Improvements of rolling circle amplification (RCA) efficiency and accuracy using *Thermus thermophilus* SSB mutant protein

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ABSTRACT

Rolling circle amplification (RCA) of plasmid or genomic DNA using random hexamers and bacteriophage phi29 DNA polymerase has become increasingly popular in the amplification of template DNA in DNA sequencing. We have found that the mutant protein of single-stranded DNA binding protein (SSB) from *Thermus thermophilus* (*Tth*) HB8 enhances the efficiency of amplification of DNA templates. In addition, the *Tth*SSB mutant protein increased the specificity of phi29 DNA polymerase. We have overexpressed the native and mutant forms of *Tth*SSB protein in *Escherichia coli* and purified them to homogeneity. *In vitro*, these proteins were found to bind specifically to single-stranded DNA. Addition of *Tth*SSB mutant protein to RCA halved the elongation time required for phi29 DNA polymerase to synthesize DNA fragments in RCA. Furthermore, the presence of the *Tth*SSB mutant protein essentially eliminates nonspecific DNA products in RCA reactions.

INTRODUCTION

Rolling circle amplification (RCA) is an isothermal method that amplifies circular DNA by a rolling circle mechanism (1–4). The reaction products are high molecular weight, linear, double-stranded, tandem repeat copies of the input template that can subsequently be used for DNA sequencing, restriction endonuclease digestion and other methods used in cloning, labeling and detection. Kits that use this method for preparation of circular DNA templates for direct use in cycle sequencing are commercially available. RCA variants have been employed for the genotyping of single nucleotide polymorphisms through ligation of circularizable DNA probes and for whole bacterial or human genome amplification via multiple-displacement amplification (5–8).

Although the utility of RCA is beginning to be accepted widely, several technical problems still remain to be solved. Perhaps the most significant is the appearance of nonspecifically amplified products. In practice, even with the most optimized reaction conditions (e.g. using modified random oligonucleotides) (9), nonspecific RCA products cannot be completely eliminated, particularly in instances where complex and/or very low amounts of the template DNA are used. Nonspecific RCA products are most likely to be derived from false priming at sites where primer dimers are formed.

Recently, a heat stable SSB protein from a thermophilic bacteria, *Thermus thermophilus* (*Tth*), has been isolated and fully characterized (10–12). Bacterial SSB proteins are required for DNA replication and repair (13,14). Most SSB proteins bind nonspecifically to single-stranded DNA (ssDNA) and prevent the formation of secondary structures. Wild-type and mutant SSB-encoding genes from *T.thermophilus* HB8 were cloned and the proteins they encode have undergone preliminary characterization (J.I. Inoue, manuscripts in preparation). We attempted to eliminate nonspecific RCA products by employing these heat-stable SSB proteins. We speculated that the presence of SSB protein could greatly stimulate polymerization (isothermal strand-displacement), and therefore reduce the formation of primer dimer and lead to the elimination of nonspecific RCA products. Mutated SSB proteins would be ideal candidates for such an application owing to their predicted ssDNA binding efficiency.

In this report, we demonstrate that a mutant SSB protein from *T.thermophilus* strain HB8 (*Tth*SSB) increases the effi-

ciency of DNA synthesis and the accuracy of RCA using the bacteriophage phi29 DNA polymerase.

MATERIALS AND METHODS

Cloning of the *ssb* wild-type gene

T.thermophilus genomic DNA was purchased from Takara-Bio. Amplification of the *ssb* gene was performed by PCR with the genomic DNA, *Nde-ssb* (5′-GCGGATCCAT ATGGCTCGAG GCCTGAACCG-3′), *Bam-ssb* (5′-TAAG-GATCCT TATTAAAACG GCAACTCCTC CTCCG-GCGGA AA-3′) and *Kod* DNA polymerase (Toyobo). The primers included *Nde*I and *Bam*HI restriction sequences (underlined) for cloning purposes and were based on the sequence of the gene encoding the *T.thermophilus* HB8 *ssb* gene (GenBank accession number NC006461). A single DNA fragment of the expected size (792 bp) was obtained. For construction of the expression plasmid, the PCR product was digested by *Nde*I and *Bam*HI, purified and inserted into plasmid pET17b. The resulting plasmid was named pET17b-*ssb*.

Cloning of the *ssb* mutant gene

For construction of the expression plasmid of the mutant protein carrying one amino acid replacement (F255P), the *ssb* mutant gene (nt 763–764, TT > CC) was prepared by PCR. The primers used are *Nde-ssb* and *Bam-ssbm* (5′-ACAGGATCCT TATTAAAACG GCAACTCCTC CTC-CGGCGGA **GGG**TCTTCCA AGCCTTCGTC-3′). The mis-matched bases are shown in bold.

Expression and purification of proteins

Purification of the SSB protein and its mutant were carried out as follows. The cells grown to an OD_{600} of 0.5 were trea-ted with isopropyl b-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM for 4 h. The harvested 1 g cells were resuspended in 10 ml of the buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol and 100 mM NaCl, and were sonicated. After removing debris by centrifugation (76 000× *g* for 60 min), the supernatant was heated for 20 min at 70°C and centrifuged (76 000× *g* for 60 min) to remove the precipi-tate. The supernatant was applied to the Heparin Sepharose 6 Fast Flow column (Amersham Biosciences, 8 ml) that had been equilibrated with the buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol and 100 mM NaCl. The SSB protein was eluted with a linear gradient of NaCl (0.1–1.0 M). Fractions containing the SSB protein, detected by SDS–PAGE, were collected and final concentration of 1 M $(NH_4)_2SO_4$ was added to it. The solu-tion was applied to the BUTYLl-TOYOPEARL 650S column (TOSOH, 4 ml) that had been equilibrated with the buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol and 1.0 M $(NH_4)_2SO_4$. The SSB protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.0–0 M). Fractions containing the SSB protein were cor-rected and diluted with the buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA and 10 mM b-mercaptoethanol to low salt concentration (<0.6%). The solution was applied to the RESOURCEQ (Amersham Biosciences, 6 ml) that

had been equilibrated with the same buffer for the Heparin Sepharose 6 Fast Flow column. The SSB protein was eluted with a linear gradient of NaCl (0.1–1.0 M). Fractions containing the SSB protein were corrected and concentrated to 200 μl by Centriprep Ym-10 (Amicon, COSMO-Bio). After the addition of equal amounts of glycerol, the sample was stored at −20°C until use. The concentration of the SSB protein was determined using a molar absorption coefficient of 50 370 M^{-1} cm^{-1} at 280 nm.

ssDNA binding assay using fluorophotometry with etheno-modified ssDNA

ssDNA binding assay by etheno-modified ssDNA (εDNA) was performed as described previously (15). Fluorescence measurements were carried out on a spectro-fluorometer (Perkin Elmer LS 55 Luminescence Spectrometer) equipped with a temperature regulated holder. All fluorescence mea-surements were performed in 200 μl buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, 100 mM KCl and 10 μM εDNA at 25°C. The excitation and emission wavelengths were 305 and 440 nm, respec-tively. Fluorescence changes of εDNA upon protein binding were measured and plotted against the concentration of SSB proteins. Apparent dissociation constants for the binding of SSB proteins to ssDNA were determined by fitting the actual fluorescence values to the theatrical curve. A computer program, Igor (WaveMetrics, Inc), was used for calculating the parameters based on the non-cooperative binding of SSB proteins to εDNA.

PCR and Multiplex PCR

Primers used for PCR were purchased from Sigma-Aldrich Japan. PCR was performed using DNA polymerase from *Thermus aquaticus* (*Taq*) according to the manufacturer's instructions (QIAGEN HotSartTaq DNA polymerase kit and QIAGEN Multiplex PCR kit, QIAGEN). Primers were used at concentrations of 0.4 μM. PCR thermal cycles used were 15 min at 95°C for DNA denaturation, followed by 35 cycles of 30 s at 94°C, 90 s at 60°C and 90 s at 72°C. PCR products were purified by a kit (DNA purification kit, Amersham Bio-sciences). Multiplex PCR was performed in the presence of *T.thermophilus* RecA as described previously (16).

Gel electrophoresis of DNA and protein

DNA was electrophoresed on 1.2% agarose gels and visual-ized by staining with ethidium bromide. Loading buffer con-tained 60 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 5 mM EDTA and 0.01% bromophenol blue (BPB). The samples and the DNA size marker (*Bst*BI digested Lambda DNA frag-ments) were run for 1 h on an agarose gel.

Protein samples were heated for 3 min at 85°C before being loaded onto the gel. Proteins were electrophoresed on 12.5% polyacrylamide gels containing 0.1% SDS under reducing conditions and visualized by staining with Coomas-sie brilliant blue R-250 (CBB). Loading buffer for gels con-tained 30 mM Tris–HCl pH 6.8, 5% (v/v) glycerol, 1% SDS, 2.5% β-mercaptoethanol and 0.01% BPB. The samples and the protein markers were run for 1 h on SDS–PAGE to ensure that all proteins had achieved mobility according to their respective sizes.
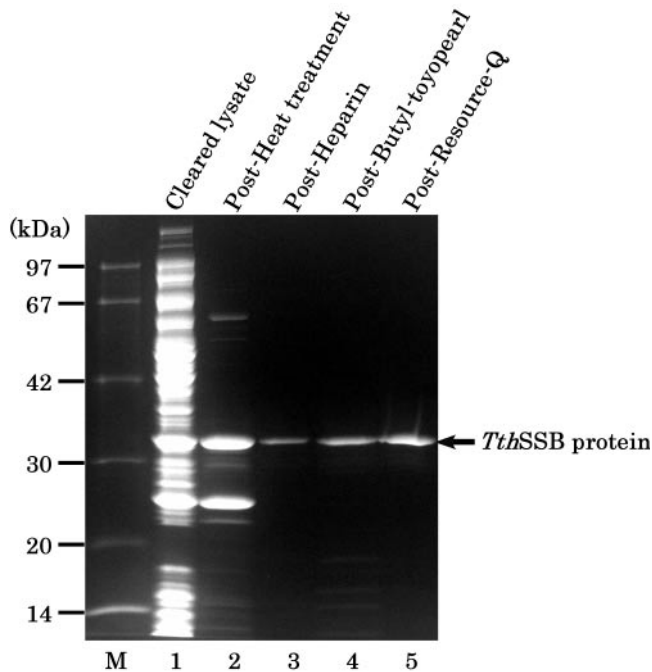
Figure 1. Analysis of *Tth*SSB protein by SDS–PAGE. The fractions obtained during the purification of *Tth*SSB protein were electrophoresed on an SDS–polyacrylamide gel (12.5%) and stained with CBB. Molecular mass markers (in kilodaltons) are indicated to the left. The arrow indicates the *Tth*SSB protein.

Standard RCAs

RCAs were performed with DNA polymerases from phi29 in accordance with the manufacturer's instructions (Amersham Biosciences). We used 1 ng pUC19 DNA (included in the kit as the control DNA template) as template. The RCA conditions used were 3 min at 95°C for denaturation, followed by ~24 h at 30°C. An enzyme inactivation step was performed for 10 min at 65°C.

RCA whole genome amplification

We used 10 ng of human genomic DNA (Promega) as templates in the genomic DNA amplifications. Template DNA was mixed with 0.5 μl hexamers (400 ng/μl, Sigma Genosys) and 0.5 μl binding buffer (400 mM Tris–HCl at pH 8.0 and 160 mM KCl) and denatured at 95°C for 4 min. The denatured DNA was amplified using 0.3 μl Phi29 DNA polymerase (10 U/μl, New England Biolabs) complemented with 2 μl of 10× Phi29 DNA polymerase buffer, 0.2 μl of 100× BSA, 3.2 μl of 2.5 mM dNTP and 1 μl of 20% DMSO (Sigma Aldrich) in a volume of 20 μl at 30°C for ~24 h. The phi29 DNA polymerase was inactivated at 65°C for 10 min and the amplification product was purified using a spin-column (Sephadex G-50, Amersham Biosciences) to eliminate the un-reacted primers.

DNA spot hybridization

To perform DNA spot hybridizations, DNA samples were mixed with 30 μl of DNA denaturation solution. The mixtures were then incubated for 5 min at 65°C and cooled on ice. Equal volumes of 20× SSPE (0.2 M sodium phosphate, pH 7.4, 3.0 M NaCl and 0.02 M EDTA) was added to each
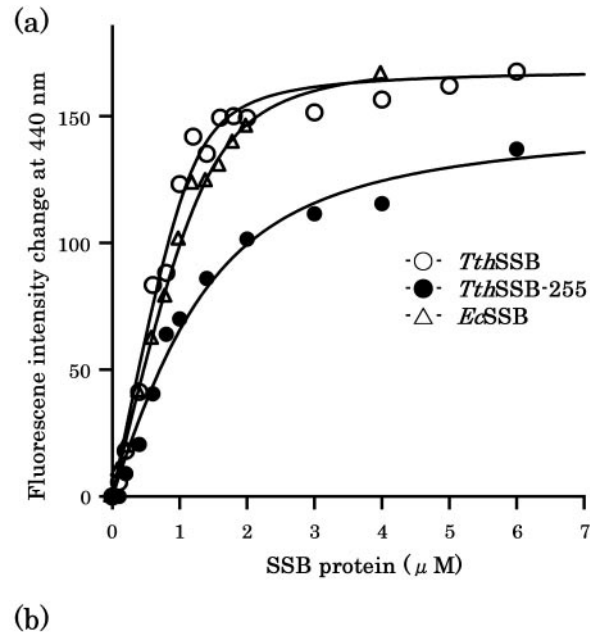

Figure 2. ssDNA binding of SSB proteins. (**a**) Fluorescence spectral changes. Each indicated concentration of SSB protein was incubated at 25°C for 5 min with 10 μM εDNA. The emission spectra excited by 305 nm were measured using a 5 mm × 5 mm cell at 25°C. The ordinate represents the difference in the fluorescence emission intensity at 440 nm between εDNA in the presence and absence of each SSB. The solid lines represent the theoretical curves. (**b**) The dissociation constants for the binding of SSB proteins to ssDNA. Apparent dissociation constants were determined by fitting the actual fluorescence values to the theatrical curves.

sample, which were spotted onto a nylon membrane. The DNA was then fixed to the membrane by ultraviolet irradiation. The membrane was incubated for 2 h at 68°C in 20 ml of prehybridization solution in a hybridization chamber. The radiolabeled probes were prepared with [γ-^{32}P]dCTP (6000 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK), a kit (BcaBEST^{TM} Labeling kit, Takara Bio) and pUC19 or a human gene site (3121 bp). Following addition of the radiolabeled probes, the membrane was incubated at 68°C for a further 12 h. After hybridization, the membrane was washed with 200 ml 0.5× SSC (7.5 mM sodium citrate pH 7.0 and 75 mM NaCl), 0.1% SDS at 68°C. Finally, the membrane was dried and exposed to an imaging plate (BAS2000 Image analyzer, Fuji Photo Film) for 24 h.

RESULTS

Purification of wild-type and mutant *Tth*SSB protein

The wild-type *T.thermophilus* SSB and its mutant were prepared as described in Materials and Methods, which were
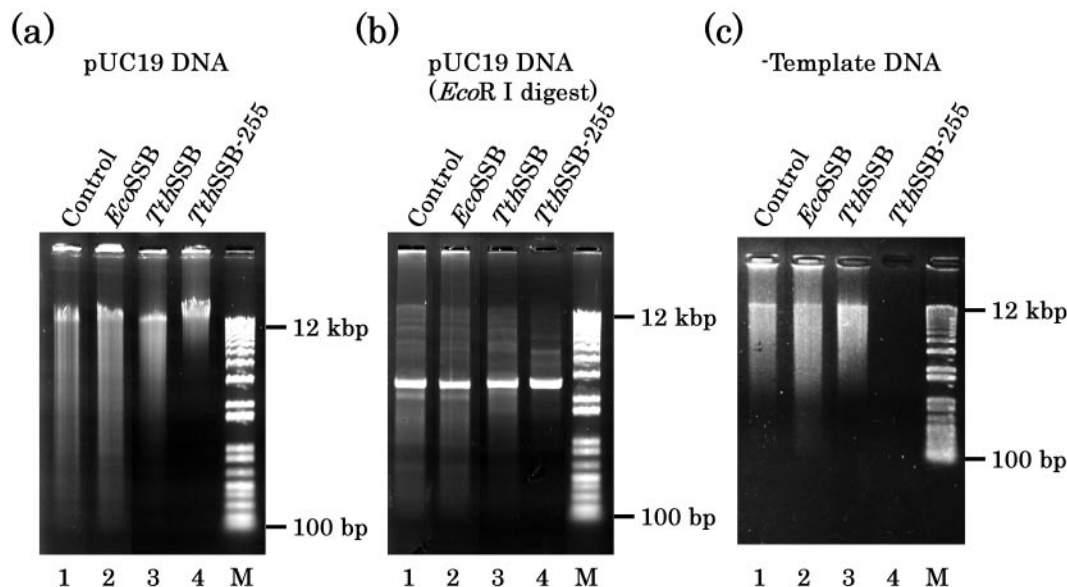
Figure 3. Effect of *Tth*SSB-255 protein on RCA assays. (**a**) RCAs were carried out in the absence or presence of the indicated SSB proteins using pUC19 DNA as a template and phi29 DNA polymerase. (**b**) Same as (a), except for using linearized (*Eco*RI) pUC19 DNA as the template. (**c**) Same as (a), except that the amplifications were carried out in the absence of template DNA. Lane M: molecular weight markers (100 and 12 kb).

named *Tth*SSB and *Tth*SSB-255 protein, respectively. Each step of the purification of *Tth*SSB was analyzed by 12.5% SDS–PAGE with CBB staining (Figure 1). Almost all proteins from *E.coli* could be removed by heat treatment. The *Tth*SSB-255 mutant protein was also purified using the same strategy (data not shown). The final products were shown to be 99% pure.

Properties of *Tth*SSB-255 protein

The *Tth*SSB-255 protein has a single amino acid replacement (F255P) and the same pI (5.06) as the wild-type protein. The strategies of purification for both proteins were identical. Therefore, the mutant would not be expected to have significantly different properties compared with the wild-type. To assess the effect of the F255P mutation on the DNA binding properties of SSB, we determined the dissociation constants for the binding of *Tth*SSB-255 to ssDNA. As shown in Figure 2a, the *Tth*SSB-255 protein showed weak binding to ssDNA in comparison with the wild-type *Tth*SSB and *Ec*SSB proteins. Figure 2b summarizes the dissociation constants for the binding of each SSB protein to ssDNA.

Effect of SSBs on RCA

To examine whether the presence of either wild-type or *Tth*SSB-255 or *Ec*SSB was able to reduce the formation of nonspecific RCA products and thus affect the RCA reaction, we compared the patterns of RCA products obtained in the presence (or absence) of these proteins. As shown in Figure 3a, high molecular weight DNA is generated as a result of isothermal strand-displacement amplification. However, RCA produces nonspecific amplification artifacts (Figure 3c, lane 1 for no protein, lane 2 for *Ec*SSB and lane 3 for *Tth*SSB) in the absence of input template DNA. In contrast, RCA products in the presence of the *Tth*SSB-255 protein were free of such nonspecific amplification

products (Figure 3c, lane 4) and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 3a and b, each of lane 4).

Effects of the DNA synthesis in the presence of *Tth*SSB-255 protein

Since the above effects on DNA synthesis were observed with short elongation times, we examined whether the *Tth*SSB-255 protein was able to affect the mean rate of DNA synthesis of DNA polymerase. Conventional RCA and RCA in the presence of the *Tth*SSB protein produce nonspecific amplification artifacts in the absence of input template DNA (Figure 4b and d). Therefore, the RCA electropherogram is not indicative of successful amplification. In contrast, RCA in the presence of the *Tth*SSB-255 protein was free of such nonspecific amplification products (Figure 4f) and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 4e). From the results of spot hybridization analysis, with 30 min elongation times, the DNA fragment was not amplified in the absence (Figure 4a) or presence of the *Tth*SSB protein (Figure 4c). In contrast, the DNA fragment was amplified in the presence of *Tth*SSB-255 protein (Figure 4e). These results support the idea that the DNA polymerase at least doubles the mean rate of DNA synthesis in the presence of *Tth*SSB-255 protein.

Whole genome amplification

Figure 5 demonstrates the amplification products obtained following whole genome amplification via RCA, using intact human genomic DNA. High molecular weight DNA is generated as a result of isothermal strand-displacement amplification. However, conventional RCA and RCA in the presence of *Tth*SSB protein produced nonspecific amplification artifacts (Figure 5a and b, each of lanes 3 and 4) in the absence
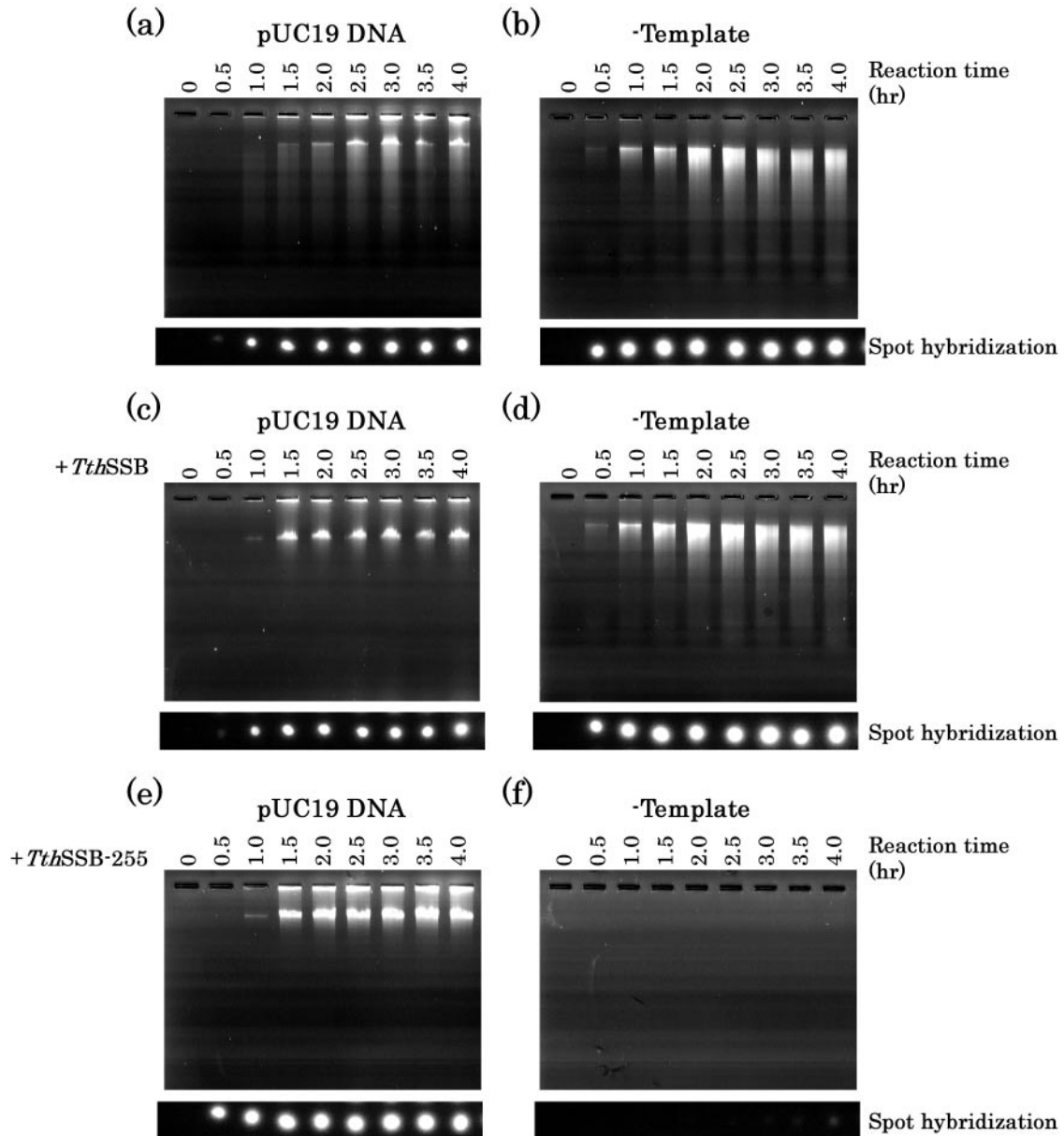
Figure 4. Effect of *Tth*SSB-255 protein on the efficiency and specificity of RCA. (**a**) Top: RCAs were performed in the absence of the SSB proteins using pUC19 DNA as template and phi29 DNA polymerase for the indicated reaction times. Bottom: Signals of spot hybridization of the same samples. (**b**) Top and Bottom: same as (a), except for the absence of template DNA. (**c**) Top and Bottom: same as (a), except for the presence of the *Tth*SSB protein (3.0 µg/20 µl reaction volume). (**d**) Top and Bottom: same as (c), except for the absence of template DNA. (**e**) Top and Bottom: same as (a), except for the presence of the *Tth*SSB-255 protein (3.0 µg/20 µl reaction volume). (**f**) Top and Bottom: same as (e), except for the absence of template DNA.

of input genomic DNA. In contrast, RCA in the presence of *Tth*SSB-255 protein was free of such nonspecific amplification products (Figure 5c, lanes 3 and 4) and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 5c, lanes 1 and 2).

The effect of template concentration

We expected that RCA in the presence of *Tth*SSB-255 protein would proceed with a considerably lower concentration of template DNA than that required for conventional RCA. As shown in Figure 6, high molecular weight DNA is generated as a result of isothermal strand-displacement amplification.

However, RCA produces nonspecific amplification artifacts (Figure 6a, lane 5) in the absence of input genomic DNA. In contrast, RCA in the presence of *Tth*SSB-255 protein was free of such nonspecific amplification products (Figure 6b, lane 5), and the presence of high molecular weight DNA is a clear indication of template-specific amplification (Figure 6b, lanes 1–4).

The results were further validated by Southern hybridization. Figure 6a shows that the amount of RCA products decreased drastically as template concentration was reduced to 1/10 in the control experiment (without *Tth*SSB-255, Figure 6a, bottom). In contrast, in the presence of *Tth*SSB-255, substantial amounts of RCA products were still obtained
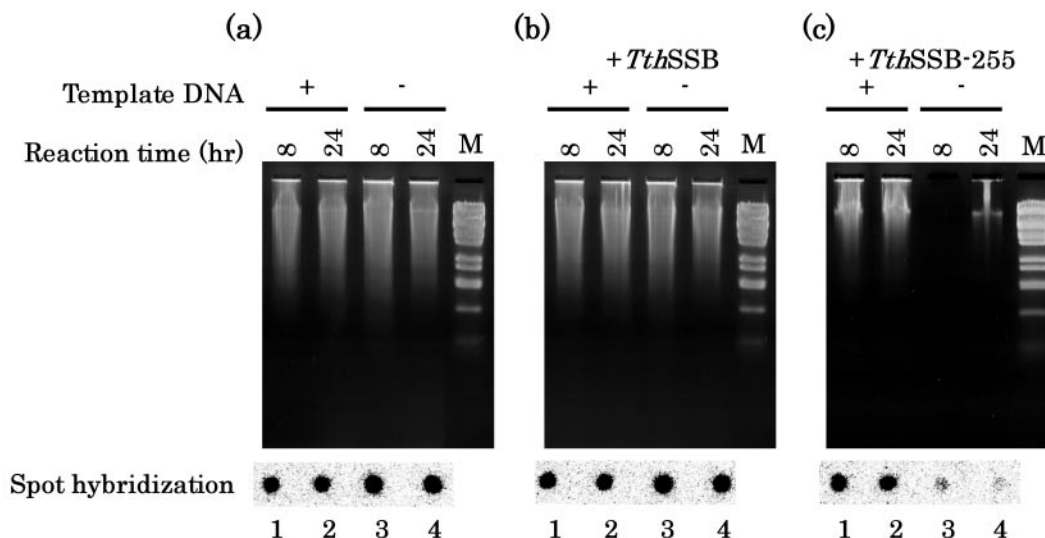
Figure 5. Effect of *Tth*SSB-255 protein on the efficiency and specificity of whole genome amplification via RCA. (**a**) Top: RCAs were carried out in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of human genomic DNA as template for the indicated reaction times. Bottom: signals of spot hybridization of the same samples. (**b**) Same as (a), except the presence of *Tth*SSB protein (3.0 µg/20 µl reaction volume). (**c**) Same as (a), except for the presence of *Tth*SSB-255 protein (3.0 µg/20 µl reaction volume).
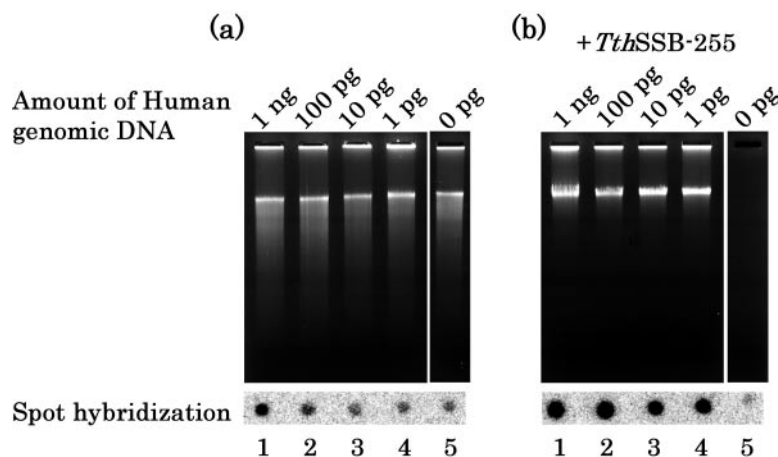

Figure 6. The effect of template concentrations on RCA products. (**a**) Top: RCA was performed with different amounts of template DNA in the absence of SSB protein. RCA products at serially diluted template DNA concentrations are shown. The products were electrophoresed on an agarose gel and stained with ethidium bromide. Bottom: signals of spot hybridization of the same samples. (**b**) Top and Bottom: same as (a), except for the presence of the *Tth*SSB-255 protein.

even when the template concentration was reduced to 1/1000 of the original concentration (Figure 6b, bottom). It is clear that *Tth*SSB-255 greatly stimulates polymerization (isothermal strand-displacement), particularly at lower template concentrations, indicating that in the presence of *Tth*SSB-255, the pairing of primers to template sequences occurs with high efficiency. Consequently, considerably lower template concentrations are enough to perform the RCA in the presence of *Tth*SSB-255.

PCR examinations of RCA-amplified whole genome

To estimate the quality of DNA obtained following RCA of human genomic DNA, a comparison was made between conventional RCA and RCA in the presence of *Tth*SSB-255 protein. To validate the effect of the *Tth*SSB-255 protein, a region in human genomic DNA was equally subdivided

into six 1346 bp sites (designated a-1–a-6, see Figure 7a). Using primers (20 bp) complementary to the terminal sequence of the subdivided site, PCR was performed. When we employed the DNA obtained by conventional RCA as a template, no amplification or nonspecific PCR products were observed (Figure 7b). As seen in Figure 7d, the employment of the amplified DNA in the presence of *Tth*SSB-255 eliminated nonspecific PCR products in most of the six PCR sites examined. These results suggest that the products by RCA in the presence of *Tth*SSB-255 protein are suitable for following PCR.

Multiplex PCR examinations of RCA-amplified whole genome

Encouraged by the elimination of nonspecific PCR products and the particularly low concentrations of template DNA
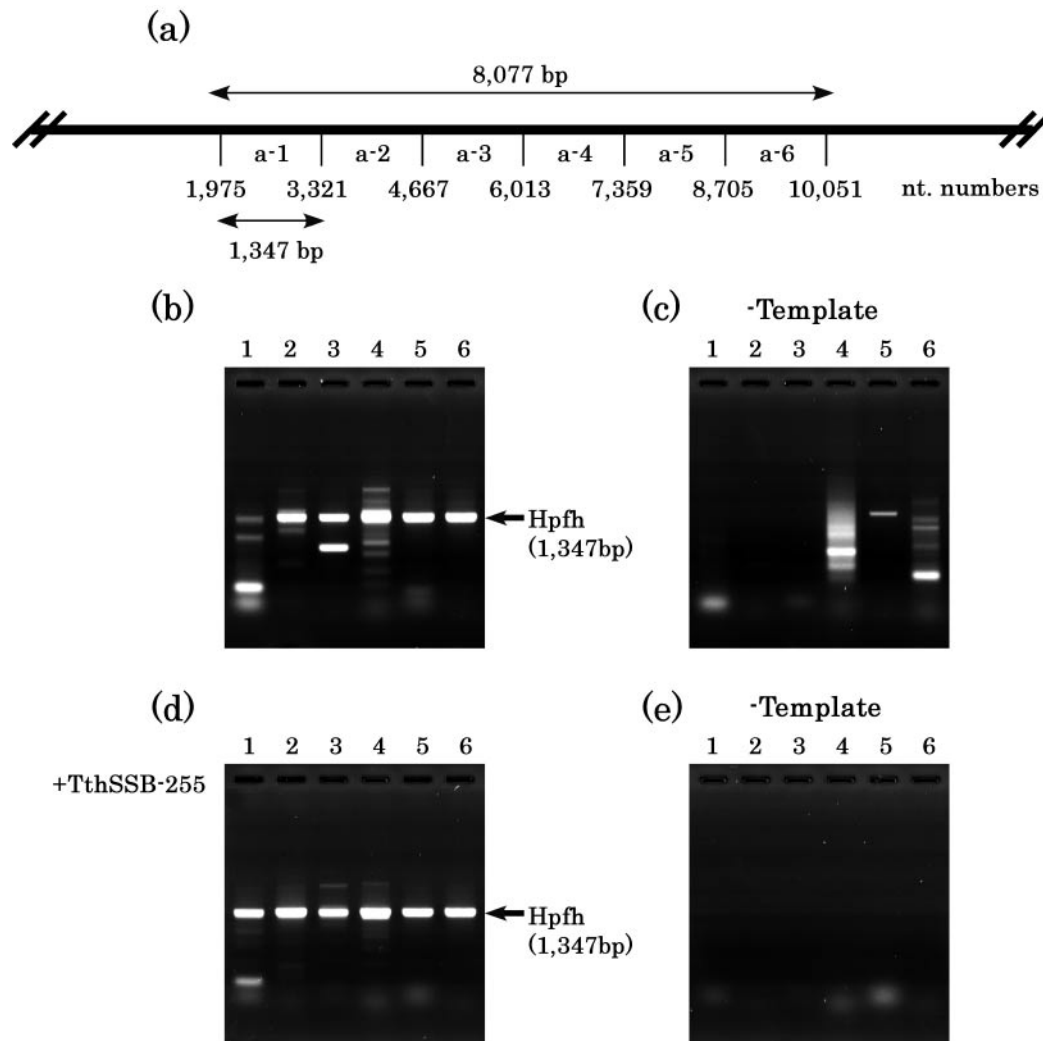
Figure 7. PCR examinations of RCA amplified whole genome. PCR amplifications were carried out on 0.5 µl samples of RCA products using human genomic DNA as the template. The products were electrophoresed and stained with ethidium bromide. (**a**) A diagrammatic representation of the subdivided region (a-1–a-6, 8077 bp in GenBank Acc. No. X91835). Nucleotide (nt) numbers correspond to registries in GenBank. The PCR amplifications using the RCA products from the reaction shown in Figure 5a lane 1 (**b**), Figure 5a lane 3 (**c**), Figure 5c lane 1 (**d**) and Figure 5c lane 3 (**e**). Throughout (b–e), the six subdivided sites are indicated as lanes 1–6. Locations of the specific PCR products are indicated by arrows.

required for RCA in the presence of *Tth*SSB-255 protein, we further examined the effect of the protein in RCA amplification from very small DNA amounts. To estimate the extent of amplification obtained following RCA of human genomic DNA, multiplex PCR examinations were performed using serially diluted template DNA concentrations.

We show the results of multiplex PCR for the randomly selected 12 human genes (derived from different chromosome) ranging from 57 to 360 bp in which 12 parallel PCR corresponding to each gene were conducted in the same reaction mixture (Figure 8a). The multiplex PCR were carried out on 0.5 µl samples of RCA products using human genomic DNA as the template in the absence or presence of *Tth*SSB-255 (Figure 8b and c). While the amount of PCR products amplified from RCA products decreased drastically as template concentration in the RCA was reduced to 1/1000 of that in lane 1 (Figure 8b), in the presence of *Tth*SSB-255 substantial amounts of PCR products were still obtained even

when the template concentration was reduced to 1/100 000 of the original concentration (Figure 8c).

It is clear that *Tth*SSB-255 protein greatly stimulates polymerization particularly at lower template concentrations. This indicates that in the presence of *Tth*SSB-255, elimination of nonspecific DNA products in RCA reactions occurs with high efficiency, resulting in a considerably lower template concentration requirement and homogeneous amplification of template than conventional RCA.

DISCUSSION

A thermophilic SSB protein and its mutant form from *T.thermophilus* HB8 were purified and its effects on the activity of phi29 DNA polymerases were assayed. The presence of *Tth*SSB-255 mutant protein shortened the elongation time required to synthesize a DNA fragment by phi29 DNA polymerase. Such stimulation could be the result of direct
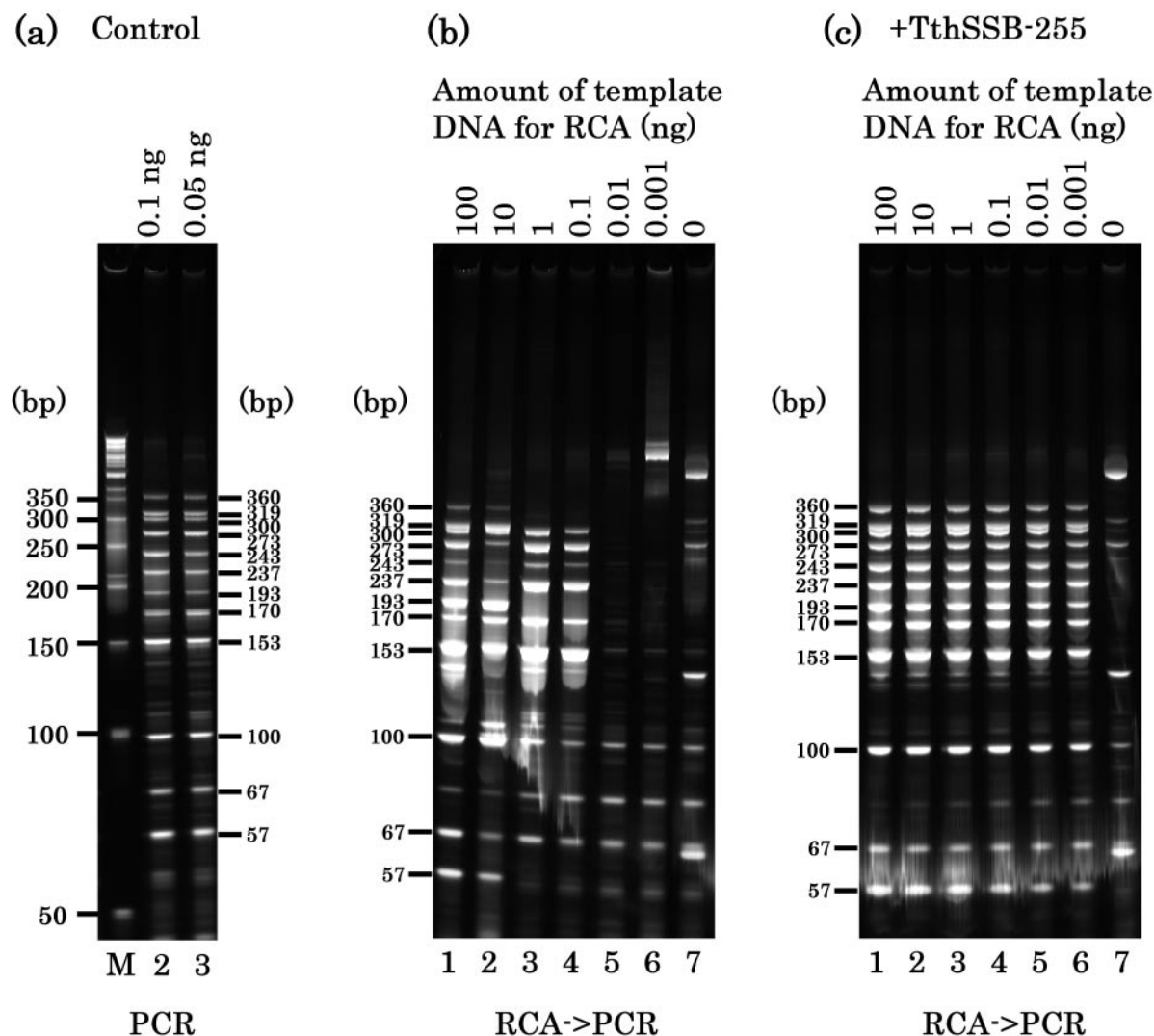
Figure 8. Multiplex PCR examinations of RCA amplified whole genome. (**a**) Control, multiplex PCR amplifications for the 12 randomly selected human genes using human genomic DNA as the template. Amount of the template DNA for the PCR is indicated. Multiplex PCR amplifications of the 12 human genes using RCA products as the template in the absence (**b**) or presence (**c**) of *Tth*SSB-255 protein. Amount of the template DNA for RCA is serially diluted as indicated. Throughout (a–c), all samples were amplified with primers at the same primer pair concentrations (0.1 µM per pair). Aliquots of 5 µl volume were electrophoresed through 12.5% acrylamide gel in Tris–borate/EDTA buffer, and stained with SYBR Green (SYBR Green I, Novagen). The signals were detected using a Fluoro Imager (Fluoro Imager 595, Molecular Dynamics). Product sizes (from 57 to 360 bp) are indicated on the right or left side of each panel. The oligonucleotide sequences used for the primers are as follows. Primer sets 1 (chromosome 11), 5'-GGGCA GAGCC ATCTA TTGCT TACA-3', 5'-GGTTG CTAGT GAACA CAGTT GTGTC A-3' ; Primer sets 2 (chromosome 16), 5'-GCACT CTTCT GGTCC CCACA GA, 5'-TTGGT CTTGT CGGCA GGAGA CA-3' ; Primer sets 3 (chromosome 8), 5'-GTCCT TCCCC CGCTG GAAAC-3', 5'-GCAGC AGAGA TCATC GCGCC-3'; Primer sets 4 (chromosome 7), 5'-CACAG ATTTC CAAGG ATGCG CTG, 5'-CGTGC TCTGT TCCAG ACTTG-3'; Primer sets 5 (chromosome 10), 5'-CGTCT GGCGA TTGCT CCAAA TG-3', 5'-GGGCA GTTGT GATCC ATGAG AA-3'; Primer sets 6 (chromosome 17), 5'-GCCTC TGATT CCTCA CTGAT TGCTC T, 5'-TGTCA ACCAC CCTTA ACCCC TCC-3'; Primer sets 7 (chromosome 20), 5'-TTGGA GGGGT GGGTG AGTCA AG, 5'-GGAGG GGTGG GGGTT AATGG TTA-3'; Primer sets 8 (chromosome 13), 5'-GGAAC AAGAC ACGGC TGGGT T-3', 5'-AGCAA GGCAG GGCAG GCAAG T-3'; Primer sets 9 (chromosome 3), 5'-CGGTC CCATT CTCAG GGAAT CT-3', 5'-GCCCA GAGGA AGAAG AAGGA AA-3'; Primer sets 10 (chromosome 1), 5'-GCCCC CACCC AGGTT GGTTT CTA-3', 5'-ATGCC TTCAT CTGGC TCAGT GAA-3'; Primer sets 11 (chromosome 6), 5'-GCTCA GCATG GTGGT GGCAT AA-3', 5'-CCTCA TACCT TCCCC CCCAT TT-3'; Primer sets 12 (chromosome 22), 5'-GACTA CTCTA GCGAC TGTCC ATCTC-3', 5'-GACAG CCACC AGATC CAATC-3'.

interactions between *Tth*SSB and DNA polymerase. However, as an increase in DNA synthesis was also observed when a *Bst* DNA polymerase was used (Supplementary Figure S1), it follows that the effect of *Tth*SSB-255 was not due to specific interactions with a DNA polymerase, but was most probably due to an indirect consequence of its binding to ssDNA.

There are several putative explanations for such an indirect effect on DNA synthesis stimulation since the *Tth*SSB-255 protein showed weak binding to ssDNA. For example, the

*Tth*SSB-255/ssDNA complexes could avoid the formation of folded ssDNA structures, which are generally considered to block the progression of DNA polymerase. In addition, the primers could hybridize not only free ssDNA region but also ssDNA on the *Tth*SSB-255/ssDNA complexes. Then, *Tth*SSB-255 could prevent non-productive binding of DNA polymerase to ssDNA, and direct it to specific dsDNA regions where the primers hybridize, thus allowing more effective strand-displacement in the RCA reaction.

Although several groups have reported that DNA binding proteins, such as T4gene 32, enhance RCA reactions, there are no reports on the application of SSB protein for RCA where the protein reduces nonspecific RCA products (17,18). Under the conditions we employed here, the inclusion of wild-type SSB protein either from *E.coli* or *T.thermophilus* in the reaction mixture for RCA neither reduced nonspecific products nor template concentrations required for RCA. *Tth*SSB-255 could only enhance RCA reactions drastically, which showed weak ssDNA binding affinity. This characteristic of the protein would be critical for reduction of nonspecific products. As shown in Supplementary Figure SP2, *Tth*SSB has a significant cluster consisting of acidic residues in its C-terminus although the role has not been cleared. Since F255P substitution is in the cluster, it may be related to a function of ssDNA binding in spite of acidic environment.

The utility of RCA-based whole genome amplification has been demonstrated for a variety of uses including quantitative PCR, SNP genotyping, Southern blot analysis of restriction fragments, chromosome painting (FISH), subcloning and DNA sequencing. The usefulness of whole genome amplification depends on its ability to give complete coverage of the genome with little regional bias, which is critical when the product is to be used for SNP genotyping (19,20). At extremely low template concentrations, regions of the genome are rarely lost, resulting in loss of one allele despite amplification of the locus (21). At a considerably lower template concentration, RCA in the presence of *Tth*SSB-255 protein could amplify template homogeneously than conventional RCA. Therefore, this method may be applicable to a genotyping application.

Finally, the homology between *Tth*SSB and *Taq*SSB proteins is very high especially in the C terminal domain (see Supplementary Figure S2) (22). Therefore, we strongly suspect that the same effects will be achieved by the *Taq*SSB mutant protein, which has the mutated amino acid at the same site.

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