

Escherichia coli single-stranded DNA-binding protein alters the structure of intramolecular triplexes in plasmids

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The ability of the *Escherichia coli* single-stranded DNA-binding protein (SSB) to recognize structural features associated with intramolecular triplex formation in oligopurine · oligopyrimidine (pur · pyr) inserts in recombinant plasmids was evaluated. The SSB protein binds to supercoiled plasmids and causes a site-preferential increase in OsO₄ reactivity of the pyrimidine strand involved in the formation of the Hy-3 isomer of the triplex structure. The *E. coli* RecA protein showed no reaction with triplexes in similar studies. This behavior is consistent with SSB-mediated unpairing of the H-DNA-forming region.

Triple-stranded DNA; Supercoiling; SSB protein; Chemical probe; Unpaired base

1. INTRODUCTION

DNA is a conformationally flexible molecule; appropriate segments can adopt several types of non-B structures (reviewed in [1–4]). Extensive studies were reported on left-handed Z-DNA (occurring mainly at alternating purine–pyrimidine sequences), cruciforms (at inverted repeat sequences) and bent DNA. Triplexes occur at oligopurine · oligopyrimidine (pur · pyr) sequences in negatively supercoiled plasmids at pHs 4.5–7.5 (reviewed in [5]). The remarkable feature of the triplex (also termed H-DNA) [6,7] is that one half of the purine strand (usually the 3' half) interacts with both the 5' and the 3' halves of the pyrimidine stretch which is folded back to give T · A · T and C · G · C⁺ triads (Hy-3 isomer). The formation of triplexes containing exclusively G · G · C and A · A · T triads has also been reported [8,9].

Intramolecular triplexes contain several regions that are single-stranded. These regions include a single-stranded loop located in the center of the strand that

folds back into the major groove of the duplex, half of the purine or pyrimidine strand (depending on whether Hy or Hu isomers are formed) and the junction regions [5,10,11]. Hypersensitivity to S1 and P1 nucleases, OsO₄, DEPC, BAA, DMS, as well as other chemical probes, justify these conclusions. Whereas the triplex model fits the available data well for a number of inserts which were investigated [5,10–17], other types of structures may be favored by certain other sequences (reviewed in [5]).

Numerous proteins have been characterized that bind single-stranded DNA with a high preference and show no apparent base or sequence specificity (reviewed in [18,19]). The *E. coli* single-stranded binding protein (SSB) and T4 gene 32 protein are the most thoroughly investigated examples. The 20,000 Da SSB protein is an essential protein which is required for replication, homologous recombination, and the SOS pathway of DNA repair [20–26]. It exists in solution as a stable asymmetric tetramer which covers approximately 70 nucleotides of single-stranded DNA polymer and forms nucleosome-like beads [27,28]. The mode of binding of SSB to single-stranded oligonucleotides reveals variable stoichiometries depending on the length and type of oligomer [27,29,30]. In general, the SSB protein has approximately 10³ greater affinity for single-stranded DNA than for double-stranded DNA [29].

Herein, we demonstrate that the SSB protein binds to supercoiled plasmids containing triplexes and causes a change in the conformation as revealed by chemical reactivity with resolution at the bp level.

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Abbreviations: BAA, bromoacetaldehyde; DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate; py, pyridine; SSB, single-stranded DNA binding protein; Hy, an isomer of H-DNA in which half of the pyrimidine strand is donated as a third strand; Hu, an isomer of H-DNA in which half of the purine strand is donated as a third strand.

2. MATERIALS AND METHODS

2.1. Plasmids

The inserts in the plasmids used in these studies are shown in Fig. 1. pRW1707 was a derivative of pRW791 [31]; pRW1410 was derived from pRW790 [10] whereas pPP1 was a derivative of pUC18 [12]. All inserts were cloned into the *Bam*HI site in the polylinkers in the vectors. Topoisomeric samples were prepared as described [32].

2.2. Proteins

The *E. coli* SSB protein, the *E. coli* RecA protein, and the T4 gene 32 protein were purchased from US Biochemical Corp. Samples of RecA were also generously provided by Drs. I.R. Lehman (Stanford University) and Jack Griffith (University of North Carolina) and a homogeneous preparation of the T4 gene 32 protein was generously provided by Dr. Peter von Hippel (University of Oregon). The determination of the specific activity of the RecA protein was described [33]. Bovine serum albumin was from Sigma.

2.3. Chemical modification studies

Typical OsO_4 reaction mixtures (30 μl) contained 3 μg of plasmid DNA in 50 mM Tris-HCl buffer or 50 mM sodium acetate buffer (at the appropriate pH as indicated), 0.5% pyridine and the indicated amount of SSB protein. After 30 min at 22°C, the modification reaction was initiated by the addition of OsO_4 to a final concentration of 0.9 mM. After 30 min at 22°C, the DNA was ethanol precipitated, digested with restriction enzymes to release the insert containing pur · pyr sequence, and labeled. After acrylamide gel purification step, the insert was cleaved with piperidine and analyzed on the sequencing gels [10]. The effect of SSB binding to plasmids containing triple-stranded DNA were performed using DEPC, KMnO_4 or DMS under conditions identical to those described for OsO_4 modification except that pyridine was omitted. 3 μl of DEPC, 1 μl of DMS, 8 mM KMnO_4 was used in reactions performed at room temperature. The DEPC reaction was stopped by ethanol precipitation after 30 min of incubation, whereas KMnO_4 and DMS reactions were terminated by the addition of mercaptoethanol (final concentration 0.5 M) after 3 min of modification. Further workup was as described for OsO_4 -treated samples.

2.4. S1 nuclease studies

The effect of binding of SSB on the S1 nuclease reactivity of the inserts in the triplex state were performed as follows: 3 μg of plasmid DNA was mixed with increasing amounts of SSB (see legend to Fig. 2) in 30 μl of 50 mM sodium acetate buffer (pH 4.5), 1 mM ZnSO_4 , and 6 U of S1 nuclease were added; the digestion was continued for 10 min at 22°C and the reaction was stopped by phenol extraction and ethanol precipitation. DNA was analyzed by electrophoresis on a 1% agarose gel after digestion with *Ava*II as described [10,13].

2.5. Other methods

Agarose gel electrophoresis studies and DNA sequencing determinations were performed as described previously [10,12,13,16,33–37]. Bands on gels were quantitated as described previously [17,38].

3. RESULTS

3.1. SSB binds to supercoiled DNA at acidic pH and the DNA–protein complex is not destroyed by OsO_4 reagent

The pur · pyr inserts containing different sequence arrangements and base compositions which are employed in these studies are shown in Fig. 1. Many oligopurine · oligopyrimidine sequences, including those listed in Fig. 1, form Hy-3 isomers at acidic pH [5,10,14,39]. Thus, in order to assess the effect of SSB binding on the structure of the intramolecular triplex-

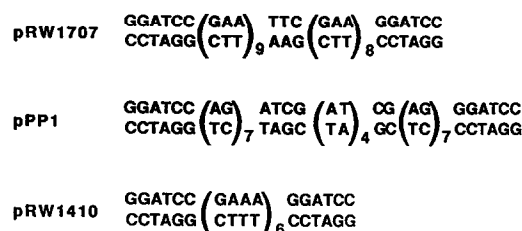


Fig. 1. Inserts in the plasmids used in these studies.

forming sequence in supercoiled plasmids, it was necessary to define a range of pH conditions at which the SSB–DNA complexes were stable. It was also necessary to identify a base-specific and structure-sensitive probe that did not destroy this interaction. For this analysis we used an agarose gel retardation assay [40].

A typical example of agarose gel electrophoretic determinations to evaluate the binding of SSB protein to the recombinant plasmids is shown in Fig. 2A. The conditions employed for the preparation of this protein–DNA complex were identical to those employed to study the effect of the SSB binding on the conformation of the triplex (described in Fig. 2B). As the concentration of SSB was increased, a retardation of the DNA migration was found (Fig. 2A, lanes 2–5). However, no change was found in the electrophoretic mobility of the nicked DNAs. At the highest concentration of SSB employed, slight retardation of the nicked DNA was observed occasionally. Thus, with this assay and under these conditions, the SSB protein binds to the supercoiled but not to the relaxed plasmids. Identical results were also observed with pPP1 and pRW1410.

The binding of SSB protein to the vector DNA showed results similar to those in Fig. 2A. Thus, the SSB binding does not appear to be insert specific in this assay. Sensitivity of the agarose gel retardation method most likely does not allow us to make a distinction between preferential interactions. This result was anticipated since this protein is known to bind to plasmids which do not contain intramolecular triplexes [40]. The results presented below in Fig. 2B and Fig. 3 demonstrate that the triplex conformation may be a preferred binding site for the protein.

The protein–DNA complex can be demonstrated under a broad range of pHs (4.5–8.5) using the agarose band-shift assay. This is especially important since all plasmids listed in Fig. 1 form intramolecular triplexes at acidic pH [10,12,13,31]. The presence of 5 mM OsO_4 , 8 mM KMnO_4 , or DEPC (saturated conditions) completely inhibit the interaction. The complex formation is not substantially inhibited, however, by the presence of 0.9 mM OsO_4 , 0.5% pyridine or 0.5% DMS. Initial results showed that the DMS reagent was not effective in footprinting SSB–DNA complexes. Thus, among the probes tested, OsO_4 was the only chemical suitable for probing the conformation of the triplex-forming se-

quence in supercoiled DNAs complexed with the SSB protein. We found the agarose gel retardation method extremely useful in determining whether the protein–DNA interaction takes place or is abolished at the given set of experimental conditions.

3.2. Effect of SSB on the triplex-forming sequence containing 75% AT bp (pRW1410)

OsO₄ in the presence of the ligand (i.e. pyridine) adds to the 5,6 double bond of thymine forming an osmate ester. Thymine residues in duplex DNA are not reactive. However, single-stranded DNA or unpaired regions of the duplex molecule are highly sensitive to this probe [41]. Accordingly, the single-stranded regions of the triplex structure that contain thymine residues were identified and mapped [10–12,17,31], providing important evidence for formation of H-DNA in supercoiled molecules.

Fig. 2B shows the effect of SSB on the OsO₄ reactivity of the triplex-forming sequence of pRW1410 ($-\sigma = 0.06$). At pH 5.0 and in the absence of SSB, the insert of this plasmid adopts an Hy-3 isomer of the triplex [10], resulting in strong OsO₄ reactivity of the center of the pyrimidine strand (loop region) and at the 3' end of the pyrimidine tract (3' junction region) (lane 1). Upon addition of the SSB protein (0.045 μ g/3 μ g of plasmid), the 3' half of the pyrimidine strand, which serves as a Hoogsteen paired third strand of the triplex, becomes much more reactive compared to the 5' half of the same strand. This result can be interpreted as formation of the melted bubble of about half of the pur·pyr tract. Further increase in SSB concentration renders the entire (TTTC)₆ sequence evenly hypersensitive to OsO₄. This behavior most likely reflects the unpaired state of the entire pur·pyr block.

It is worth noting that uniform OsO₄ sensitivity, which is SSB dependent, of the entire oligopyrimidine sequence correlates with a diminishing of intensities at the T residues at the 3' junction (indicated by arrowheads). In contrast, the thymines located further outside the pyr·pyr block, i.e. at the 5' end of the sequence, reveal a slight increase in accessibility to the chemical probe at high concentrations of SSB (indicated by arrows on Fig. 2B).

In a control experiment we addressed the question as to whether SSB-induced changes were restricted to acidic conditions under which the H-DNA structure forms within the pur·pyr region of pRW1410. At neutral pH this sequence can not adopt a triple-stranded conformation [10]. An experiment similar to that shown in Fig. 2B was conducted except that sodium acetate buffer, pH 5.0, was replaced with Tris-HCl buffer, pH 7.3. As expected, there was no increase in reactivity of T residues within the (TTTC)₆ strand in the presence of SSB. Thus, the elevation in OsO₄ sensitivity coincides with H-DNA formation within the pur·pyr sequence at pH 5.0.

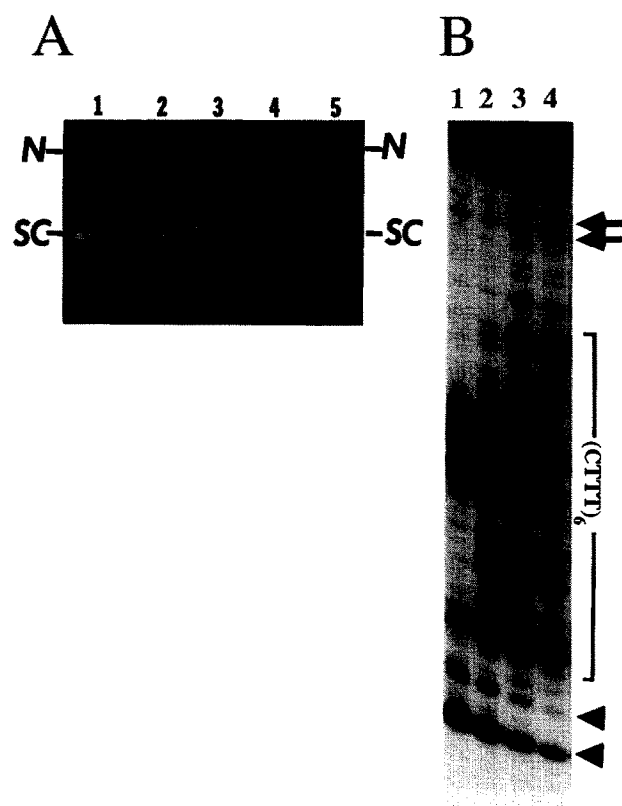


Fig. 2. (A) Agarose gel electrophoresis of pRW1707 ($-\sigma = 0.060$) complexed with *E. coli* SSB protein. 3 μ g of the plasmid in 50 mM sodium acetate buffer (pH 5.0), 0.5% pyridine was supplemented with 1 μ l of SSB solution containing 0.045 μ g (lane 2), 0.225 μ g (lane 3), 0.45 μ g (lane 4) or 2.5 μ g (lane 5) of the protein. The sample shown in lane 1 was supplemented with 1 μ l of the buffer in which SSB was stored (0.2 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5, 50% glycerol) (no protein added). The total volumes of the reaction mixtures were 30 μ l. After 30 min at 22°C, OsO₄ was added to a final concentration of 0.9 mM. Modification proceeded for another 30 min and 1/10 of the volume was loaded on a 1% agarose gel made in 50 mM sodium acetate buffer, pH 5.0. N, nicked DNA; SC, supercoiled DNA. (B) Effect of SSB on OsO₄ reactivity of pRW1410 insert. 3 μ g of pRW1410 ($-\sigma = 0.06$) was complexed with the SSB protein (0, 0.045, 0.225 and 0.45 μ g of SSB in lanes 1–4, respectively) in 50 mM acetate buffer, pH 5.0, and treated with OsO₄ reagent as described in A. The reaction was stopped by ethanol precipitation. The plasmid was digested with *EcoRI*–*PstI*, labelled at the 3' end, and the radioactive insert was purified by acrylamide gel electrophoresis. After piperidine treatment (90°C, 30 min) the sample was resolved on the sequencing gel. The same number of counts was loaded on each channel.

3.3. Effect of SSB binding on the triplex structure of pRW1707 and pPP1

The SSB protein binds single-stranded oligo(dT) with the greatest specificity [27,29]. Binding constants to other single-stranded polymers are somewhat lower. Thus, we were interested to extend the OsO₄ mapping studies to the supercoiled plasmids with pur·pyr inserts containing 66% (pRW1707) or 50% AT bp (pPP1).

Fig. 3A demonstrates the effect of SSB binding to native pRW1707 on the OsO₄ modification pattern as analyzed by the bp sequencing procedure developed

previously [10,34]. In the absence of SSB protein, the insert adopts different types of triplex structures as a function of pH [31]. At pH 7.0 (lane 6), the insert forms a standard Hy-3 isomer [5] with strong OsO_4 modification sites in the center (loop region) and at the 3' end of the pyrimidine strand (junction region). However, at pH 5.0 (lane 1), this insert seems to adopt a structure simultaneously containing two triplexes [31].

In the presence of increasing concentrations of SSB protein, an increased reactivity of OsO_4 at the T residues which are involved in the T·A·T triplex cores (indicated by brackets in Fig. 3) were observed at both pH values employed. A somewhat higher SSB concentration is required to cause these effects as compared to pRW1410 (Fig. 2B). Densitometric quantitation of the data in Fig. 3 reveals an increase in reactivity of 10- to 50-fold at the highest concentration of SSB. Similar experiments were performed using native ($-\sigma = 0.06$) pPP1 plasmid (data not shown). The insert of this plasmid adopts a triple-stranded conformation at pH 5.0 [12]. Uniform increase in OsO_4 reactivity of T residues of the triplex core was found at the protein:DNA ratio

of $0.225 \mu\text{g}$ SSB per $3 \mu\text{g}$ of pPP1. Thus, the behavior of the triplex structure of the insert of pPP1 in response to SSB was the same as the structural change observed for inserts of other plasmids.

Studies were conducted to evaluate the specificity of the increased reactivity in the triplex region caused by binding of the SSB protein. The behavior of T residues located 20–110 bp away from the pur·pyr block was studied according to the method described in Fig. 3. In virtually all cases, only background reactivity was found; however, in a few cases, small (2–3 fold) increases in OsO_4 accessibility were observed (data not shown). Thus, we conclude that over the examined region of the plasmid sequences the triplex stretch is the preferred site of SSB-induced enhancement in OsO_4 reactivity.

A control study was performed to determine the potential effect of SSB binding to single-stranded DNA on its OsO_4 reactivity. The synthetic oligomer 5'-GATC-CGAA(TTC)₉G was incubated with various amounts of SSB (up to 60/1 SSB monomer to DNA) at 22° for 30 min. In all cases, all thymines were modified; no differ-

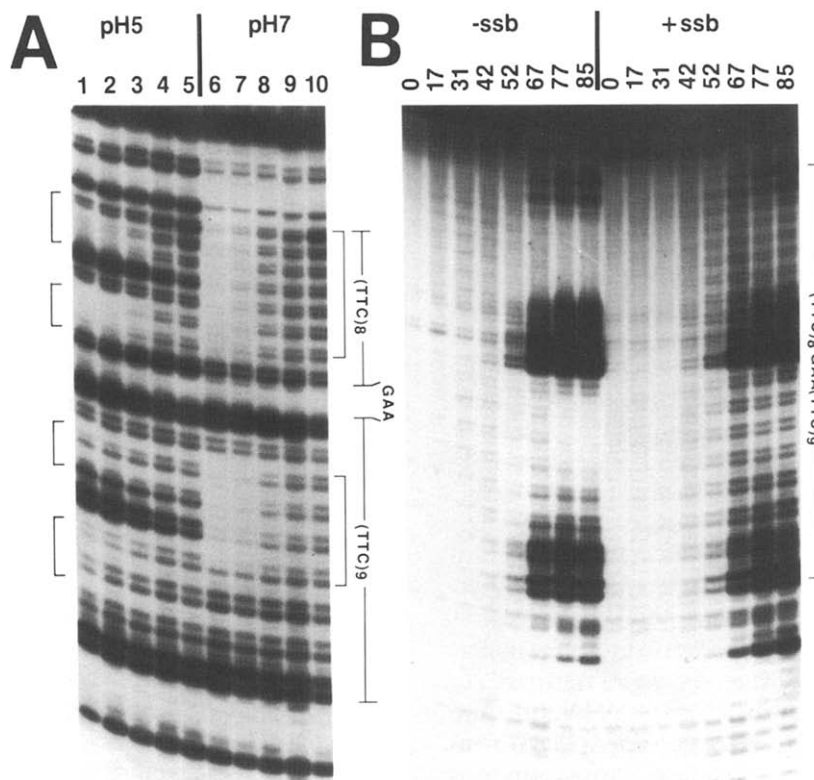


Fig. 3. The effect of SSB binding to pRW1707 on OsO_4 reactivity of T residues within the pur·pyr insert. (A) Lanes 6–10: the pRW1707–SSB complex was prepared and modified with OsO_4 at pH 7.0. The plasmid was at an average negative supercoil density of approximately $-\sigma = -0.060$. The modifications of the pRW1707–SSB complex shown in lanes 1–5 were performed under the same conditions, except that the 50 mM Tris-HCl buffer was replaced with 50 mM sodium acetate buffer, pH 5.0. Arrow bars indicate T residues within the pur·pyr block, whereas the brackets show regions of the sequence in which reactivity of T's increase upon increasing the protein:DNA ratio. Other details as in the legend to Fig. 2. (B) OsO_4 modification was performed in 50 mM Tris-HCl buffer, pH 5.0. Topoisomeric samples ($-\sigma \times 10^3$ as indicated) of pRW1707 were prepared by treating plasmid with topoisomerase in the presence of various concentrations of ethidium bromide [32]. The samples in the first 8 lanes were treated with SSB dilution buffer, whereas the samples shown in the rightmost 8 lanes were supplemented with $0.225 \mu\text{g}$ of SSB before OsO_4 modification. Further workup was identical to that described in (A). The bracket indicates the pur·pyr block.

ence was found between the free oligomer and the protein-DNA complexes (data not shown). This result is consistent with the assumption that the increased OsO_4 reactivity of the triplex-forming sequences in plasmids is due to the SSB-induced changes in the structure of this region.

3.4. Stoichiometry of binding

The most dramatic effect of the SSB protein was found for the insert in pRW1410 at pH 5.0. A very pronounced enhancement in modification of T residues forming the triplex core was observed at the lowest DNA:protein ratio employed. Specifically, 0.045 μg of SSB per 3 μg of plasmid was sufficient to cause the effect as compared with 0.225 μg of SSB for the same amount of plasmid required in the case of pRW1707 (Fig. 3, lanes 3 and 8) as well as for pPP1. pRW1410 contains an insert 24 bp long of 75% AT content. The AT-richness of the insert may be responsible for this behavior. Since this general effect translates to approximately 5 monomer SSB molecules (or 1.25 tetramers) per plasmid molecule, we conclude that the SSB protein interacts with preference at the triplex region.

Except for pRW1410 (pur·pyr insert of 75% AT) conditions were not reached where 100% of the triplex DNA was destroyed, even at the highest SSB concentrations employed (55 SSB monomers per 1 plasmid molecule (see Fig. 3A, lanes 5 and 10)). Such behavior clearly depends on the sequence composition of the triplex forming insert (% of AT). The vector serves as a 70- to 100-fold excess of competitor DNA in these determinations. Furthermore, other sequences may adopt cruciforms and other types of non-B DNA structures. These structures are likely to compete more effectively for SSB protein with triplexes containing higher content of C·G·C⁺ triads. Prior work [42] showed that *E. coli* SSB protein bound to supercoiled DNA containing the *Drosophila melanogaster* histone gene repeat by stabilizing denaturation bubbles that map near the gene boundaries.

3.5. Specificity for SSB

Studies were conducted with other proteins to evaluate the specificity of this behavior (Figs. 2 and 3) to the SSB protein. Substitution of the SSB protein with the *E. coli* RecA protein (up to 5.4 μg per 3 μg of supercoiled pRW1707, with or without ATP) did not result in an enhancement of the OsO_4 -modified Ts as compared to the control where the protein was omitted. Hence, this important recombination protein, which binds single-stranded DNA, cannot replace the SSB protein in this system. Control experiments revealed that the RecA protein survived the OsO_4 treatment, as revealed by the ability of the treated protein to bind and retard plasmids during agarose gel electrophoresis. Also, substitution of the SSB protein with bovine serum albumin (up to 4 μg per 3 μg of the supercoiled

pRW1707) showed no change from the control in the absence of protein. Studies with the T4 gene 32 protein were not possible since the OsO_4 reaction rendered the protein inactive.

3.6. SSB-dependent OsO_4 reactivity correlates with the supercoil-induced triplex formation

Investigations were conducted with topoisomers of pRW1707 to determine if the enhanced OsO_4 reactivity of T residues requires the pur·pyr sequence to be in the H-DNA state. Topoisomeric samples of pRW1707 were modified with OsO_4 under standard conditions (pH 7.0) and the resulting modification patterns of the pyrimidine strand (Fig. 3B) were determined in the absence and presence of a fixed amount of SSB protein. As expected, in the absence of SSB, the formation of the triplex occurs in the insert at or above supercoil densities of -0.067 ; enhanced sensitivity of thymines located mainly in the center or at the 3' end of the pyrimidine strand was found. However, in the presence of SSB the OsO_4 hyper-reactivity is generated at approximately the same supercoil density as the formation of the triplex. Relaxation below the $-\sigma$ value necessary to stabilize H-DNA renders the triplex sequence insensitive to OsO_4 , whether or not the SSB is present. Therefore, we conclude that the H-DNA formation is responsible for the observed effect.

3.7. Other probes

As discussed earlier, DEPC, which reacts with the single-stranded 5' half of the purine strand of the Hy-3 isomer of the triplex, readily destroyed the SSB supercoiled DNA complexes as demonstrated by agarose gel electrophoresis. In accord with this observation, SSB caused no change in the DEPC modification pattern of the triplex-forming sequences. For the same reason, 8 mM KMnO_4 was found to be ineffective.

DMS, which does not destroy the complex, also failed to detect changes in the modification patterns of G residues on the purine strands of pRW1707 or pPP1 after complex formation with SSB or the T4 gene 32 protein. Furthermore, we were unable to demonstrate any effect of SSB binding on S1 nuclease reactivity of triplexes, although the protein binds readily to the plasmids in the S1 buffer (sodium acetate, pH 4.5) used. The S1 nuclease sensitivity of the triplex-forming plasmids remained unchanged upon addition of the SSB protein (data not shown).

4. DISCUSSION

The pur·pyr stretches capable of forming H-DNA are frequently found in both prokaryotic and eukaryotic genomes and have been implicated in numerous biological events, such as replication, recombination and gene regulation [5,17,39]. The formation of H-DNA in *E. coli* cells has been reported by numerous

laboratories [43–45]. One striking feature of the intramolecular triplex structure is its partially non-paired segment. These segments are reactive to single-strand-specific probes, including the OsO_4 reagent [9–12,15–17,43].

The *E. coli* single-stranded DNA-binding protein is essential in replication and methyl-directed mismatch repair processes, and constitutes an important component of the homologous recombination reaction catalyzed by RecA protein [46,47]. It forms a stable tetramer in solution which seems to be an active form of the protein that interacts with single-stranded DNA in at least four different modes [46,47]. These modes differ by the number of nucleotides that are occluded per SSB tetramer.

The protein also binds to supercoiled DNA. Previously, it has been suggested that SSB molecules may melt supercoiled DNA at specific sites. For example, it was shown that plasmid DNA containing *Drosophila melanogaster* histone genes forms a denaturation bubble upon complexation with the SSB protein [42]. Using a dynamic light scattering method, Langowski et al. [40] have demonstrated that binding of SSB to pBR322 or pUC8 plasmids causes relaxation of the molecules. No formation of a denaturation region(s) was evident from these determinations.

In this work, we tried to assess the effect of the SSB protein binding on the supercoil and pH-induced intramolecular triplexes. We found that the *E. coli* SSB protein interacts with supercoiled plasmids containing intramolecular triplexes to generate an enhanced reactivity to OsO_4 of thymines in the triple-stranded core. This effect can be demonstrated using plasmids containing different base compositions in their triplex-forming pur · pyr inserts. Less protein was required for the plasmid containing a 75% AT-rich pur · pyr segment to generate the OsO_4 -enhanced reactivity, as compared with other more GC-rich substrates. Moreover, SSB-induced OsO_4 sensitivity of the triplex core correlates with the supercoil-induced transition of the oligopurine element into the triple-stranded state.

Although the triplex constitutes only a tiny (1–2%) portion of each plasmid, it seems to be a preferred site for the protein-dependent increase in OsO_4 reactivity. We have not analyzed the behavior of all T residues around the vector sequence, but those which were located 20–100 bp away from the inserts revealed no or very small enhancement in OsO_4 sensitivity. S1 nuclease mapping studies showed no SSB-induced sensitivity within the vector sequence.

Binding of the SSB protein to the circular molecules removes superhelical turns [40,42]. In one report it has been postulated that one SSB tetramer may unwind as many as 12.5 turns of the helix [40]. Such unwinding caused by a single tetramer would absorb almost all the free energy (ΔG) necessary to support H-DNA formation in the plasmids employed in this work. Clearly, the

SSB binding must be highly metastable, as suggested before [42]. Treatment of the complex with topoisomerase, even at the highest protein:DNA ratio employed, readily and completely relaxes the DNA (not shown). Alternatively, the SSB dissociation rate may be fast enough so that within the frame of our OsO_4 modification analysis (30 min) a portion of plasmid molecules remains supercoiled and contains the inserts in the triplex state. This, in combination with the lower affinity of SSB to the inserts containing less than 75% AT bp's, would explain why, in studies of pRW1707 and pPPI, we were not able to remove 100% of H-DNA, even at the ratio of 13 SSB tetramers per 1 plasmid molecule (2.5 μg SSB/3 μg of plasmid).

Based on the above data, we believe that the SSB protein may recognize the supercoil-induced triplex structure to form metastatic single-strand unpaired regions that are substantially enhanced in OsO_4 reactivity. This interpretation is in perfect accord with the prior hypothesis which assumed that the protein removes secondary folds or other secondary structures from DNA [48]. Manor and co-workers [49,50] have recently reported a phenomenon called 'replication arrest' that takes place during DNA polymerization. DNA polymerase stops were observed near the center of the oligopurine or oligopyrimidine templates due to the folding process that results in triple-stranded DNA formation. The replication arrest could be prevented by adding the SSB protein to the extension reaction mixture since it removes the folded-back structure [50]. Our analysis performed on supercoiled DNA is in perfect agreement with this interpretation. The SSB protein in our system also removes triplexes from plasmids. This removal of H-DNA is not a result of SSB-induced relaxation of superhelical turns. As demonstrated in the course of these studies (see Fig. 3), relaxation of plasmids with topoisomerase prevents SSB from inducing OsO_4 reactivity within the pur · pyr inserts.

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