The binding-site sizes of *Escherichia coli* single-stranded-DNA-binding protein and mammalian replication protein A are 65 and ≥ 54 nucleotides respectively

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The electrophoretic mobilities of complexes formed with single-stranded (ss) DNA and tetrameric *Escherichia coli* ssDNA-binding protein (*EcoSSB*) or mammalian replication protein A (RPA) were analysed. The electrophoretic mobilities of the complexes in a native polyacrylamide gel increased as the lengths of the DNA increased from 28 to 70 nt, thus revealing paradoxical

‘descending-staircase’ patterns. Increases in the electrophoretic mobilities of *EcoSSB*·ssDNA complexes were observed when the lengths of the bound DNA were increased by 1 nt. Quantitative analyses of the complexes suggested that the binding-site sizes of *EcoSSB* and RPA were 65 and ≥ 54 nt respectively. The binding-site size for RPA is at least 24 nt larger than previously reported.

INTRODUCTION

Single-stranded (ss)-DNA-binding proteins (SSBs) [1] play a general role in lagging strand synthesis by binding to ssDNA, resulting in the removal and/or prevention of secondary structures. These SSBs also stimulate the activities of their associated primases allowing for co-ordinated synthesis of leading and lagging strands. Perhaps the most thoroughly studied SSB is that of *Escherichia coli* (*EcoSSB*), which forms a tetrameric complex which binds co-operatively to ssDNA [2]. *In vitro* studies have demonstrated that ssDNA wraps around *EcoSSB* to form nucleosome-like complexes, which are presumably required for DNA replication and/or recombination [2].

A number of eukaryotic SSBs required for DNA replication have also been identified, such as adenovirus-encoded DNA-binding protein [3,4] and human replication protein A (human RPA; also known as ‘replication factor A’ or ‘human SSB’) [5]. *In vitro* replication of simian-virus-40 (SV40) DNA requires virus-encoded large T antigen and seven cellular proteins, including two DNA polymerases (α and δ), two polymerase accessory proteins, topoisomerases I and II and human RPA [6]. SV40 replication, like mammalian chromosomal replication, proceeds in a bidirectional manner.

The interactions of various SSBs with ssDNA have been analysed by a variety of techniques, such as filter binding, tryptophan-fluorescence quenching, electron microscopy and, most recently, electrophoretic-mobility-shift assays (EMSAs) [2,5,7,8]. The expectation of this type of analysis is that, once the length of the nucleotides is sufficient for the binding of two RPA molecules, the electrophoretic mobility of the (RPA)₂·ssDNA complex will be much lower compared with that of the (RPA)₁·DNA complex [9,10]. On the basis of decreased electrophoretic mobilities of RPA·ssDNA complexes, it has been concluded that the binding-site size of RPA was 8 and 30 nt in low [10] and high [9] salt respectively.

In the present study we examined the electrophoretic mobilities of *EcoSSB*·ssDNA and RPA·ssDNA complexes as a function of nucleotide length. We report that the electrophoretic mobilities

of the SSB·ssDNA complexes increased with increased DNA length up to 70 nt. The results of these studies provide a new interpretation of how SSB proteins interact with DNA and also assign a larger nucleotide-binding-site size for RPA.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer (Foster City, CA, U.S.A.) with the trityl group on and purified with oligonucleotide purification cartridges (Cruachem, Glasgow, Scotland, U.K.). Sequences of oligonucleotides and their corresponding complements (in the 5′-to-3′ direction) were:

(ATC)_n: GATCC(ATC)_nTAGA

(GAT)_n: AGCTTCTA(GAT)_nG

(where $n = 1-10, -15$ and -19). d(T)_{29,30,31,32,40,50,60} contained only thymine residues.

Labelling of synthetic single-stranded oligonucleotides

A 5 µg portion of column-purified synthetic oligonucleotide containing poly d(T)_n was 5′-end-labelled with [γ -³²P]ATP by incubation in buffer containing 1 mM dithiothreitol (DTT), 1 µl of 7000 Ci/mmol [γ -³²P]ATP (ICN), 50 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 0.1 mM spermidine, 0.1 mM EDTA and 20 units of T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ -³²P]ATP was removed by size-exclusion chromatography (NucTrap column; Stratagene).

Labelling of DNA cleaved from plasmid

Various numbers of d(ATC)_n·(GAT)_n repeats were cloned into the *Bam*HI/*Hind*III sites of the pBLCAT2 vector [11] as previously described [12]. Oligonucleotides were labelled on the d(ATC)_n-containing strand only and were liberated from plasmid as previously described [12].

Abbreviations used: ss, single-stranded; (*Eco*)SSB, (*Escherichia coli*) ss-DNA-binding protein; RPA, replication protein A; EMSA, electrophoretic-mobility-shift assay; M_{rel} , relative electrophoretic mobility; SV40, simian virus 40; DTT, dithiothreitol.

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Figure 1 EMSA of *EcoSSB*·DNA complexes containing 30–60 nucleotides of $d(T)_n$

Binding reactions were performed as described in the Materials and methods section and contained 7.5, 6.6, 8.7, and 5.1 nM $d(T)_{30}$, $d(T)_{40}$, $d(T)_{50}$, or $d(T)_{60}$ respectively and 16 nM *EcoSSB* (where indicated). **(A)** EMSA in the absence of competitor DNA. Complexes were formed in the absence (left-hand panel) or presence (right-hand panel) of *EcoSSB* and applied to a 4% polyacrylamide gel. Symbols used to denote the position of the respective complexes are shown to the left of the Figure. The plus sign refers to the complex formed with $d(T)_{30}^*$, and not $d(T)_{30}$. The nucleotide length of the labelled DNA is indicated at the top of the Figure. **(B)** EMSA in the presence of competitor $d(T)_{30}$. Binding-reaction mixtures contained various concentrations of unlabelled $d(T)_{30}$ ranging from 0 to 5600 nM. Shown above are the results of electrophoretic analysis of three of the 15 reactions. The concentration of polyacrylamide in the gel was 6%. The unfilled symbols to the right of the Figure correspond to those shown in **(A)**. The filled symbols correspond to complexes not observed in **(A)**. **(C)** Competition analysis. The autoradiographs from **(B)** were scanned three times with a PDI (Huntington Station, NY, U.S.A.) densitometer to determine relative binding. Background subtracted from the scanned images was 0.15 A unit. Relative binding is defined as the amount of radioactivity in a given complex divided by the total radioactivity. Symbols correspond to the same complexes shown in **(A)** and **(B)**. The molar fold excess of unlabelled $d(T)_{30}$ required for 50% inhibition of a singly liganded complex (open symbol) containing $d(T)_{30}^*$, $d(T)_{40}^*$, $d(T)_{50}^*$, or $d(T)_{60}^*$ was 8.0, 12, 55, or 210 respectively.

Extracts

NG108-15 mouse neuroblastoma × rat glioma hybrid cells [13] were maintained in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, U.S.A.) with 10% fetal-bovine serum. Nuclear extracts were prepared from subconfluent NG108-15 cells by the method of Dignam et al. [14]. Cytosolic extract prepared from HeLa cells [15] was generously given by Dr. Marc Wold, University of Iowa College of Medicine.

EMSA

Binding reactions were performed in 10 μ l and contained 4×10^4 d.p.m. (≈ 7.0 fmol) of DNA, 8% glycerol, 0.125–0.20 M NaCl, 18 mM Hepes, pH 7.40, 1 mM EDTA, 1 mM DTT, and 0.16–0.64 μ M *EcoSSB* (USB, Cleveland, OH, U.S.A.). The reaction mixtures were incubated at room temperature or 37 °C for 20 min. A 1 μ l portion of loading dye (50% glycerol/0.4% Bromophenol Blue) was added to the DNA samples prior to gel electrophoresis. During electrophoresis, 15 °C tap water was circulated through a Hoefer (San Francisco, CA, U.S.A.) SE 600 series unit. Electrophoresis was performed in 1 × TBE buffer (45 mM Tris/borate/1 mM EDTA) through a 4 or 6% poly-

acrylamide gel at 25 mA/gel. Gel plates were 14 cm long × 16 cm wide × 1.5 mm thick. Electrophoresis was stopped when the Bromophenol Blue marker had migrated 10 cm. Dried gels were placed between two intensifying screens (Du Pont) and exposed to Fuji RX film for 2–48 h at –80 °C.

Purified proteins

EcoSSB was obtained from United States Biochemical Corp. (Cleveland, OH, U.S.A.). Recombinant human RPA [16] was generously given by Dr. Marc Wold.

RESULTS

In the absence of *EcoSSB*, the mobilities of 5'-end-labelled synthetic oligonucleotides containing $d(T)_{30}$, $d(T)_{40}$, $d(T)_{50}$, or $d(T)_{60}$ were rapid and correlated inversely with nucleotide length (Figure 1A). Incubation of $d(T)_{30}$, $d(T)_{40}$, or $d(T)_{50}$ with 0.16 μ M *EcoSSB* (125 mM NaCl) resulted in the formation of *EcoSSB*·DNA complexes that were much slower-migrating compared with free DNA. However, the mobilities of the complexes were more-or-less directly dependent upon nucleotide length (Figure 1A). Binding of $d(T)_{60}$ to *EcoSSB* resulted in the

Figure 2 EMSA of *Eco*SSB-DNA complexes containing 29–32 nucleotides of d(T)

Binding reactions were performed as described in the Materials and methods section and contained 16 nM *Eco*SSB and 8.1, 7.3, 7.4, and 6.0 nM d(T)₂₉, d(T)₃₀, d(T)₃₁ or d(T)₃₂. The nucleotide length of the labelled DNA in the binding reaction is indicated at the top of the Figure. Products were applied to a 4% polyacrylamide gel. Shown are the *Eco*SSB-DNA complexes only. In order to maximize resolution of the complexes, electrophoresis was performed for 4 h, during which time the free probes migrated out of the polyacrylamide gel.

formation of two complexes; one complex migrated faster than those formed with the other oligonucleotides, while the second migrated at a position equivalent to the complex formed with d(T)₃₀.

To investigate the interactions of *Eco*SSB and ssDNA further, end-labelled oligonucleotides containing d(T)₃₀, d(T)₄₀, d(T)₅₀ or d(T)₆₀ [hereafter denoted with an asterisk (*) to indicate the ³²P label] were incubated in the presence of various amounts of unlabelled d(T)₃₀. In theory, at high concentrations of unlabelled d(T)₃₀, *Eco*SSB could become 'doubly-liganded' such that the electrophoretic mobility of a complex containing d(T)₃₀* · d(T)₃₀ · *Eco*SSB might equal the electrophoretic mobility of the rapidly migrating complex containing d(T)₆₀*.

In the presence of 175 nM d(T)₃₀, the complex formed with d(T)₃₀* was completely competed, while the complex containing d(T)₆₀* was not competed (Figures 1B and 1C). These data demonstrate that the affinity of *Eco*SSB for d(T)₆₀* was higher compared with d(T)₃₀*. In support of this conclusion, an 8.0-fold molar excess of unlabelled d(T)₃₀ was required for 50% inhibition of the complex containing d(T)₃₀*, whereas a 210-fold molar excess of d(T)₃₀ was required for comparable inhibition of the complex containing d(T)₆₀* (Figure 1C).

In the presence of 175 nM and 350 nM d(T)₃₀, significant amounts of complexes exhibiting high mobility were formed with d(T)₄₀* and d(T)₅₀* respectively (Figures 1B and 1C). The mobilities of the rapidly migrating complexes were comparable with that of the complex formed with d(T)₆₀* (results not shown). These results provide evidence that doubly liganded *Eco*SSB complexes can be formed with d(T)₄₀* and d(T)₅₀*. The results of the competition experiments also provide evidence that *Eco*SSB is in the tetrameric form which binds 65 nt [(SSB)₆₅ binding mode], rather than the dimeric form which binds 35 nt [17]. If *Eco*SSB were in the (SSB)₃₅ binding mode, the addition of unlabelled d(T)₃₀ to an *Eco*SSB · d(T)₄₀* complex should not result in the formation of a new complex with increased mobility. The conclusion that *Eco*SSB is in the (SSB)₆₅ binding mode is also supported by additional experiments described below.

A 1 nt change is sufficient for alteration of the electrophoretic mobility of an *Eco*SSB-DNA complex

To determine the minimal nucleotide length necessary for a change in the electrophoretic mobility of an *Eco*SSB-DNA complex, an EMSA was performed with d(T)₂₉, d(T)₃₀, d(T)₃₁ and d(T)₃₂. The electrophoretic mobilities of the complexes increased with increasing d(T)_n (Figure 2), indicating that the

Figure 3 EMSA of *Eco*SSB-DNA complexes containing fragment 2

EMSA was performed as described in the Materials and methods section. The oligonucleotide probes were prepared by restriction-enzyme digestion with *Hind*III and *Eco*RI, instead of *Hind*III and *Bam*HI. See the text for the nucleotide sequence of the probe DNA. The nucleotide lengths of the probes, and the number of ATC repeats within them, are indicated in the Figure. Binding reactions were the same as those described in the legend to Figure 2. The polyacrylamide concentration of the gel was 6%. In the absence of protein, probe DNA was present in both single- and double-stranded forms (results not shown). The signal at the bottom of the gel represents free probe DNA only in the double-stranded form. Single-stranded DNA containing the ATC repeats, which migrates slower than its double-stranded form [22], is only present in the shifted complexes. Values at the top of the Figure represent the numbers of ATC repeats contained in fragment 2.

minimal value of *n* necessary for altering the electrophoretic mobility of an *Eco*SSB-DNA complex was 1.

Quantitative analyses of complexes formed with *Eco*SSB reveal a binding-site size of 65 nt

To investigate further the possibility that *Eco*SSB was in the 65 nt-binding mode, EMSAs were performed with ssDNA of the sequences d[GATCC(ATC)_nTAGAAGCT] (fragment 1) and d[AATTCGCCAATGACAAGACGCTGGCGGGGCCGATCC(ATC)_nTAGAAGCT] (fragment 2). Binding of *Eco*SSB to fragments 1 (results not shown) and 2 (Figure 3) resulted in the production of shifted complexes whose electrophoretic mobility increased with increased repeat length (up to ≈ 76 nt). When the length of the fragment was increased to 91 nt, a complex of slower mobility was observed, indicating that the complex contained two *Eco*SSB tetramers (Figure 3).

The binding-site size of *Eco*SSB was determined by plotting the relative amount of protein fluorescence (*F_r*) as a function of the ratio (nt of ssDNA)/(*Eco*SSB tetramer):

$$F_r = (F_0 - F)/F_0$$

where *F₀* is the amount of fluorescence of the protein in the absence of DNA [17]. In an analogous manner, the binding-site size of *Eco*SSB can be obtained by plotting the relative electrophoretic mobility (*M_{rel}*) of *Eco*SSB-ssDNA complexes as a function of nucleotide length. In this case:

$$M_{rel} = (M_0 - M)/M_0$$

Figure 4 Binding-site-size determination of *EcoSSB* and mammalian RPA

Diagonal lines represent the linear regression of data points ≤ 70 nts (*EcoSSB*) and ≤ 43 nts (RPA) obtained by using Cricket graph software (Computer Associates International, Islandia, NY, U.S.A.). Data for $\circ$ and $\blacktriangledown$ are from Figures 3 and 6 respectively; other data sets are not shown. See the text for other details.

where M_0 is the mobility of protein in the absence of ssDNA and M is the distance the *EcoSSB* complex migrated from the well origin. To obtain an estimate for the value of M_0 , the M_{rel} of the *EcoSSB* complex formed with 70 nt was arbitrarily set to 1.00. The plots of M_{rel} as a function of nucleotide length for *EcoSSB* complexes formed with fragments 1 and 2 are shown in Figure 4. The nucleotide fragment length corresponding to the point where the line of linear regression intersects the maximum M_{rel} is the estimated binding-site size for *EcoSSB*. The value obtained is 65, which is in good agreement with that obtained in previous studies [2].

The binding-site size of mammalian RPA is ≥ 54 nt

RPA expressed in HeLa cells, rodent cells or a recombinant gene expressed in *E. coli* was incubated with fragment 1 and subjected to EMSA analysis. In the HeLa-cell extract (results not shown) the complex that contained RPA was identified by interaction with an antibody to its 70 kDa subunit (Figure 5). Results obtained with an antibody to the 34 kDa subunit of RPA yielded identical results (not shown). Only one protein in the rodent extract formed a complex that exhibited electrophoretic properties similar to those of the HeLa RPA (complex A; Figure 6).

Figure 5 Antibody to the 70 kDa subunit of human RPA supershifts complex A formed with human HeLa-cell extract

EMSA and probe preparation were as described according to the legend in Figure 3. Probe DNA contained 58 nts and 15 ATC repeats. Where indicated, $2 \mu\text{l}$ of a 1:50 dilution of antibody to the 70 kDa subunit of RPA was incubated with human HeLa-cell extract at 37°C for 15 min prior to the addition of probe DNA. The complex that migrates slower than A only appeared with fragments ≥ 58 nts and presumably represents an RPA dimer. The polyacrylamide concentration of the gel was 4%.

Therefore it was assumed that this complex contained rodent RPA.

The M_{rel} values for monomeric RPAs expressed in the three systems were similar (Figure 4). To obtain an estimate of the binding-site size of RPA, the M_{rel} data were analysed in a manner similar to that described for *EcoSSB* (Figure 4). Although binding studies were performed with an 82 nt fragment and RPAs expressed in the three systems, there were no data corresponding to this point. Further, at 70 nt, data were only available for RPA expressed in *E. coli*. The lack of RPA data for the larger fragment sizes was due to formation of complexes containing RPA multimers, which migrated at rates much lower than the monomer. Since (i) data were not available for the larger fragments and (ii) the M_{rel} of the RPA•ssDNA formed with the 70 nt fragment significantly deviated from the line of linear regression, we assumed that the maximal electrophoretic mobility of an RPA complex occurred with the 70 nt fragment. Consequently, the binding-site size of mammalian RPA is estimated to be a minimum of 54 nt.

DISCUSSION

In the present paper we have documented that, at DNA fragment lengths insufficient for protein dimerization, the electrophoretic mobilities of *EcoSSB*•ssDNA and RPA•ssDNA complexes increased as the lengths of the DNAs were increased up to 70 nt, thus revealing paradoxical descending-staircase patterns. This result was unexpected, since the masses of the SSB•ssDNA complexes increased (and hence should migrate more slowly) as the lengths of the DNA increased. Since the binding-site size derived from M_{rel} measurements in this study (Figure 4) parallel the results obtained from previous tryptophan-fluorescence-quenching studies [17], the anomalous electrophoretic mobilities of the *EcoSSB*•ssDNA complexes likely result from an interaction between *EcoSSB* and ssDNA.

Figure 6 EMSA of rodent extract and fragment 1

EMSA was performed as described in the legend to Figure 3. The polyacrylamide concentration of the gels was 4%. Binding reactions contained 10 μ g of NG108-15 rodent nuclear extract. Complex A contains RPA. Complex D was exclusively formed with extract prepared from bovine mitochondria (results not shown) and therefore presumably contains mitochondrial SSB.

The descending-staircase pattern may be due to protein-induced changes in charge density of the DNA

Although the biophysical basis for the acceleration of electrophoretic mobility as a function of nucleotide length is not known, it likely arises from either DNA-induced changes in protein conformation or protein-induced changes in the charge density of the DNA. Record et al. [18] have estimated that there is 0.76 univalent cation 'thermodynamically bound' per phosphate in ssDNA. The dominant factor driving formation of SSB·ssDNA complexes is, not surprisingly, the entropic contribution from the release of cations from the DNA [18]. In the case of *Eco*SSB·ssDNA interactions, it has been calculated that 16 ($\pm$ 4) univalent cations are released from ssDNA upon binding an *Eco*SSB tetramer [19]. The release of these cations, in theory, would unshield 21 phosphates of the DNA backbone, thus causing an increase in its negative charge upon binding *Eco*SSB, and a concomitant increase in its electrophoretic mobility.

The binding-site size of mammalian RPA determined by electrophoretic analyses is larger than that determined by tryptophan fluorescence quenching

*Eco*SSB forms a stable homotetramer which binds with high affinity to ssDNA [2]. Owing to the symmetric nature of this protein, many of the tryptophan residues are in similar or identical environments. Thus, if one disregards potential negative-co-operativity [20] effects, the tryptophan residues of *Eco*SSB are anticipated to be uniformly quenched as the length of the bound DNA is increased. However, fluorescent quenching of heterotrimeric RPA may not be uniform, which may lead to a low estimate of its binding-site size. In the present study we have utilized a technique that does not rely upon tryptophan-fluorescence quenching and provide evidence that the binding-site size of monomeric RPA is at least 54 nt in length, a value much larger than that reported by Wold and colleagues [9,21].

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