Functional Analysis of Multiple Single-stranded DNA-binding Proteins from *Methanosarcina acetivorans* and Their Effects on DNA Synthesis by DNA Polymerase BI*

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Single-stranded DNA-binding proteins and their functional homologs, replication protein A, are essential components of cellular DNA replication, repair and recombination. We describe here the isolation and characterization of multiple replication protein A homologs, RPA1, RPA2, and RPA3, from the archaeon *Methanosarcina acetivorans*. RPA1 comprises four single-stranded DNA-binding domains, while RPA2 and RPA3 are each composed of two such domains and a zinc finger domain. Gel filtration analysis suggested that RPA1 exists as homotetramers and homodimers in solution, while RPA2 and RPA3 form only homodimers. Unlike the multiple RPA proteins found in other Archaea and eukaryotes, each of the *M. acetivorans* RPAs can act as a distinct single-stranded DNA-binding protein. Fluorescence resonance energy transfer and fluorescence polarization studies revealed that the *M. acetivorans* RPAs bind to as few as 10 single-stranded DNA bases. However, more stable binding is achieved with single-stranded DNA of 18–23 bases, and for such substrates the estimated $K_d$ was 3.82 ± 0.28 nM, 173.6 ± 105.17 nM, and 5.92 ± 0.23 nM, for RPA1, RPA2, and RPA3, respectively. The architectures of the *M. acetivorans* RPAs are different from those of hitherto reported homologs. Thus, these proteins may represent novel forms of replication protein A. Most importantly, our results show that the three RPAs and their combinations highly stimulate the primer extension capacity of *M. acetivorans* DNA polymerase BI. Although bacterial SSB and eukaryotic RPA have been shown to stimulate DNA synthesis by their cognate DNA polymerases, our findings provide the first in vitro biochemical evidence for the conservation of this property in an archaeon.

Single-stranded DNA-binding proteins (SSB) play essential roles in cellular DNA transactions including DNA replication, repair, and recombination. In DNA replication, the function of SSB is to protect and stabilize the intermediate ssDNA that is generated by the unwinding action of a DNA helicase at the replication fork. In addition, SSBs prevent the formation of secondary structures by single-stranded template DNA. In bacteria, such as *Escherichia coli*, SSB is composed of a single polypeptide that oligomerizes in solution to form a homotetramer (2). In contrast, eukaryotic SSB or replication protein A (RPA) is a stable complex of three distinct subunits, with the human RPA being composed of 70-, 32-, and 14-kDa polypeptides (1). Although the SSBS in eukaryotes and bacteria differ in subunit composition, they are composed of four single-stranded DNA-binding domains, also known as oligonucleotide/oligosaccharide binding (OB) folds: there is one in each monomer of the homotetrameric bacterial SSB (2), three in RPA70 and one in RPA32 of the human (3).

In the Archaea, which together with Bacteria and Eukarya constitute the three domains of life, single-stranded DNA-binding proteins have been described for only thermophilic and hyperthermophilic organisms (4–8), although this domain includes numerous mesophilic genera. As observed with other proteins involved in information processing (9), SSB or RPA in Archaea exhibit more similarity to eukaryotic RPAs than to bacterial SSBS (10). It is worth noting, however, that the organization of RPAs in the currently cultivable Archaea, namely the Euryarchaeota and Crenarchaeota (11), differs. In the Euryarchaeota, single-subunit RPAs containing multiple OB folds and a putative zinc finger motif have been characterized from *Methanococcus jannaschii* (Mja) (4) and partially characterized from *Methanothermobacter thermoautotrophicus* (Mth) (5), while in *Pyrococcus furiosus* (Pfu) a heterotrimeric RPA (RPA41, RPA32, RPA14) has been reported (6). Each of the subunits of the PfuRPA contains an OB fold, and in addition PfuRPA41 contains a zinc finger-like motif. In the Crenarchaeota, an RPA has been described in only *Sulfolobus solfataricus* (Sso). Interestingly, the SsoRPA is a single polypeptide containing a single ssDNA-binding domain (7, 8). The protein, however, oligomerizes in solution to form a homotetramer similar to the *E. coli* SSB (8). Thus, as in the other domains of life, the archaeal SSBs contain single OB domains (9). However, the archaeal SSBs can oligomerize in solution to form higher order oligomers (10).

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1 The abbreviations used are: SSB, single-stranded DNA-binding protein; RPA, replication protein A; OB fold, oligonucleotide/oligosaccharide binding fold; PolBI, DNA polymerase BI; dT, Thymine; DTT, dithiothreitol; PCNA, proliferating cell nuclear antigen; EMSA, electrophoretic mobility shift assay; deDNA, double-stranded DNA; ssDNA, single-stranded DNA; FPA, fluorescence polarization anisotropy; FRET, fluorescence resonance energy transfer; PAR, 4-(2-pyridylazo) resorcinol; MMTS, methyl methanethiosulfonate; Mac, *Methanosarcina acetivorans*; Mja, *Methanococcus jannaschii*; Mth, *Methanothermobacter thermoautotrophicus*; Pfu, *Pyrococcus furiosus*; Sso, *Sulfolobus solfataricus*; Mka, *Methanothermobacter kandleri*; Tvo, *Thermococcus volcanium*; Fa, *Ferroplasma acidarmanus*; Hap, *Halobacterium sp.* NRC-1.

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all characterized archaeal RPA s exist as multiple OB fold-containing complexes.

We are investigating the mechanism of DNA replication in *M. acetivorans* (Mac), a mesophilic archaeon harboring the largest archaeal genome (5.75 Mb) sequenced to date. Here we describe the identification of three distinct genes, *rpa1*, *rpa2*, and *rpa3*, coding for multiple ssDNA binding activities, and the characterization of their gene products. The architectures of the *M. acetivorans* RPA s, which are highly conserved in *M. mazei* (5), differ from those of the characterized archaeal RPA s, and unlike the *M. thermoautotrophicus* RPA, which inhibits its DNA polymerase BI (PolBI) homolog (5), the 

## EXPERIMENTAL PROCEDURES

### Cloning of Multiple RPA-encoding Genes from *M. acetivorans*

The gene coding for *PhsRP4A1* was used in searching for RPA-like sequences in the *M. acetivorans* genome (http://www.genome.wi.mit.edu/annotation/microbes/methanosarcina/). Three different genes coding for proteins containing OB fold-like sequences were found. Based on the sequence information, three different pairs of primers (MacRP1A/ MacRP1R, MacRP2A/MacRP2R, and MacRP3A/MacRP3B), as shown in Table I, were synthesized and used to PCR amplify the genes coding for the polypeptides designated MacRP1A (Genbank accession number AAM07979, conserved hypothetical protein), MacRP2A (Genbank accession number NP_617912, hypothetical protein), and MacRP3A (Genbank accession number AAAM4934, conserved hypothetical protein), respectively. PCR conditions were 25 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 40 s, and extension at 72 °C for 1 min 30 s. The PCR mixture contained 10 pmol of each primer, 200 μM each of dNTP, 5 units of *ExTaq* DNA polymerase and other ingredients as described by the manufacturer (TaKaRa Bio Inc.).

Each PCR product was cloned into pGEM-T, a TA-cloning vector (Promega), and nucleotide sequenced to confirm the integrity of the gene. The forward and reverse primers for each gene contained an NdeI and an Xhol site, respectively. Thus, each gene was released from the TA-cloning vector with the two restriction endonucleases and ligated with buffer A. After extensive washes with the same buffer, each gene was bound to the same column. Each mixture was then analyzed by gel filtration as described for the individual proteins. The column was calibrated by running a set of protein standards (ferritin: 440 kDa, catalase: 232 kDa, aldolase: 158 kDa, albumin: 67 kDa, ovalbumin: 43 kDa, and ribonuclease A: 13.7 kDa, Amersham Biosciences) under the same conditions.

### Pull-down Assays

As further analysis for potential interactions among the RPA proteins, we designed a pull-down assay by immobilizing His6-tagged RPA1 onto a metal affinity column, as described above. The immobilized protein (bait) was then reacted with RPA2 or RPA3, or RPA2/RPA3 as prey in a buffer containing 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl. The proteins used as prey were produced as polypeptides containing N-terminal TT-tag encoded by the plasmid (Novagen). The proteins were produced in E. coli and purified as described above. Where interactions between RPA2 and RPA3 were examined, His8-tagged RPA2 was used as bait and TT-tagged RPA2 was used as prey. The bait/prey mixtures were incubated for 2 h at room temperature with gentle shaking, followed by extensive washing with the incubation buffer. The bound proteins were eluted with the same buffer containing imidazole at 150 mM concentration. Aliquots of eluted products were analyzed by Western blotting using antibodies against His6 tag (Pierce Biotechnology) and TT tag (Novagen) according to the manufacturer’s protocol.

### Electrophoretic Mobility Shift Assay (EMSA)

We used EMSA to determine whether each RPA protein binds to ssDNA and can discriminate between ssDNA and double-stranded DNA (dsDNA). The nucleotide sequence of the 42-mer oligonucleotide (MacMC-R) used as the substrate in EMSA is shown in Table I. The DNA was end-labeled with [-γ-32P]ATP and T4 polynucleotide kinase (Roche Applied Science). Each protein under investigation was incubated with 1 pmol of labeled substrate in 20 μl of binding buffer (20 mM Tris-HCl, pH 8.5, 15 mM MgCl2, 0.05 mM EDTA, 0.25 mM DTT, 0.05 μg/ml bovine serum albumin (Sigma)). Where competition experiments were carried out, unlabeled ssDNA (MacMC-R) or dsDNA in the form of pUC19 plasmid (New England Biolabs) was added at 10 and 50 pmol. The products of the reactions were resolved by 1% agarose gel electrophoresis in 0.1× TAE buffer (4 mM Tris acetate, 0.1 mM EDTA, pH 8.4), and the signals were detected using a PhosphorImager (BAS-1800, Fuji Film).
Fluorescence Polarization Anisotropy (FPA)—In order to obtain an independent measure of RPA binding to ssDNA, we employed FPA, a technique that has been extensively used to detect protein-DNA interactions. For instance, RecA filament formation on ssDNA has been studied using this technique (12). Anisotropy, as described in Lakowicz (13), can be used to measure the rotational time of a molecule tagged with a fluorescent dye. Anisotropy, r, is defined in Equation 1,

\[ r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}} \]  

(Eq. 1)

where \( I_{VV} \) and \( I_{VH} \) are the vertically polarized emission intensities upon vertically polarized excitation and horizontally polarized excitation, respectively. \( G \) is the correction factor for the equipment given by \( I_{VH}/I_{HH} \) where \( I_{VH} \) and \( I_{HH} \) are the horizontally polarized emission intensities upon vertically polarized excitation and horizontally polarized excitation, respectively. If the dye rotates substantially within its fluorescent lifetime, the anisotropy will approach 0. However, if the molecule is very rigid and immobile, the dye will not rotate much during its lifetime and anisotropy will approach the maximum of 0.4. Larger molecules will have a higher anisotropy than small molecules. The ssDNA, FL-18, used in the anisotropy measurements has the sequence shown under FPA in Table I and was HPLC-purified (Operon Technologies). Anisotropy measurements were performed in a fluorometer using the same buffer conditions as described for fluorescence resonance energy transfer (FRET) below. The sample was excited at 490 nm and emission was collected at 518 nm. Titrations of the RPA proteins were done in small increments until the anisotropy was saturated.

FRET—The oligonucleotide sequences used in FRET measurements are shown in Table I. The DNA molecules were synthesized and purified as described previously (14). FRET is a widely used method for studying changes in distance between a donor and an acceptor fluorophore pair that are attached to a biomolecule (15, 16). The energy transfer efficiency, \( E \), is determined according to Equation 2,

\[ E = \frac{1}{1 + (R/R_0)^6} \]  

(Eq. 2)

where \( R_0 \) is a constant defined by the specific dyes used (here \( R_0 = 60 \) Å) and \( R \) is the distance between the two fluorophores (17). To test for purified RPA binding to ssDNA, a DNA complex consisting of 18 dsDNA base pairs and a single-stranded poly(dT) tail (ranging from 10 to 40 bases in length) was constructed (Fig. 1). FRET measurements of this construct (the ssDNA tail) as RPA is added reveal, not only if the protein is binding to the ssDNA, but also how the conformation of the ssDNA changes upon protein binding. In accordance with the equation above, if a protein interacts with the ssDNA such that the distance between the dyes is decreased, FRET will increase; and if the distance is increased, FRET will decrease. To minimize the amount of free ssDNA after annealing, acceptor (Cy5) labeled strands were titrated against donor (Cy3) labeled strands (Fig. 1) to a final concentration of 10–20 μM in an annealing buffer (400 mM NaCl, 20 mM Tris, pH 8.0). Fluorescence measurements were performed in a buffer of 100 mM NaCl, 20 mM Tris (pH 8.0), and 2 mM DTT in a fluorometer (Cary Eclipse, Varian) at 24°C (±1°C). Cy3 was excited at 532 nm and emission was collected from 545 nm to 740 nm with peak emission intensities measured at 587 nm for Cy3 and 667 nm for Cy5. Several NaCl concentrations were tested but similar results were obtained for the range 100–300 mM (data not shown). Concentrations of RPA1, RPA2, and RPA3 were those of dimers, and titrations of the proteins were done in small increments until FRET was saturated.

**FIG. 1. DNA construct used for fluorescence measurements.** A, the single-stranded DNA tail ranged from 10 to 40 dt. The donor dye, Cy3, was attached to the end of the tail with the acceptor dye, Cy5, attached near the base of the tail. The energy transfer efficiency depends on the distance between the donor and acceptor. For the longer ssDNA strands the distance between the dyes will be greater, so FRET is less than for short ssDNA strands. If a protein interacts with the DNA such that the ends move closer together (B), FRET will be high. If the binding of a protein moves the ends apart (C), FRET will be low.

Eclipse, Varian) at 24°C (±1°C). Cy3 was excited at 532 nm and emission was collected from 545 nm to 740 nm with peak emission intensities measured at 587 nm for Cy3 and 667 nm for Cy5. Several NaCl concentrations were tested but similar results were obtained for the range 100–300 mM (data not shown). Concentrations of RPA1, RPA2, and RPA3 were those of dimers, and titrations of the proteins were done in small increments until FRET was saturated.

Spectroscopic Determination of Protein-bound Zinc in MacRPA—Unlike MacRPA1, which lacks a putative zinc finger motif, MacRPA2 and MacRPA3 contain a zinc finger-like sequence characterized by \( \epsilon_2 \) (any amino acid residue) within the C-terminal half (Fig. 2). Similar motifs are conserved in some other archaeal RPAs reported previously (Fig. 2). However, evidence showing that these proteins bind to zinc is lacking. Therefore, a spectroscopic method (18) was used to detect whether the two proteins (RPA2 and RPA3) possessing the putative zinc finger motif contained zinc. Briefly, 5 nmol of the MacRPA under investigation was incubated with 4-(2-pyridylazo) resorcinol (PAR) at a concentration of 10 mM in a buffer containing 50 mM Tris-HCl (pH 7.0) and 800 mM NaCl. The level of background zinc (free zinc) in the reaction mixture was recorded as the absorbance at 500 nm. To release the zinc bound to the protein, methyl methanethiol sulphonate (MMTS) was added to the reaction mixture, and the Zn-PAR complex formed was monitored by the increase in absorbance at 500 nm (Beckman DU 7500). All reactions were carried out at room temperature and readings were taken at 30-s intervals.
Mutational Analysis of CX₆CX₆CX₈H—In order to determine the contribution of the putative zinc finger motif in MacRPA3 to its zinc and ssDNA binding properties, we created a MacRPA3 with a C313A mutation (CX₆CX₆CX₈H → AX₆CX₆CX₈H) by the QuiikChange™ site-directed mutagenesis kit (Stratagene). The two complementary oligonucleotides (C313A-F and C313A-R) used as primers in the mutagenic PCR are shown in Table I. The template for the PCR amplification was the pGEM-T plasmid harboring rpa3, and the accuracy of the mutagenic PCR was confirmed by nucleotide sequencing. A fragment containing the mutation was isolated by digestion with HindIII (nucleotide 684–688) and XhoI and inserted into pT28/rpa3 digested with the same restriction enzymes to remove the wild type fragment. The plasmid construct was designated pT28/C313A, and its product (MacRPA3-C313A) was purified as described above for the other RPA proteins. The gel filtration analysis and the determinations of zinc binding, ssDNA binding, and dsDNA binding by MacRPA3-C313A were carried out as described above.

Primer Extension Analysis—The effect of each MacRPA protein and different combinations of the three proteins (RPA1/RPA2 or RPA1/RPA3 or RPA2/RPA3 or RPA1/RPA2/RPA3) on the primer extension capacity of MacPolBI was investigated. One pmol of a 32P-labeled oligonucleotide (Table I), complementary to positions 6205 to 6234 of the M13mp18 genome (19), was annealed to 1.0 µg of M13mp18 ssDNA by heating in DNA polymerase buffer (20 mM Tris-HCl, pH 8.8; 100 mM NaCl, 5 mM MgCl₂, and 2 mM β-mercaptoethanol) to 95 °C for 5 min and then gently cooling to room temperature. The primer extension reaction was initiated by adding 250 µM of each dNTP followed by 0.5 µg of MacPolBI. To test the effect of the RPA proteins on primer extension by MacPolBI, they were added in increasing amounts (10, 20, 30 pmol) to the reaction mixture. Where mixtures of RPAs were present in the reaction, each protein was added in equal amounts. The reaction was carried out at 57 °C for 15 min and terminated with 6 µl of stop solution (98% formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue). The products were then analyzed on 1% alkali agarose gel as previously described (20).

Protein Concentration—Protein concentrations were determined by the Bradford method using a commercially available kit (BioRad) with bovine serum albumin (New England Biolabs) as the standard.

RESULTS

Identification of MacRPA Genes—Three different genes encoding RPA-like proteins were retrieved from the genome of M. acetivorans by searching with PfuRPA41. Further searches in Genbank showed that all three genes are highly conserved in M. mazei (RPA1, RPA2, RPA3 are NP_633323, NP_632317, and NP_633775, respectively), a close relative of M. acetivorans. The amino acid sequence identities of RPA1, RPA2, and RPA3 in the two species were higher than 80%. MacRPA1 contained four putative OB folds (A, B, C, and D) or repeats of about 100 amino acids, and unlike hitherto reported euryarchaeal RPAs with functional activity, it lacked a zinc finger motif (Fig. 2). In contrast, MacRPA2 and MacRPA3 were composed of two repeats or OB folds (A and B) and a zinc finger-like motif. The repeats B and C of MacRPA1 shared 56% identity while those in MacRPA3 were only 25% identical. The estimated molecular masses of MacRPA1, MacRPA2, and MacRPA3 were 53.9, 46.7, and 49.3 kDa, respectively, and the estimated pIs were 4.4, 4.6, and 5.2, respectively.

Expression, Purification and Subunit Compositions of MacRPAs—To determine whether the products of rpa1, rpa2, and rpa3 encode functional RPAs, each gene was expressed as a fusion protein containing an N-terminal His₆ tag. Each protein was highly purified, as demonstrated by SDS-PAGE (Fig. 3, lanes 2–4), through affinity chromatography on a Co²⁺-charged resin followed by anion exchange chromatography on a HiTrap Q column. MacRPA1 and MacRPA2 eluted from the column at 250 mM NaCl concentration, while MacRPA3 eluted at 275 mM NaCl. The proteins were then subjected to gel filtration analysis to estimate their subunit organization in solution. Samples were taken from the beginning to the end of each peak for SDS-PAGE analysis, and for biochemical analysis, proteins from peak elutions were used. Two peaks were observed for RPA1 while a single peak was observed for RPA2 and also for RPA3 (Fig. 4). The native forms of recombinant RPA1, as determined from the two peaks, were 214.8 ± 0.7 kDa and 91.3 ± 0.4 kDa, where the variation is the standard error estimated from four injections. The results suggested that, in solution, RPA1 exists as homotetramers and homodimers, respectively. Interestingly, we also observed changes in the ratio of the two forms of RPA1 during gel filtration analysis: the dominant species switched from homodimers to homotetramers in a concentration-dependent manner. Thus the ratio of homotetramer/homodimer when 100 µl of protein at concentrations of 0.1 µg/µl, 0.2 µg/µl, and 0.5 µg/µl were injected were 1:2, 1:1, and 2:1, respectively. The single peak for RPA2 and RPA3 coincided with molecular masses of ~81.6 ± 1.3 kDa and 81.3 ± 2.1 kDa, respectively, suggesting that each of the two proteins exists as a homodimer in solution. The estimates are the averages and standard errors of four injections. Next, we tested whether the three RPAs formed complexes in solution, since multiple-sub-
unit RPAs in other organisms are known to exist as complexes (1, 6). To this end, we analyzed different combinations of the three RPAs by gel filtration analysis. The SDS-PAGE analysis of the collected fractions seemed to suggest a co-elution of RPA1 with either RPA2 or RPA3. However, the peaks for RPA1/RPA2, RPA1/RPA3, RPA2/RPA3, and RPA1/RPA2/RPA3 were not very different than those of the homodimeric forms of the proteins, and more importantly the peaks were broad, suggesting the presence of multiple complexes (results not shown). As further confirmation of these results, we conducted a pull-down assay to determine if any interaction exists among the three proteins. In this experiment, a His6-tagged MacRPA1 was used as bait protein (immobilized) while MacRPA2 or MacRPA3 with an N-terminal T7 tag was used as prey protein. Aliquots of eluted proteins were resolved by SDS-PAGE and analyzed by Western blotting. Although we detected the prey proteins in the eluted fractions together with the bait by Western blotting, the amount of bait protein was by far in excess and whether this constituted meaningful interactions or not could not be concluded (results not shown). However, this may complement our inference that when together in solution, the Mac-RPAs exist in the individual conformations that were detected in our gel filtration analysis. We are in the process of co-expressing the MacRPAs in an attempt to clarify this hypothesis.

**DNA Binding Properties of MacRPAs Detected by EMSA**—
Each of the highly purified proteins was used in an electrophoretic mobility shift assay to determine the ability of each individual protein to bind specifically to ssDNA. As shown in Fig. 5A, each of the proteins (RPA1, RPA2, and RPA3) retarded the mobility of the 32P-labeled ssDNA probe (Fig. 5A, lane 2). Although equimolar amounts (5 pmol) of RPA1 and RPA3 were used in the assay, RPA1 shifted all of the labeled ssDNA, while RPA2 shifted about 60%. Twice the amount of protein (10 pmol) was required by RPA2 to observe a ssDNA/RPA2 complex. Moreover, the ssDNA/RPA2 complex was very unstable as shown by the smearing of the shifted bands. To determine whether the binding of the MacRPA proteins to ssDNA was specific, fixed amounts of proteins were incubated with fixed amounts of labeled ssDNA and increasing amounts of unlabeled ssDNA or dsDNA. As shown in Fig. 5A (lanes 3 and 4), unlabeled ssDNA, as a competitor, completely inhibited the

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**Fig. 4.** Gel filtration analysis of MacRPA proteins. The native molecular masses of the RPA proteins were estimated by gel filtration on a Superdex 200 HR 10/30 column. The arrows indicate the elution positions of protein markers run as standards to calibrate the column. The numbers 1, 2, 3, 4, 5, and 6 represent ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa), respectively. The corresponding RPA proteins and their peak elution volumes are indicated in each panel. The absorbance at 280 nanometers are in milliabsorbance units.
binding of each protein to the labeled ssDNA, while excess amounts of unlabeled dsDNA (lanes 5 and 6) did not have any visible effect on binding to the labeled ssDNA. This clearly showed that each of the three proteins binds specifically to ssDNA. The binding of each RPA to ssDNA in various salt concentrations (25, 50, 100, and 200 mM NaCl) was also tested. While no difference was detected for RPA1 and RPA3, the stability of the ssDNA/RPA2 complex improved with increasing salt concentration (data not shown).

An RPA3 protein with a mutation in its putative zinc finger motif (MacRPA3-C313A) was made (Fig. 3, lane 5) to study the contribution of Cys313 to zinc binding and DNA binding. In the reaction with RPA3 alone, 5 pmol of protein bound to about 60% of the labeled ssDNA (1 pmol), and the same amount of MacRPA3-C313A bound to about 53% of the probe (Fig. 5B, MacRPA3-C313A, lane 2). However, in the case of RPA3-C313A, most of the ssDNA/protein complex was observed in the gel filtration analysis of MacRPA3-C313A protein resolved by 1% agarose gel electrophoresis followed by visualization of autoradiography. Except for RPA2, which was at 10 pmol, the other proteins under investigation were added at 5 pmol/reaction. A, individual MacRPAs; B, MacRPA3 C313A mutant. The RPA protein under investigation is indicated under each panel (for details see "Experimental Procedures"). The arrows (I) and (II) represent free DNA and protein/DNA complex, respectively.

**Fig. 5. Single-stranded DNA binding properties of MacRPAs.** A fixed amount (1 pmol) of 32P-labeled ssDNA (I) was incubated with each RPA under investigation (2), and challenged with 10 pmol of unlabeled ssDNA (3), 50 pmol of unlabeled dsDNA (4), 10 pmol of unlabeled dsDNA (5), and 50 pmol of unlabeled dsDNA (6). Each reaction mixture (20 \( \mu \)l) was incubated at 25 °C for 30 min., and the products were resolved by 1% agarose gel electrophoresis followed by visualization using autoradiography. Except for RPA2, which was at 10 pmol, the other proteins under investigation were added at 5 pmol/reaction. A, individual MacRPAs; B, MacRPA3 C313A mutant. The RPA protein under investigation is indicated under each panel (for details see “Experimental Procedures”). The arrows (I) and (II) represent free DNA and protein/DNA complex, respectively.

**Fig. 6. Anisotropy measurements reveal binding of MacRPAs to ssDNA.** Fluorescence polarization anisotropy of an 18-base long ssDNA was measured relative to protein concentration. A higher anisotropy implies the DNA is becoming less mobile. As protein binds to the ssDNA, the complex becomes larger and less mobile in solution. This experiment was performed on a 10.3 mM concentration of a heterogeneous sequence of 18 ssDNA bases (FL-18) labeled with fluorescein (for details see “Experimental Procedures”).

**Table II**

| MacRPA | Hill coefficient (n) | Dissociation constant (Kd) (nm) |
|--------|----------------------|---------------------------------|
| 1      | 1.39 ± 0.13          | 3.52 ± 0.28                     |
| 2      | 0.69 ± 0.07          | 173.61 ± 105.17                 |
| 3      | 1.46 ± 0.08          | 5.92 ± 0.33                     |

**Fluorescence polarization anisotropy—** Fluorescence polarization anisotropy is a reliable biophysical method that has been used to study molecular orientation and mobility and the processes that modulate them. We used this method to study the interaction of the MacRPAs with a ssDNA substrate (FL-18) composed of 18 heterogeneous bases. Anisotropy of FL-18 was low (−0.07) in the absence of any protein (Fig. 6). Upon protein addition, anisotropy increased indicating that the protein binding decreased the rotational mobility of the fluorophore. RPA1 and RPA3 showed significant binding with RPA1 showing slightly stronger affinity. RPA2 showed the weakest binding among the three, complementing the poor binding shown in the electrophoretic mobility shift assays (Fig. 5A). This data was fitted to a Hill binding model (21) resulting in dissociation constants and Hill coefficients that can be found in Table II. The large errors in the dissociation constant for RPA2 confirms that RPA2 does not bind well, and this model is not adequate to describe its behavior.

**ssDNA Binding of MacRPAs Detected by Fluorescence Resonance Energy Transfer**—We used a more sensitive method, FRET, to detect the binding of the MacRPAs to ssDNA and to obtain information on their binding site sizes and binding geometry. FRET efficiency was approximated experimentally

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2 J. B. Robbins, M. C. Murphy, T. Ha, and I. K. O. Cann, unpublished observations.
by the intensity of the acceptor emission divided by the total emission of the acceptor and donor,
\( I_A / (I_A + I_D) \) (Fig. 7A). Here, we used the peak intensities for emission. All tail lengths examined showed binding of RPA, however, with small tails such as T10 (10 thymines), it required more protein to approach the saturation of FRET (Fig. 7B). Even for RPA1, which seems to bind well with T10, it required more than five times the amount of protein to DNA to saturate FRET. RPA2 again showed the weakest binding among the three MacRPAs. Similar results were also found for T15, although RPA3 showed almost no change at all (Fig. 7C). For the other lengths of ssDNA, RPA1 and RPA2 showed similar decreasing FRET behavior, but RPA3 showed a unique FRET curve. Fig. 7, D and E show RPA3 exhibiting a different behavior than the other MacRPAs in that FRET does not only decrease as more protein is added; it initially increases then decreases. The effect is most pronounced in the longest DNA tested, T40, but can also be seen in T23. The change in FRET for these cases may indicate two different binding modes for RPA3 and longer ssDNA. The first mode suggests some compacting of the DNA around the protein to bring the two ends of the tail closer together (Fig. 1B). The second mode at higher protein concentrations acts to straighten out the ssDNA and thus lower FRET. This could be due to a dimer of protein binding first to the ssDNA and then as the concentration of protein in solution is increased, another protein can interact with the protein/DNA complex. From the FPA experiments, we know that RPA3 does indeed bind to the DNA, but exactly how it effects the DNA is not clear. Interestingly, this behavior of MacRPA3 is seen with Methanopyrus kandleri RPA, which also has a two OB fold and one zinc finger domain architecture. Further experiments would be needed to determine the exact binding process, but it is clear that RPA3

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)
has a unique behavior that may be shared with other archaeal RPAs. The FRET data for RPA1 was also fitted to a Hill model binding curve and the results can be found in Table III. RPA2 and RPA3 data were not fitted to a model since RPA2 did not reach saturation in FRET and RPA3 showed a behavior that is too complex to use a simple Hill binding model for. Overall, the biophysical data suggested a high affinity binding of RPA1 and RPA3, but low affinity binding of RPA2.

**Binding of Zinc by MacRPAs**—The MacRPAs were investigated for the presence of zinc in each protein. Individual proteins were incubated with PAR for 5 min to determine background levels of zinc by monitoring the absorbance at 500 nm. Addition of MMTS, which released the bound zinc from the proteins, resulted in an increase in absorbance as the freed zinc was bound by PAR to form a Zn-PAR complex. As shown in Fig. 8, addition of MMTS did not result in an increase in absorbance in the case of the zinc finger-free RPA1. In contrast, addition of MMTS to either RPA2 or RPA3, which harbored putative zinc finger motifs, resulted in large increases in absorbance, reflecting an increase in the release of zinc from each protein. To investigate the contribution of the conserved zinc finger-like motif to zinc binding, one of the conserved cysteines in this motif in RPA3 was mutated to alanine. The C313A mutation in RPA3 led to a drastic decrease in the Zn-PAR complex formed (Fig. 8), suggesting that the first cysteine in the highly conserved CX2CX8CX2H motif (Fig. 9) is important for zinc binding.

**Effect of MacRPAs on Primer Extension Activity of PolBI—** Eukaryotic RPAs (22) and E. coli SSB (23) have been shown to stimulate DNA synthesis by their cognate DNA polymerases.

### Table III

| MacRPA | ssDNA tail length | Hill coefficient | Kd (nm) |
|--------|------------------|-----------------|--------|
| 1      | 10               | 1.42 ± 0.09     | 10.87 ± 0.54 |
| 1      | 15               | 1.81 ± 0.16     | 3.91 ± 0.23  |
| 1      | 23               | 1.67 ± 0.18     | 5.34 ± 0.26  |
| 1      | 40               | 1.52 ± 0.10     | 4.21 ± 0.20  |

However, in the Archaea this property of single-stranded DNA-binding proteins has yet to be demonstrated. In *M. thermocautrophus*, the only archaeal organism in which an RPA homolog has been tested for its effect on DNA polymerase activity, the protein rather inhibited DNA synthesis by the DNA polymerase (5). We, therefore, investigated whether this was a common feature of archaeal RPAs. Thus, each of the *M. acetivorans* RPAs was tested for its ability to stimulate or inhibit the family B DNA polymerase (PolBI) found in this organism. Recombinant MacPolBI was highly purified as a protein with an N-terminal His6 tag (Fig. 3, lane 6). As the template/primer, a 32P-labeled oligonucleotide annealed to M13mp18 ssDNA was used. In the reaction with PolBI alone, the end-labeled primer was extended to ~500 nucleotides (Fig. 10A, lane 5). Increasing amounts of individual RPAs (10–30 pmol) stimulated primer extension by PolBI and completely replicated products (~7.2-kb fragments) were observed (Fig. 10A, lanes 6–14). The complete products represented a 14-fold increase in the maximum length of products synthesized. In this case also, RPA2 had the least effect and complete products were only clearly seen at 30 pmol of protein added. We used different combinations of the MacRPA proteins to determine if any of them will inhibit DNA synthesis by PolBI. However, similar to the individual proteins, all combinations of MacRPAs (RPA1/RPA2 or RPA1/RPA3, RPA2/RPA3, and RPA1/RPA2/RPA3) also highly stimulated primer extension by PolBI (Fig. 10, B and C) and no inhibition was detected. It is, however, very important to note that RPA2 and RPA3 are sensitive to long term storage at 4 °C. These proteins are from a strict anaerobe and they may be very sensitive to oxygen.

**DISCUSSION**

The thermophilic methanogens studied so far have been shown to harbor a single gene coding for a functional RPA homolog (4, 5). However, our search for genes encoding RPA-like sequences in the genome of the mesophilic *M. acetivorans* yielded three candidates (24). To investigate whether this finding was true for other members of this genus, the genes were used in searching the genome of *M. mazei*, which intriguingly contains 1.7 megabases less genomic information than *M. acetivorans* (Fig. 9). In the case of RPA1 in the *M. barkeri* strain, the first cysteine in the putative zinc finger motif (CX2CX8CX2H) in RPA3 is essential for zinc binding. The presence of zinc was determined by incubating 5 nmol of proteins with PAR and measuring the release of Zn by MMTS as the increase in absorbance at 500 nm. Zinc was not detected in RPA1, but in RPA2 and RPA3. Mutating the first cysteine in RPA3 to alanine resulted in a drastic decrease in zinc binding by RPA3-C313A.
genome, a frameshift in the sequence of the gene has resulted in the gene being annotated as two hypothetical proteins (Genbank accessions ZP_00078656 and ZP_00078657).

Although the components of most eukaryotic RPAs tend to aggregate when expressed individually (1), the M. acetivorans RPA proteins were all produced as highly soluble proteins in E. coli cells. While we initially hypothesized that the products of the three genes would form a heterotrimeric RPA, as reported in the hyperthermophilic archaeon P. furiosus (6) and eukaryotes, such as humans (1), our analyses suggested that such a complex may not be formed. Interestingly, each gene coded for a functional single-stranded DNA-binding protein. MacRPA1, the largest of the three proteins, lacked the putative zinc finger motif found in the single-subunit RPAs isolated from M. jannaschii and M. thermoautotrophicus (4, 5). A similar zinc finger motif is conserved in the largest subunit of PfuRPA (6) and human RPA (1, 10). The MjaRPA was reported to exist as a monomer in solution (4, 8). In contrast, gel filtration analysis of MacRPA1 suggested multimerization of its molecules into homotetramers and homodimers. These complexes represent proteins containing sixteen and eight OB folds, respectively. It is, however, not known whether MacRPA1 binds ssDNA in these forms in vivo. It is interesting to note that although a homotetrameric RPA that lacks a zinc finger motif has been reported in the crenarchaeote Sulfolobus solfataricus, the SsoRPA contains only four OB folds (8). Thus, the SsoRPA contains only four OB folds. MacRPA2 and MacRPA3 contain two OB folds in tandem at their N-terminal half. In addition, both proteins contain zinc finger motifs in the C-terminal region. The sequence of this motif is different from those reported previously in archaeal RPAs (4–6). Each of the two proteins oligomerized in solution to form homodimers, suggesting that they may bind to ssDNA with four ssDNA-binding domains as observed for MjaRPA (4), SsoRPA (8), and eukaryotic RPA (1). However, this also suggests two zinc finger motifs in the homodimeric MacRPA2 and MacRPA3.

The gel filtration analysis failed to provide clear evidence for interactions between the MacRPAs. Furthermore, our pull-down assay also suggested that, under the conditions tested,
the majority of the proteins were in their homomeric forms when the different RPAs were mixed together. Thus, each of the three RPAs is likely to function as an individual RPA with, perhaps, different roles in the Methanosarcinaceae. Indeed, the only gene encoding an RPA-like protein in the hyperthermophilic euryarchaeote, M. kandleri (Mka), codes for a protein with a similar architecture to that of RPA2 and RPA3. It is noteworthy that a gene coding for a MacRPA1-like protein is not found in the genome of this archaeon. The MkaRPA gene (Genbank™ accession number: NP_614724) overlaps with that for MkaRadA recombinase (Genbank™ accession number: NP_614725), a protein whose function is enhanced by RPA (6).
The MkaRPA is similar in size (48.2 kDa) and pI (4.7) to MacRPA2 and MacRPA3, and our preliminary results showed that MkaRPA protein also binds specifically to ssDNA. Thus, aside from the genus *Methanosarcina*, the two-OB fold and one zinc finger domain RPA occurs in another archaeal genus. The structural organizations of the MacRPAs, being different than those hitherto reported (1–7) may suggest novel forms of RPA.

In the eukaryotic RPA70 there is an evolutionarily conserved zinc finger motif posterior to the ssDNA-binding domains. This motif has been shown to be essential for DNA replication and mismatch repair (26), and similar motifs have been found in MjaRPA and PfuRPA (Fig. 2). As shown in Fig. 8, we effectively released the zinc in MacRPA2 and MacRPA3 with MMTS, a thiol modification reagent, suggesting the involvement of cysteine ligands in zinc binding in both proteins. A C313A mutation in RPA3 drastically reduced the amount of zinc bound by RPA3-C313A. The three cysteines and one histidine found in the zinc finger motif are highly conserved (Fig. 9). This may imply that each of these residues is important for the stable coordination of zinc. Thus, the residual zinc found in RPA3-C313A may suggest an incomplete disruption of the zinc finger. Further mutational analysis within this motif is currently underwaoran in our laboratory. MacRPA1 did not contain zinc, as predicted from its polypeptide (Fig. 2). The absence of a zinc finger motif in a protein that exhibits a similar architecture to MjaRPA and MtbRPA (10), suggests either a loss of this motif from MacRPA1 or the other two proteins, which come from thermophiles, might have gained this additional information after a split from a common ancestor with the *Methanosarcinae*.

The overall measurements using FRET and FPA indicated that RPA1 and RPA3 bind tightly to ssDNA as long as the DNA is longer than 15 bases. All three RPAs also bind to shorter DNA, down to 10 bases long, but the affinity is significantly reduced. This may suggest that the RPAs might have two different binding modes, strong and weak, where the strong binding requires interaction with longer ssDNA. RPA2 showed weak binding with all lengths of ssDNA tested. The Hill binding model allows for positive cooperativity of the protein binding to the DNA, and is indicated by a Hill coefficient greater than one (21). For all calculations reported for RPA1 and RPA3, the Hill coefficient is greater than one implying that more than one protein is interacting with the ssDNA tail. The dissociation constants resulting from these measurements indicate the RPA1 binds most readily to ssDNA between 18–23 bases. Though the binding site size seems to be around 20 bases, the positive cooperativity even for shorter ssDNA may indicate some protein–protein interaction. *E. coli* SSB has been shown to decrease the end-to-end distance of a 70-mer ssDNA tail with one homotetramer, but increase the end-to-end distance when bound to two homotetramers. This indicates a wrapping of the DNA around one tetramer, which is consistent with the crystal structure, but only binding to half of each of the two tetramers in the low FRET conformation (27, 28). In contrast, binding of RPA1 and RPA2 decreased FRET for all ssDNA under the conditions tested. ssDNA is a reasonably flexible molecule and its end-to-end distance is substantially reduced compared with a fully stretched form. It is likely that the binding of RPA1 and RPA2 straightens a section of the ssDNA and thus reduce FRET. RPA3 however, shows a possible similarity to the *E. coli* SSB by having a high FRET and a low FRET binding mode, although it is not clearly exactly if a monomer, dimer, or tetramer is binding to the ssDNA. Three significant gaps are seen in RPA2 when aligned with RPA3, and this is reflected in the difference in their molecular masses (46.7 kDa versus 49.3 kDa). The difference in the behavior of RPA2 and RPA3, although they both have the two-OB fold and one zinc finger domain architecture, may be attributed to amino acid deletions in RPA2 or insertions in RPA3. Furthermore, the difference in the identity of their two OB folds (59 and 25% for RPA2 and RPA3, respectively) may also influence their ssDNA-binding behavior.

We examined the effect of the different forms of the MacRPAs on DNA synthesis by a *M. acetivorans* DNA polymerase. The archaeal family B DNA polymerases have low processivities. As an example, the *Thermococcus litoralis* PolBI is reported to synthesize seven nucleotides per binding (29). It was hypothesized that processivities of these polymerases will increase in the presence of accessory factors (30), such as proliferating cell nuclear antigen (clamp), replication factor C (clamp loader), and replication protein A. The enhancement of primer extension of archaeal PolBIs by their cognate clamps and clamp loaders was later demonstrated (31–34). In this study, as shown in Fig. 10, A–C, the MacRPAs in all combinations clearly enhanced the primer extension capacity of *M. acetivorans* PolBI. This finding shows, for the first time, that the ability of ssDNA-binding proteins to enhance the DNA synthesis capacity of a cognate DNA polymerase is conserved in a member of the third domain of life.

Methanogens play a key role in the efficient anaerobic decomposition of organic matter, and the *Methanosarcina* are recognized as the most physiologically and metabolically versatile group. They are capable of growth on any methanogenic substrate (H₂/CO₂, acetate, methanol, and methylated amines) with the exception of formate. Apart from its importance in the global carbon cycle, methane is also a significant greenhouse gas (35). The elucidation of the DNA replication mechanism of the methanogenic Archaea is, therefore, a very important theme for not only basic research but also for industrial applications. We have assigned DNA replication-related function to three genes coding for proteins designated hypothetical proteins in the *Methanosarcina*. Since most organisms thrive on one ssDNA-binding protein, the implications of more than one RPA in the *Methanosarcina*, at present, is not known. The availability of a genetic system for manipulating *M. acetivorans* will enable us to inactivate each rpa gene from the genome, in the future, to determine their individual roles in DNA replication, repair and recombination.

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Functional Analysis of Multiple Single-stranded DNA-binding Proteins from *Methanosarcina acetivorans* and Their Effects on DNA Synthesis by DNA Polymerase BI

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