Ramification Amplification: A Novel Isothermal DNA Amplification Method

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We have developed a novel isothermal DNA amplification method with an amplification mechanism quite different from conventional PCR. This method uses a specially designed circular probe (C-probe) in which the 3′ and 5′ ends are brought together in juxtaposition by hybridization to a target. The two ends are then covalently linked by a T4 DNA ligase in a target-dependent manner, producing a closed DNA circle. In the presence of an excess of primers (forward and reverse primers), a DNA polymerase extends the bound forward primer along the C-probe and displaces the downstream strand, generating a multimeric single-stranded DNA (ssDNA), analogous to the "rolling circle" replication of bacteriophages *in vivo*. This multimeric ssDNA then serves as a template for multiple reverse primers to hybridize, extend, and displace downstream DNA, generating a large ramified (branching) DNA complex. This ramification process continues until all ssDNAs become double-stranded, resulting in an exponential amplification that distinguishes itself from the previously described nonexponential rolling circle amplification. In this report, we prove the principle of ramification amplification. By using a unique bacteriophage DNA polymerase, Ø29 DNA Polymerase, that has an intrinsic high processivity, we are able to achieve significant amplification within 1 hour at 35°C. In addition, we applied this technique for *in situ* detection of Epstein-Barr viral sequences in Raji cells.

Key words: circular probe, rolling circle, PCR, *in situ* amplification.

We previously showed that application of PCR to a circularized probe (C-probe or padlock probe) generates multimeric DNA products, resulting in a greater amplification power than conventional PCR [1]. We termed this method ligation-dependent PCR (LD-PCR) because PCR can be initiated only when both ends of the probe are connected. We have extended these findings and present a novel isothermal nucleic acid detection method based on the amplification of a ligase-dependent C-probe (Fig. 1). Because the power to amplify is derived from the generation of multiple ramifying (branching) DNAs, we have coined the term ramification amplification (RAM).

The RAM assay uses a C-probe and a DNA polymerase with high processivity and displacement activity. The C-probe contains three regions: two target complementary sequences at the 5′ and 3′ termini (25 nucleotides each) and an interposed generic linker region (71 nucleotides) with several primer-binding sites. Once the C-probe binds to the

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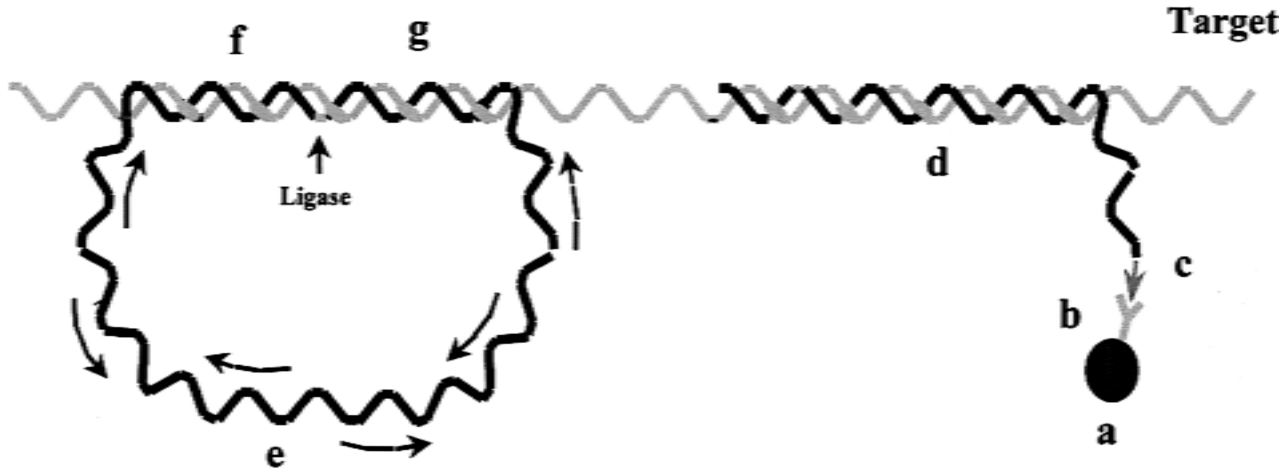

Fig. 1. Schematic representation of capturing target and circular probe (C-probe). The target bound to the complementary region of the capture probe (d) is retained on paramagnetic beads (a) through the binding of the biotin moiety (c) of the capture probe to streptavidin (b) coated on the beads. C-probe is also captured by binding to the target through their complementary regions (f, g). Hybridization to the target brings the 5′ and 3′ ends of the C-probe in juxtaposition, which then can be linked by a T4 DNA ligase to form a covalently closed C-probe. Sequence at the noncomplementary region (e) of the C-probe is generic for the binding of primers.

target, the 5′ and 3′ regions are brought into juxtaposition (Fig. 1). A closed circular molecule can be generated by incubating the probe with T4 DNA ligase [1,2]. After ligation, both forward and reverse primers and bacteriophage Ø29 DNA polymerase are added to the reaction. Forward primers bind to the linker region of the ligated C-probe (Fig. 1) and are extended by the polymerase. As the DNA polymerase reaches the downstream forward primer, these primers and their extended sequences are displaced [3]. Multimeric single-stranded DNAs (ssDNAs) are produced by continuously rolling over the closed circular DNA (Fig. 2). Multiple reverse primers then bind to the nascent strands. Extension from these reverse primers results in displacement of downstream-bound reverse primers with their extended sequences. Displaced ssDNAs serve as templates for further primer extension and amplification (Fig. 2). Like the constant unfurling of streamers, multiple primer extensions are simultaneously in progress, resulting in a large ramified complex. The reaction end-products are multimeric double-stranded DNAs (dsDNAs) of various lengths, including such smaller units as monomers, dimers, and trimers. Because the displaced DNAs are single stranded, allowing primers to bind to them at a constant temperature, the need for thermocycling to generate ssDNA for the binding of PCR primers is obviated. In addition, because RAM is an isothermal amplification method and large multimeric products are generated, cell morphological characteristics are preserved while amplification products are better localized in the cells, making this method ideal for *in situ* amplification.

Materials and Methods

Various concentrations of synthetic Epstein-Barr virus encoding RNA (EBER) DNA targets were hybridized with 10^{11} molecules phosphorylated C-probe and 10^{12} molecules biotinylated capture probe (Table 1) in 120 μL hybridization buffer containing 2 M guanidine thiocyanate (Sigma, St. Louis, MO), 0.5% bovine serum albumin (Sigma) 80 mM EDTA, 400 mM Tris HCl (pH 7.5), and 0.5% sodium-*N*-lauroylsarcosine (Sigma) at 37°C for 1 hour [1,4] (Fig. 3). Twenty micrograms streptavidin-coated paramagnetic beads (Dynal, Lake Success, NY) was added to the reaction and incubated at 37°C for an additional 30 minutes to capture the probe-target hybrids through the biotin-streptavidin interaction. The beads were then washed twice with 180 μL washing buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40 [Sigma]) to remove nonhybridized probes. During each wash, the beads were drawn to the wall of the assay tube by placing the tube on a Magnetic Separation Stand (Dynal), enabling the supernatant to be removed by aspiration. Then, 1 μL 5-U/μL ligase (Roche Molecular Bio-

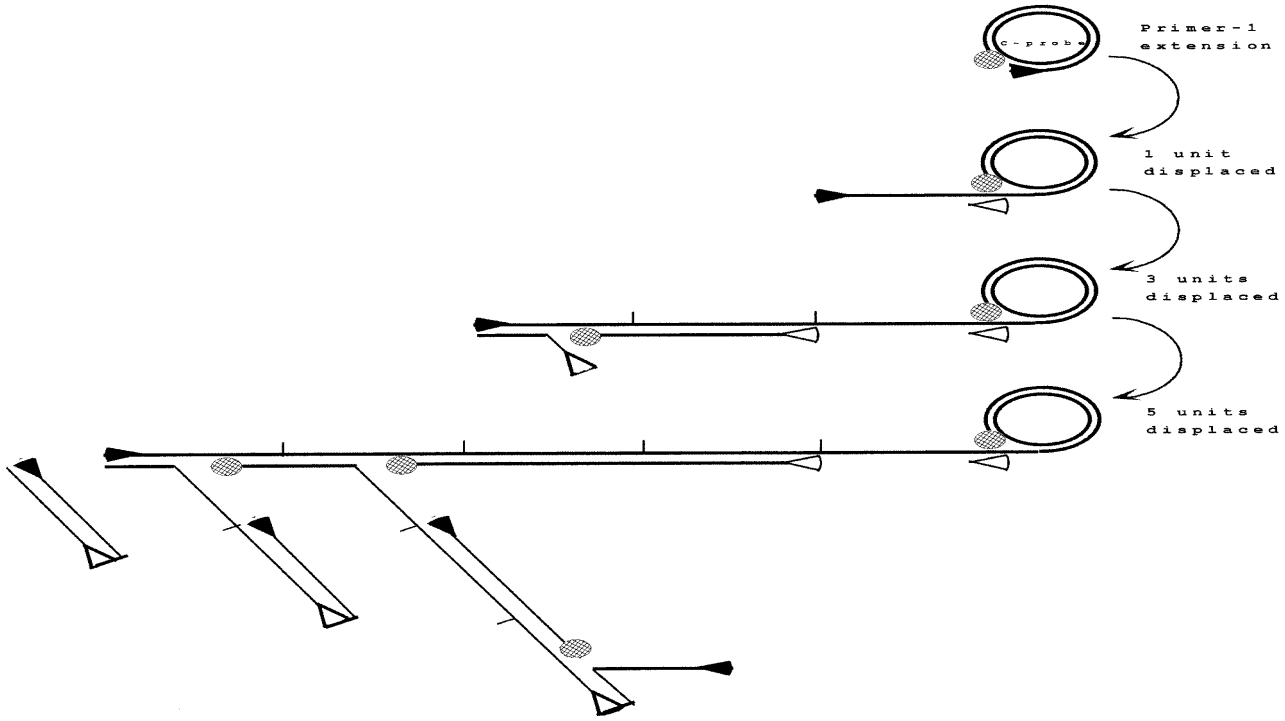

Fig. 2. Schematic representation of ramification amplification of ligated circular probe (C-probe). On addition of primers and Ø29 DNA polymerase, forward primer (▶) hybridized to the C-probe and was extended by the polymerase (⋘). The polymerase continues to extend after one round of synthesis by displacing the bound forward primer and its extended product, generating a long multimeric ssDNA, a process analogous to the rolling circle replication of bacteriophage *in vivo*. As it grows, multiple reverse primers (◁) can bind to the nascent ssDNA as their binding sites are available. Each bound reverse primer will extend and displace the downstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primer to bind and extend, thus forming a large ramifying DNA complex. This process results in exponential amplification of the C-probe sequence.

chemicals, Indianapolis, IN), 20 μL 1-mM $MnCl_2$, 66 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM ATP, and 5 mM $MgCl_2$ were added to the beads and incubated at 37°C for 1 hour. This step allows the formation of a covalently linked C-probe.

A typical RAM reaction was performed in 50 μL containing 90 ng Ø29 DNA polymerase (courtesy of Dr. John Cunniff, Pharmacia, Cleveland, OH); 10^{13} molecules each primer; 66 mM Tris-HCl (pH 7.5); 1 mM DTT; 6 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$; and 80 μM each dATP, dCTP, dGTP, and dTTP, together with 20 μL ligation products. The reaction was incubated at 30°C for 1 to 2 hours. RAM products (10 to 20 μL) were incubated with 10 U restriction enzyme *Eco*R I (New England Biolabs, Beverly, MA), 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, and 100 mM NaCl at 37°C for 1 hour to digest multimeric DNA into monomer. The products were examined as previously described by electrophoresis through an 8% polyacrylamide gel [1].

The *in situ* RAM reaction was performed by combined techniques of *in situ* hybridization of C-probe and the previously described RAM assay. Briefly, cultured Raji cells infected with Epstein-Barr virus (EBV) and EBV-negative NB4 cells were placed on silane-coated slides by cytospin

Table 1. Sequences of Circularizable Probe, Hemiprobes, Capture Probes, and PCR Primers

Probes (no. of nucleotides)	Sequence (5′–3′)
C-probe (121)	TCACCACCCGGGACTTGTACCCGG|GACT***GTCTGTGT***|***ATCTGCTAAC****CA*|*AGAGCAACTACA*|
	|<--------RAM-1--------|-------- RAM-2 ---->|<--------RAM-3--------|
	*CGAA***TTC****TCGAT*|**TAGGTTACTG**CG|ATTAGCACAAGC|GGGAAGACAACCACAGACACCGTCC
	|--------RAM-4-------->|<-------- RAM-5 --------|-------- RAM-6 -------->|
EBER-target (95 nt)	GTCCCGGGTACAAGTCCCGGGTGGTGA|GGACGGTGTCTGTGGTTGTCTTCCCAGCTTCGGTCAA GTACCAGCTGGTGGTCCGCATGTTTTGATCC
Capture probe (45)	Biotin-AAGAGGATCAAAACATGCGGACCACCAGCTGGTACTTGACCGAAG
PCR primer 1 (18)	GTTAGCAGATACACAGAC (forward)
PCR primer 2 (18)	CAAGAGCAACTACACGAA (reverse)
PCR primer 3 (18)	TTCTCGATTAGGTTACTG (reverse)
RAM primer 1 (12)	ACACAGACAGTC (forward)
RAM primer 2 (12)	ATCTGCTAACCA (reverse)
RAM primer 3 (12)	TGTAGTTGCTCT (forward)
RAM primer 4 (12)	CGAATTCTCGAT (reverse)
RAM primer 5 (12)	CGCAGTAACCTA (forward)
RAM primer 6 (12)	ATTAGCACAAGC (reverse)

Single underlines indicate sequences complementary to EBER-1, dotted underlines indicate restriction enzyme *Eco* R I recognition site, bold italic letters indicate the binding regions for PCR primer 1, italic letters indicate the binding region for PCR primer 2, and bold letters indicate the binding regions for PCR primer 3. Dash lines indicate RAM primer-binding site, and arrowheads indicate the direction of primer extension. For EBER target, the single underlines indicate C-probe binding region, the vertical bar indicates contact sites of the 3′ and 5′ ends of the C-probe, and double underline indicates the capture probe-binding region.

C-probe, circular probe; EBER, Epstein-Barr virus encoding RNA; RAM, ramification amplification.

centrifugation at 750 rpm for 5 minutes, then fixed by immersion in a 3:1 solution of methanol acetic acid at 20°C for 20 minutes. Cells were digested with 25% pepsin (1 N HCl) at 37°C for 10 minutes to increase cell-membrane permeability and washed with phosphate-buffered saline (pH 7.2; PBS) for 5 minutes. The phosphorylated C-probe (10^{12} molecules) dissolved in 50 μL hybridization buffer containing 50% formamide, 1% polyvinylpyrrolidone, 2% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 5× SSPE, and 100 μg salmon sperm DNA was added to the slides and incubated at 95°C for 10 minutes and 37°C for 2 hours to allow the formation of RNAC-probe hybrids. The slide was washed once with 2× SSC/0.1% SDS to remove unbound probes and twice with 1× ligase buffer to remove 2× SSC. Finally, 50 μL ligation solution containing 66 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM ATP, 1 mM $MnCl_2$, 5 mM $MgCl_2$, and 1 U T4 DNA ligase was applied to the slide and incubated at 37°C for 1 hour to allow the formation of covalently linked DNA. After ligation, 50 μL RAM reaction containing 90 ng Ø29 DNA polymerase; 10^{13} molecules each primer (one forward primer, one reverse primer); 66 mM Tris HCl (pH 7.5); 1 mM DTT; 6 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$; 100 μM each dATP, dCTP, and dGTP; 65 μM dTTP; and 35 μM digoxigenin-11-dUTP (Roche Molecular Biochemicals) was applied to the slides and incubated at 30°C for 2 hours. After RAM, slides were washed with 0.2× SSC to remove nonincorporated digoxigenin-11-dUTP. The amplification products were detected by colorimetric method using alkaline phosphataseconjugated antidigoxigenin antibody and NBT/BCIP substrate (Roche Molecular Biochemicals) per manufacturer's instruction. Results of *in situ* RAM were compared with those of *in situ* hybridization using digoxigenin-labeled EBER probe cocktail (Kreatech, Amsterdam, The Netherlands) per manufacturer's recommendation.

Results

Rolling Circle Primer Extension of DNA Polymerases

Because the limiting factor for the RAM reaction is the length of the initial ssDNA generated from the closed circular DNA, we compared three polymerases lacking exonuclease activity and having good displacement activity: Vent (exo–) [5] (New

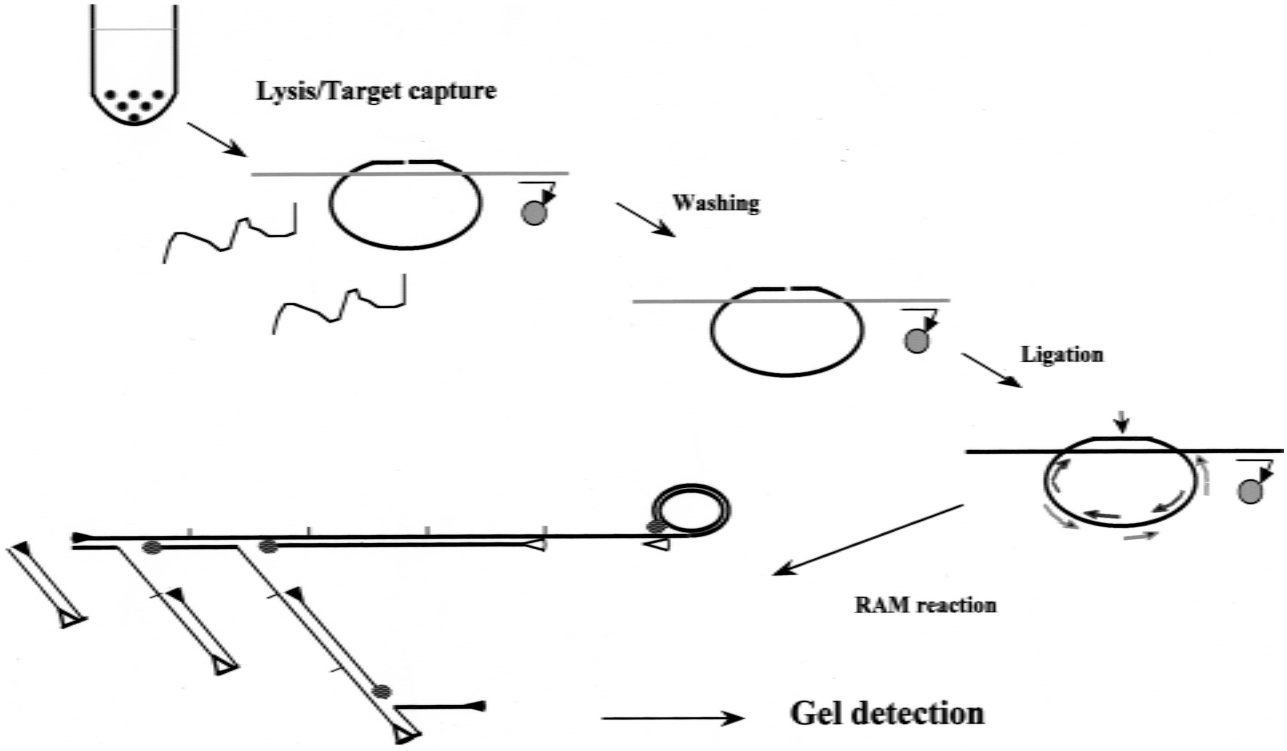

Fig. 3. Schematic representation of magnetic isolation, target-specific ligation, and ramification amplification (RAM). Target DNA, capture probe, circular probe (C-probe), and paramagnetic bead are added to hybridization buffer to allow the formation of a hybrid composed of target, C-probe, and capture probe. The hybrid is captured on a paramagnetic bead and held back during extensive washing to remove unbound C-probe. The C-probe aligned on the target is linked together by T4 DNA ligase. RAM is then performed by the addition of forward and reverse primers and Ø29 DNA polymerase. RAM products are examined by gel electrophoresis.

England Biolabs), Klenow fragment (exo–) of *Escherichia coli* DNA polymerase I [6] (US Biochemicals), and bacteriophage Ø29 DNA polymerase of *Bacillus subtilis* [7], by establishing their primer extension and displacement ability. Each polymerase was incubated with 10^{11} molecules ligated C-probe and 10^{13} molecules one forward primer in the appropriate buffer provided with the polymerase per manufacturers' instructions. Our results show poor processivity and displacement activity of both Vent and Klenow polymerases (Fig. 4). Vent polymerase produced 600-nucleotide long ssDNA (i.e., six rounds along the C-probe) at 72°C (data not shown). Klenow polymerase produced longer ssDNA at 37°C; however, the yield was much poorer than that with Vent. We found that Ø29 DNA polymerase generated ssDNA longer than 8,000 nucleotides from the ligated C-probe. In addition, large multimeric ssDNAs are generated that could not migrate into the gel because of their size (Fig. 4). These results confirmed Ø29 DNA polymerase as the enzyme of choice for RAM assay.

RAM

To show that ramification-type amplification occurs, the ligated C-probe was incubated with one, two, and three PCR primers (Fig. 5) on the assumption that ramification requires the addition of reverse primers. The experiment was performed by hybridizing 10^{11} molecules synthetic DNA target and 10^{11} molecules C-probe. After ligation, one PCR primer (forward), two PCR primers (one forward primer, one reverse primer), or three PCR primers (one forward primer, two reverse primers) were added to each reaction, together with Ø29 DNA polymerase. The products without *Eco*R I digestion were examined on an 8% polyacrylamide gel to determine the presence of large DNA fragments. As expected, the DNA generated by one primer (Fig. 5, lane A) was multimeric ssDNA with some products unable to migrate into the gel because of their large size. The addition of the second reverse primer significantly increased the amount of products (Fig. 5, lane B). The addition of a third reverse primer (Fig. 5, lane C) further increased

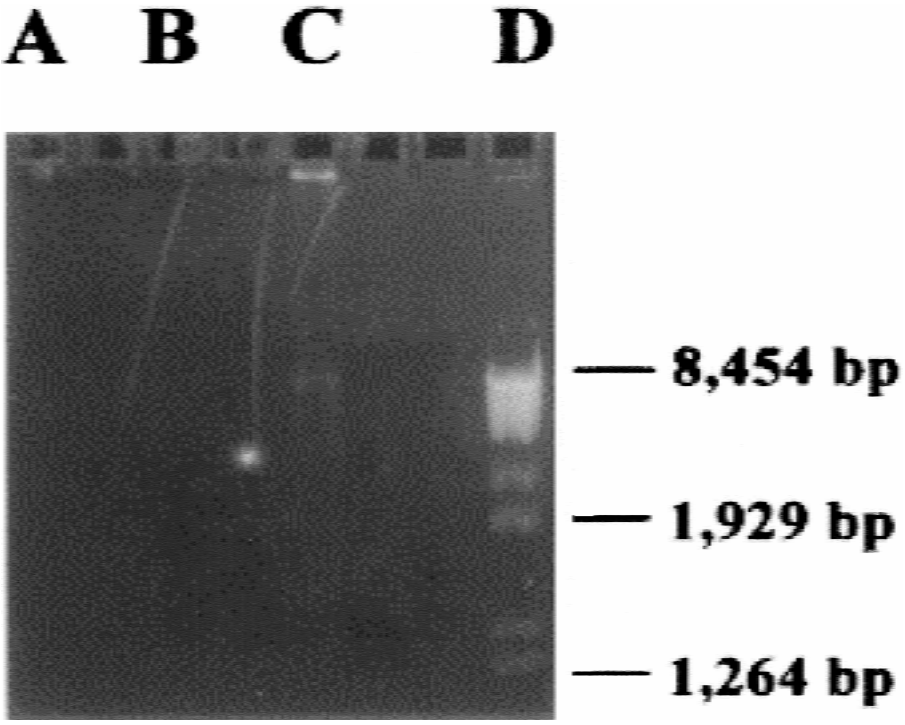

Fig. 4. Comparison of various polymerases for rolling circle primer extension: 10^{12} molecules of EBER targets were hybridized with 10^{12} molecules of C-probes and capture probes. After washing and ligation, each polymerase was incubated with 10^{11} molecules ligated C-probe and 10^{13} molecules one primer in their appropriate buffers and temperatures (72°C for Vent, 37°C for Klenow, 35°C for Ø29 DNA polymerase) for 2 hours. The products were examined on 1.2% agarose gel without *Eco*R I digestion. Lane A, Vent (exo-); lane B, Klenow fragment (exo-); lane C, Ø29 DNA polymerase; lane D, DNA size marker (1 μg Lamda DNA digested with *Bst*E II). Results show that only Ø29 DNA polymerase generated ssDNA longer than 8,000 nucleotides from ligated C-probe, as well as even longer ssDNA that cannot migrate into the gel (lane C).

DNA production. These results indicate that ssDNA generated from the C-probe can serve as a template for reverse primers to bind, extend, and displace upstream DNA under isothermal conditions, proving that RAM occurred.

Optimization of Assay Condition

To further increase amplification efficiency, we modified the reaction condition by adding bacteriophage T4 Gene 32 protein (Roche Molecular Biochemicals), an ssDNA-binding protein, to the reaction. Gene 32 protein destabilizes dsDNA helices [8] and has been used to enhance PCR [9]. The RAM assay was performed as described with 10^{10} molecules DNA target/reaction, and Gene 32 protein was added to the reaction in decreasing concentrations from 5 μg to 0.5 ng/reaction. Figure 6A shows marked enhancement of DNA production at 0.5 ng/reaction. Further dilution of Gene 32 protein nullified this enhancement at 0.05 ng/reaction (data not shown). Dimethyl sulfoxide (DMSO) was added to the RAM reaction because it destabilizes dsDNA helices while stabilizing polymerase-template complexes [10]. Figure 6B shows a significant increase in DNA production at 6% to 10% concentration of DMSO in the absence of Gene 32 protein, and no product was seen in the absence of DMSO or Gene 32 protein (Fig. 6B, first lane). The greater molecular-weight DNAs present in high-yield lanes represented incompletely digested DNA products.

Detection of DNA Target

In addition, we increased the number of primer-binding sites on the C-probe to six (three forward primers, three reverse primers) by reducing the primer length from 18 to 12 nucleotides (Table 1) because we showed that the greater the number of primer-binding sites in the C-probe, the greater the amplification achievable (Fig. 5). The RAM reaction proceeded with these additional improvements: 10% DMSO, 0.5 ng Gene 32 protein, and six in-

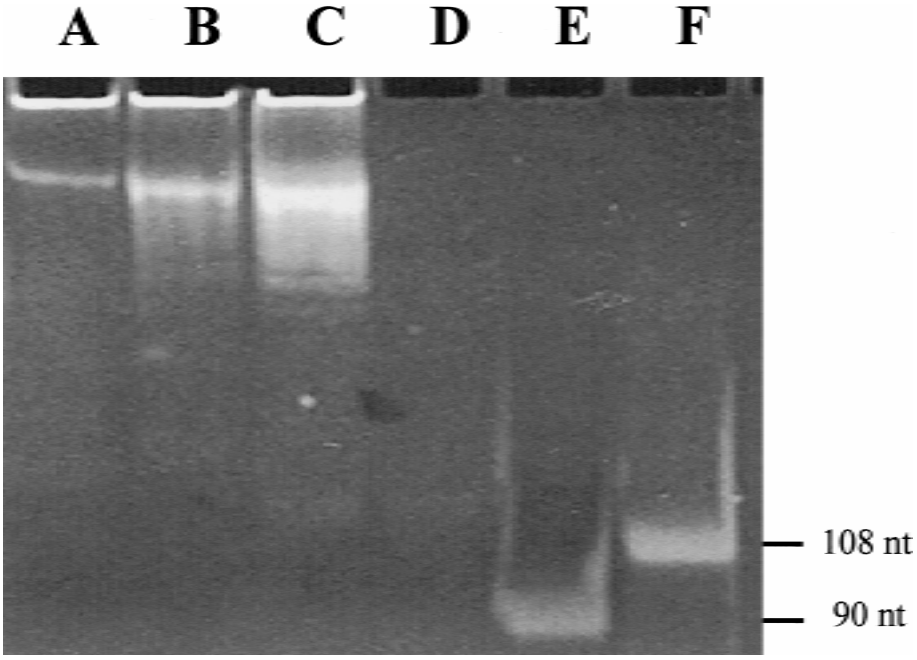

Fig. 5. Ramification amplification reaction: 10^{11} molecules synthetic DNA target were hybridized with 10^{11} molecules circular probes (C-probes) and 10^{12} capture probes. After washing and ligation, 10^{13} molecules PCR primer 1 (lane A), PCR primers 1 and 2 (lane B), or PCR primers 1, 2, and 3 (lane C) were added to each reaction with Ø29 DNA polymerase. Lane D, absence of target; lane E, synthetic EBER target (90 nucleotides); lane F, unligated C-probe (108 nucleotides). Products were examined on an 8% polyacrylamide gel without *Eco*R I digestion. Results show a significant increase in DNA product when reverse primers were added.

stead of two RAM primers (10^{13} molecule each; Fig. 7). Serial dilutions of the target showed our ability to detect 10^4 target molecules per reaction, although the resultant band appeared faint on the photographic image. C-probes derived from the same ligation reaction were also amplified by LD-PCR using two primer pairs (PCR primer 1 and 2; Table 1). The detection of 10^4 molecules EBER targets confirmed that RAM and LD-PCR had equivalent sensitivity (data not shown).

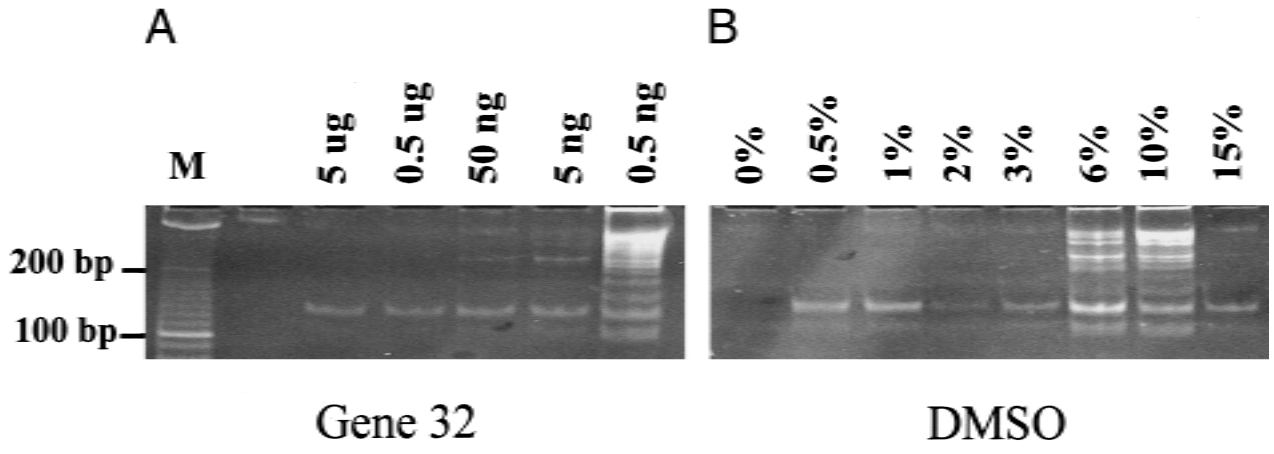

Fig. 6. Effect of Gene 32 protein and dimethyl sulfoxide (DMSO) on ramification amplification (RAM) reaction: 10^9 molecules DNA target were hybridized with 10^{11} molecules circular probed (C-probes) and 10^{12} capture probes. After washing and ligation, RAM reactions were initiated with 10^{13} three PCR primers (one forward primer, two reverse primers) and various concentrations of (A) Gene 32 protein or (B) DMSO. RAM products were examined on an 8% polyacrylamide gel after digestion with restriction enzyme *Eco*R I. Results show that both Gene 32 and DMSO stimulate the RAM reaction significantly, with optimal concentrations at 0.5 ng for Gene 32 and 6% to 10% for DMSO.

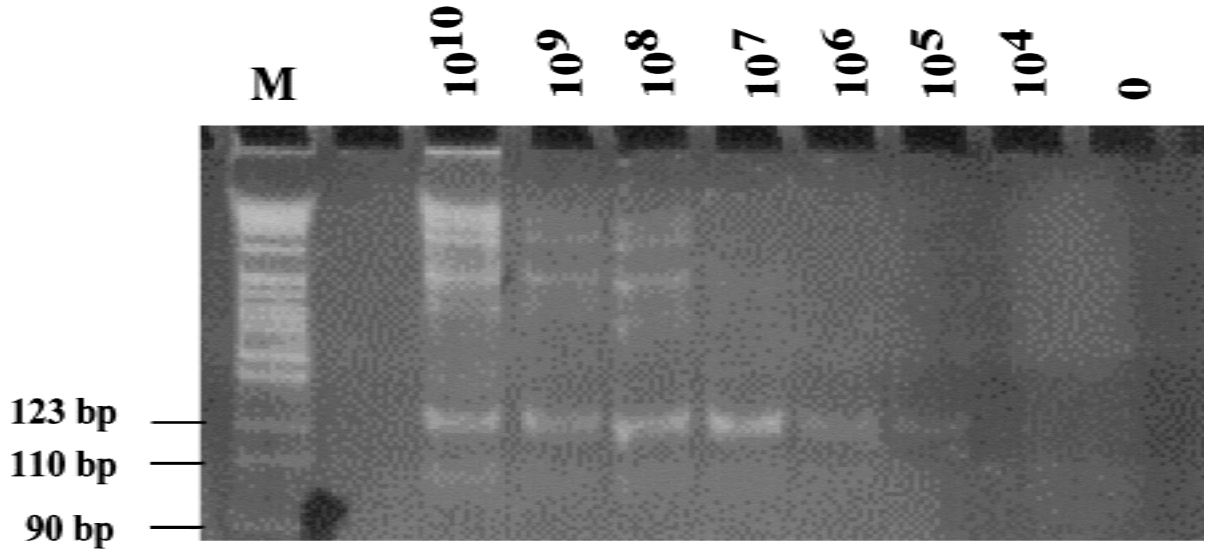

Fig. 7. Detection of EBER targets by ramification amplification (RAM). Serials of tenfold dilution of targets from 10^{10} to 10^4 molecules were hybridized with 10^{11} molecules circular probes (C-probes) and 10^{12} capture probes. After washing and ligation, 10^{13} molecules each RAM primer (three forward primers, three reverse primers) were added to each reaction with Ø29 DNA polymerase, 6 mM $(NH_4)_2SO_4$, 10% DMSO, and 0.5 ng Gene 32 protein. RAM products were examined on an 8% polyacrylamide gel after digestion with *Eco*R I. Results show a decrease in RAM products with the decrease in EBER targets and as few as 10^4 molecules targets were detected. The presence of higher molecular-weight DNAs was caused by incomplete digestion. Lane M, DNA size marker (1 μg PBR322 DNA digested with *Msp* I); lane 0, no target present.

Although the RAM reaction was performed at 35°C, the specificity of the assay was not compromised. No DNA was produced in the absence of target (Fig. 7). Furthermore, products could be digested with specific restriction endonuclease (*Eco*R I) located in the linker region (Table 1), yielding the correct bands (121 and 103 bp; Fig. 7). No RAM product was observed in the presence of nonspecific targets, even in high concentration (10^{10} molecules, data not shown).

In Situ Detection of EBER

As shown in Fig. 8, both *in situ* hybridization and *in situ* RAM detected EBV in Raji cells. However, RAM (Fig. 8B) detects more positive cells than *in situ* hybridization (Fig. 8A), indicating significant improvement of sensitivity. No signals were seen in NB4 cells, indicating target-dependent amplification (Fig. 8C).

Discussion

This report describes RAM, a novel isothermal DNA amplification method based on primer extension, displacement, and ramification. RAM generates variable-length fragments of repeat units of a C-probe. The amplification process can be expressed as x^U, where U is number of repeats generated from the closed C-probe and x is the number of primers used. For example, if two primers are used (one forward primer, one reverse primer), the formula is 2^U, indicating that RAM is an exponential amplification and therefore equivalent to PCR. With six primers (three forward primers, three reverse primers), the formula is 6^U, indicating that RAM represents a superexponential amplification. This formula also indicates that the limiting factor of the RAM assay is the length of the initial ssDNA generated from the closed C-probe; at least 20 repeats (i.e., 2,420 nucleotides long) of ssDNA from the closed C-probe are required to achieve one millionfold amplification.

This minimal length of the initial ssDNA requires that DNA polymerases show the following properties: (1) no 5′ to 3′ exonuclease activity because this will degrade rather then displace downstream strands; (2) strong displacement activity to separate downstream DNA from the template strand; and (3) high processivity (i.e., incorporating nucleotides continuously on a given primer without dissociating from the template), allowing the polymerase to synthesize at least 2,000-nucleotide long ssDNA from

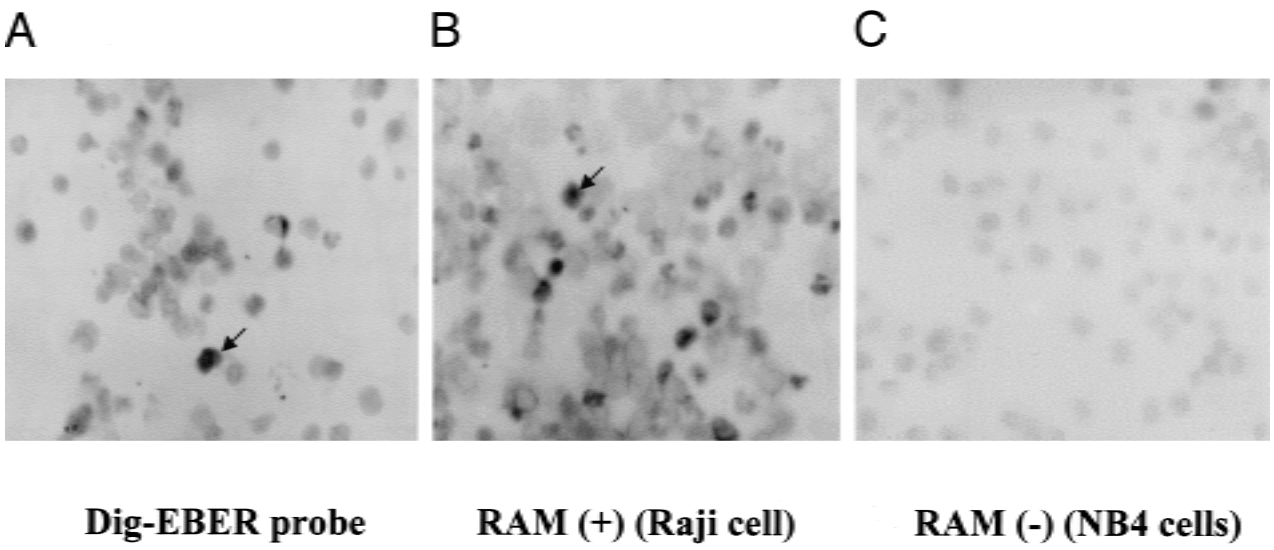

Fig. 8. *In situ* detection of EBER in Raji cell. Raji cells infected with Epstein-Barr virus (EBV) and NB4 cells negative for EBV were spotted on a slide by cytospin. EBER is detected by (A) *in situ* hybridization and (B) *in situ* hybridization followed by ramification amplification (RAM). Positive signals are seen as dark precipitation in cells (arrows). (C) No signal was seen in NB4 cells.

the ligated C-probe, achieving one millionfold amplification. Most DNA or RNA polymerases will dissociate from the template after reaching certain lengths (i.e., poor processivity) [11,12]. Also, most DNA polymerases lack intrinsic displacement activity and show some 5′ to 3′ exonuclease activity [11]; thus, they are unsuitable for downstream DNA displacement in the RAM assay. Our results show that only Ø29 DNA polymerase generated ssDNA longer than 8,000 nucleotides from the ligated C-probe and even longer ssDNA, unable to migrate into the gel (Fig. 4). This indicated that at least 60 repeats were present in the initial ssDNA.

Several other factors affect the rate of amplification. The two most important factors are the stability of the DNA-polymerase complex and accessibility of primer to its binding sites as soon as they are available. It is possible that formation of the large ramifying DNA-polymerase complex may interfere with the interaction of primers to their binding sites and disrupt the DNA-polymerase complex. Therefore, we added the organic solvent DMSO (which stabilizes the DNA polymerase [10]) and a protein cofactor T4 Gene 32 protein (which stabilizes single-stranded regions of DNA [8]), thus enhancing the binding of the primer to the growing ssDNA and stabilizing the DNA polymerase complex. As expected, the addition of these agents significantly stimulates DNA synthesis and amplification. It is noteworthy that even with all these efforts, assay sensitivity was still limited to 10^4 molecules, equivalent to 40 cycles of LD-PCR, perhaps a reflection of the intrinsic property of the DNA polymerase used in this assay. Ø29 DNA polymerase is very efficient for rolling circle primer extension [13], but less suited for the ramification reaction [14]. We recently found that the large fragment of Bst DNA polymerase of *Bacillus stearothermophilus* is much more efficient than Ø29 DNA polymerase. We hope that Bst DNA polymerase will allow us to achieve better sensitivity.

The RAM assay offers several advantages over other amplification techniques: (1) the primers readily bind to ssDNAs displaced by the DNA polymerase, enabling the reaction to be performed under isothermal conditions, obviating the need for a thermocycler; (2) generic primers amplify all probes with equal efficiency, resulting in better multiplex capability than conventional PCR [1,4]; both ends of the probe can be ligated regardless of the nature of target (DNA or RNA), eliminating the need for reverse transcription for detecting RNA and creating a uniform assay format for both RNA and DNA detection [4]; and (4) ligation requires that both probe termini hybridize with perfect matching, permitting the detection of a single-nucleotide polymorphism.

In situ RAM is an ideal technique for intracellular target detection. Hybridization of a C-probe to its target and ligation of the two ends results in a covalently locked-on probe because of the helix formed between the C-probe and target [2]. This

allows for stringent washings to remove unbound probes, reducing background signals [2]. The large multimeric products (>8,000 nucleotides long) generated during RAM prevent amplification products from diffusing out of the cells, a problem inherent to *in situ* PCR [15], thus providing better signal localization. The mild isothermal amplification condition of RAM preserves tissue morphological characteristics. Last, because the C-probe hybridizes to only a small region on the target (50 nucleotides) and no primer extension on the target nucleic acids is required, cross-linking of the target nucleic acids caused by the fixation process has a minimal effect on the target-independent aspect of the RAM reaction [16].

In summary, we have shown a novel concept of DNA amplification and used this method to detect target nucleic acids in a test tube and *in situ*. We predict that the inherent simplicity of this method will allow RAM to be used in wide variety of clinical applications.

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