; however, recent results suggest that it might be, directly or indirectly, involved in DNA repair and/or protection (7,8).

SSBs are involved in most DNA transactions including DNA repair and recombination. In most cases, their main role is to modulate the activity of other proteins or protein complexes, either by direct protein–protein interactions or, indirectly, by binding to ssDNA [(32) and references therein].

To study the interaction of reverse gyrase with an SSB, we needed two cognate well-studied proteins. The choice of reverse gyrase from *S.shibatae* and SSB from *S.solfataricus* appeared a reasonable compromise because the two proteins have been characterized in great detail and these two *Sulfolobus* strains are evolutionarily very close, and many proteins from the two organisms are almost identical or share a high degree of sequence identity.

We have shown that the *S.solfataricus* SSB activates *S.shibatae* reverse gyrase activity. Our results indicate that the protein stimulates all steps of the reaction, i.e. binding of the enzyme to DNA, cleavage of the template and strand passage. If ATP is present, the overall reaction, e.g. positive supercoiling, is stimulated. In the absence of ATP, the enzyme is only able to relax DNA, and this reaction is also stimulated by SSB, although with important differences (see below). Because reverse gyrase binds optimally to single-strand DNA regions flanked by double-strand regions, the simplest explanation of our results is that SSB stabilizes the single-strand regions required for reverse gyrase binding. We propose a possible model in which SSB traps the ssDNA produced by the low unwinding activity of reverse gyrase, or by the spontaneous breathing of the strands, which is relevant at high temperature, thereby preventing their annealing. Thus, multiple reverse gyrase molecules simultaneously bind the same DNA molecule, producing high superhelical density. This model is also supported by our co-immunoprecipitation experiments from *S.solfataricus* cell extracts, showing that reverse gyrase and SSB do not make stable protein–protein interactions in solution, but may interact with the same DNA fragment.

Our results suggest that increased efficiency of reverse gyrase binding, by stabilization of single-strand regions, is sufficient to stimulate all subsequent steps (DNA cleavage, strand passage and ligation). A few points of this model should

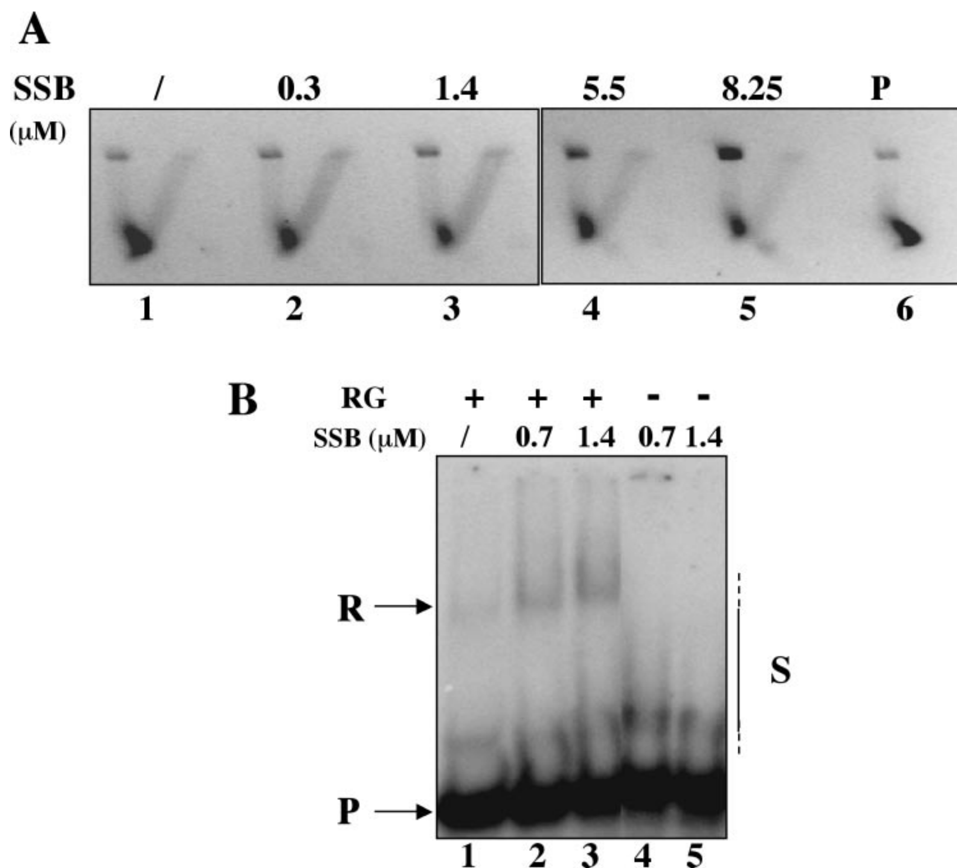

Figure 7. (**A**) Positive supercoiling activity of reverse gyrase in UV-damaged substrates in the presence of SSB. Reactions were performed as in Figure 1D, but the DNA substrate was previously irradiated with UV light (254 nm, 800 J/m^2). Each reaction contained 0.1 ng of reverse gyrase (34 pM) and the following amounts of SSB: panel 1, no SSB; panels 2–5, 0.1, 0.5, 2 and 3 μg of SSB (0.3, 1.4, 5.5 and 8.25 μM); panel 6, plasmid alone. The experiment was performed in parallel to that in Figure 1D, which can be used for comparison. (**B**) Effect of SSB on reverse gyrase binding activity of UV-damaged DNA substrates. The 3′ end-labelled RGA oligonucleotide (4×10^4 c.p.m./lane) was incubated for 10 min at 37°C with purified reverse gyrase (lanes 1–3; 1 ng, 350 pM) and the following amounts of purified SSB: lane 1, no SSB; lane 2, 125 ng of SSB (0.7 μM); lane 3, 250 ng of SSB (1.4 μM); lane 4, 125 ng of SSB (0.7 μM); lane 5, 250 ng of SSB (1.4 μM). The experiment was performed in parallel to that in Figure 3A, which can be used for comparison.

be discussed. First, we have shown that the combination of reverse gyrase and SSB is not able to induce stable strand displacement in helicase assays. Although we cannot rule out the possibility that such activity could be detected using different experimental conditions, this result suggests that even in the presence of SSB reverse gyrase-induced DNA unwinding remains strictly local and/or transient. Second, in both positive supercoiling and relaxation assays, we observe an SSB-dependent accumulation of the nicked plasmid, suggesting imbalance between the rates of cleavage and religation at high SSB concentrations. Third, in the absence of ATP, SSB stimulates relaxation activity at low concentrations, but has inhibitory effects at higher concentrations. One possible explanation of this result is that the binding of reverse gyrase and cleavage of DNA are ATP-independent. Thus SSB stimulates these steps both in the presence and in the absence of ATP. However, it is possible that for reverse gyrase to complete the reaction, SSB might be displaced to allow reannealing of the helices and religation. If DNA is saturated with SSB, reverse gyrase might be unable to displace the protein in the absence of ATP, thus the relaxation reaction is inhibited. In contrast, in the presence of ATP, reverse gyrase might be able to displace SSB and complete the reaction. In this displacement reaction reverse gyrase might use ATP as a motor force, like chromatin modelling factors. Interestingly, ATP-dependent chromatin remodelling activities can generate superhelical tension (42).

To confirm the biological relevance of our findings, we have tested the effect of SSB on reverse gyrase activity in the presence of the main component of the *Sulfolobus* chromatin, the small DNA binding protein Sul7d. We have previously shown that Sul7d inhibits reverse gyrase positive supercoiling activity, probably by stabilizing the double strand (29). SSB is able to antagonize the inhibitory effect of Sul7d, suggesting a functional interplay among these three proteins in a physiological context. SSB could be necessary to antagonize the inhibitory effect of Sul7d and the balance between the opposite effects of the two proteins might provide the means to regulate the reverse gyrase activity.

We have previously shown that, under physiological growth conditions, the great majority (about 90%) of the reverse gyrase cell content copurifies with the soluble fraction of a cell extract, whereas only about 10% is tightly associated with chromatin. This finding suggests that reverse gyrase activity is not required during the physiological growth, but the enzyme is stored in the cytoplasm until needed. In this view, SSB might be required to stabilize reverse gyrase binding and

stimulate its activity under particular conditions. For instance, reverse gyrase is specifically recruited to DNA after UV irradiation *in vivo*, and the presence of UV-induced lesions in the substrate stabilizes DNA–protein covalent intermediates (8). We have shown that addition of SSB stimulates reverse gyrase binding and cleavage, but not positive supercoiling, of UV-irradiated substrates. The stimulation of reverse gyrase binding and cleavage of damaged DNA by SSB could accelerate the trapping of the enzyme onto DNA if the catalytic cycle cannot be completed because of the lesions. It will be interesting to establish whether the enzyme recruitment to damaged DNA occurs by an active mechanism or is a consequence of the blocking of its activity. Moreover, it will be of interest to test if the enzyme covalently trapped on damaged DNA is able to recruit more binding partners, such as factors involved in repair and/or recombination.

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