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Highly Efficient Isothermal DNA Amplification System Using Three Elements of 5′-DNA-RNA-3′ Chimeric Primers, RNaseH and Strand-displacing DNA Polymerase

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We developed an efficient method of isothermally amplifying DNA termed ICAN, Isothermal and Chimeric primer-initiated Amplification of Nucleic acids. This method allows the amplification of target DNA under isothermal conditions at around 55°C using only a pair of 5′-DNA-RNA-3′ chimeric primers, a thermostable RNaseH and a DNA polymerase with strong strand-displacing activity. ICAN is capable of amplifying DNA at least several times greater than the amount produced with PCR by increasing primer concentration. This method would be applicable for on-site DNA detection including gene diagnosis, and would also be suitable for 'real time' detection when combined with a cycling probe.

Key words: chimeric primer, detection, DNA polymerase, isothermal DNA amplification, RNaseH.

Abbreviations: CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium* sp. CP4; CSVd, chrysanthemum stunt viroid; HDA, helicase-dependent amplification; ICAN, isothermal and chimeric primer-initiated amplification of nucleic acids; LAMP, loop-mediated isothermal amplification of DNA; RCA, rolling-circle amplification; SDA, strand displacement amplification; Tli RNaseH, *Thermococcus litoralis* RNaseH; TMA, transcription-mediated amplification.

Methods of amplifying nucleic acids are used widely in the life science area. At present, the polymerase chain reaction (PCR) is the most popular and useful method (*1*, *2*). Although extremely versatile and sensitive, PCR has limitations in that it requires a high-precision thermal cycler device for periodic changing of the reaction temperature. This requirement prevents PCR from being used in on-site diagnosis at bed sides of hospital or for large-scale production of nucleic acids for industrial use. To overcome this limitation, methods which do not need temperature changes for amplifying nucleic acids have been developed, including strand displacement amplification (SDA) (*3*, *4*), transcription-mediated amplification (TMA) (*5*), rolling-circle amplification (RCA) (*6*, *7*), loop-mediated isothermal amplification of DNA (LAMP) (*8*) and helicase-dependent amplification (HDA) (*9*).

SDA makes use of both the ability of a restriction endonuclease to nick the unmodified strand and the ability of a DNA polymerase with strand-displacing activity to extend a strand from the nicked site, incorporating modified nucleotides, and displacing the downstream strand. In TMA, three enzymes work simultaneously to generate cDNA in addition to RNA. One enzyme, an RNA polymerase, makes RNA from a promoter designed in the 5′ region of primers; another, a reverse transcriptase, generates complementary DNA from the RNA template; and the other, RNaseH, removes RNA from DNA/RNA hybrid molecules. In RCA, a circularized padlock probe having both ends (each about 20 bp) designed to hybridize adjacently to a specific target DNA sequence is first generated by ligation. A DNA polymerase with strand-displacement activity then extends a primer on the circularized probe (template) and generates many copies of the complementary sequence of the template linked in tandem. LAMP uses a DNA polymerase with strand-displacing activity and two sets of a pair of primers containing sequences of both target and complementary strands each derived from three distinct regions. DNA strands synthesized from inner primers are displaced by strands initiated from outer primers and a single-stranded DNA is released. This single-stranded DNA serves as a template for DNA synthesis from both hybridized primers, and a complicated stem-loop DNA is formed. This is repeated yielding the original stem-loop DNA and a new one with a stem twice as long. Finally, HDA uses a pair of primers and four components, *i.e.* DNA helicase, MutL, single-strand-binding protein and ATP for primer hybridization and subsequent primer extension by a DNA polymerase with strand-displacement activity.

Although these isothermal methods of amplifying nucleic acids have their own unique features, they are complicated compared to PCR in terms of the components of the reaction mixture or reaction products, which often limits their applications. For example, SDA and LAMP

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requires more than two primers and RCA needs a circularized probe in addition to two primers for amplification. These requirements make the design of the primers or probe both difficult and laborious. TMA and HDA need two or more enzymes in addition to the DNA polymerase, which narrows the range of parameters for the adjustment of reaction conditions. The amplified products of SDA and TMA include, in addition to natural DNA, a modified nucleotide and RNA, respectively. The amplified products of RCA or LAMP consist of concatenated target sequences. Greater caution or additional steps are often required in the handling of these products compared to simple PCR-type DNA products. Some enzymatic processing such as restriction endonuclease digestion could be hampered because of the incorporation of a modified nucleotide in the case of SDA.

We have developed an efficient method of isothermally amplifying DNA, and we termed this method, **I**sothermal and **C**himeric primer-initiated **A**mplification of **N**ucleic acids (ICAN). This method allows the amplification of target DNA under isothermal conditions at around 55°C by use of only a pair of 5′-DNA-RNA-3′ chimeric primers, RNaseH derived from a hyperthermophilic archaeon, and a polymerase with strand-displacing activity. Using this method, one can detect a target nucleic acid like PCR but without thermal cycling and amplify DNA to more than 2 μM. In this paper, we show the performance of ICAN and clarify essential factors for the amplification.

MATERIALS AND METHODS

DNA-RNA Chimeric Primer Synthesis—Chimeric primers were prepared commercially with use of columns and amidites for DNA and RNA synthesis (Sigma-Aldrich, St Louis, Missouri) by Takara Biotechnology (Dalian) Co., Ltd (Dalian, China).

Primer Sequence—Nucleotide sequences of the primers used in this study are shown below for each target. Deoxyribonucleotides and ribonucleotides are represented by capital and small letters, respectively. These sequences are designed based on the published target sequence, references of which are shown with a number in parenthesis next to the name of the target. The position of a primer on the target sequence is shown in parenthesis next to the primer sequence.

Soybean transgenic CP4 EPSPS gene (5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium* sp. CP4) (*10*)

RR-K-F1: 5′-CTCCACTGACGTAAGGGATgac-3′ (173–294)

RR-K-R2: 5′-GTGCCATTCTTGAAAGATCTgcu-3′ (304–282)

Mycobacterium bovis BCG IS6110 (*11*)

K-F-1033(68): 5′-GTACACATCGATCCGGTTCagc-3′ (1022–1043)

K-R-1133(68): 5′-GTTGATCGTCTCGGCTAGTgca-3′ (1127–1106)

human c-Ki-ras gene (*12*)

c-Ki-ras-12F: 5′-GACTGAATATAAACTTGTGG-3′ (6379–6398)

c-Ki-ras/12 FN1: 5′-GACTGAATATAAACTTGTGg-3′ (6379–6398)

c-Ki-ras/12 FN2: 5′-GACTGAATATAAACTTGTgg-3′ (6379–6398)

c-Ki-ras/12 FN3: 5′-GACTGAATATAAACTTGugg-3′ (6379–6398)

c-Ki-ras/12 FN4: 5′-GACTGAATATAAACTTgugg-3′ (6379–6398)

c-Ki-ras/12 FN5: 5′-GACTGAATATAAACTugugg-3′ (6379–6398)

c-Ki-ras/12 FN6: 5′-GACTGAATATAAACuugugg-3′ (6379–6398)

c-Ki-ras/12 FN7: 5′-GACTGAATATAAAcuugugg-3′ (6379–6398)

c-Ki-ras/12 FN10: 5′-GACTGAATATaaacuugugg-3′ (6379–6398)

c-Ki-ras-12R: 5′-CTATTGTTGGATCATATTCG-3′ (6485–6466)

c-Ki-ras/12 RN1: 5′-CTATTGTTGGATCATATTCg-3′ (6485–6466)

c-Ki-ras/12 RN2: 5′-CTATTGTTGGATCATATTcg-3′ (6485–6466)

c-Ki-ras/12 RN3: 5′-CTATTGTTGGATCATATucg-3′ (6485–6466)

c-Ki-ras/12 RN4: 5′-CTATTGTTGGATCATAuucg-3′ (6485–6466)

c-Ki-ras/12 RN5: 5′-CTATTGTTGGATCATauucg-3′ (6485–6466)

c-Ki-ras/12 RN6: 5′-CTATTGTTGGATCAuauucg-3′ (6485–6466)

c-Ki-ras/12 RN7: 5′-CTATTGTTGGATCauauucg-3′ (6485–6466)

c-Ki-ras/12 RN10: 5′-CTATTGTTGGaucauauucg-3′ (6485–6466)

chrysanthemum stunt viroid (CSVd) (*13*)

Vd1N3: 5′-CACCCTTCCTTTAGTTTCcuu-3′ (182–202)

d-Vd1: 5′-CACCCTTCCTTTAGTTTCCTT-3′ (182–202)

Vd2N3: 5′-CGTTGAAGCTTCAGTTGTuuc-3′ (288–268)

d-Vd2: 5′-CGTTGAAGCTTCAGTTGTTTC-3′ (288–268)

Template DNA and cDNA/RNA Hybrid—A 1.1-kb fragment of soybean transgenic CP4 5-enol-pyruvylshikimate-3-phospate synthase class 2 precursor (EPSPS) gene including cauliflower mosaic virus 35S promoter sequence plus flanking non-translational region (accession no. AB209952) was amplified by PCR from soybean powder, certified reference material (Sigma-Aldrich). PCR was performed using a forward primer [5′-ATCGT TGAAGATGCCTCTGC-3′] and a reverse primer [5′-TCC GTATGATCGCACGTCAT-3′] under the following conditions: 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min. The amplified PCR fragment was cloned into the TA-cloning site of the pT7-BlueT vector (Novagen, San Diego, California) to obtain pT7Blue-GMO. For preparation of *Mycobacterium bovis* BCG genomic DNA, freeze-dried BCG vaccine purchased from Japan BCG Seizo (Tokyo, Japan) was dissolved in 2 mg/ml lysozyme solution containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA and 0.5% SDS. After incubation of above solution at 37°C for 2 h, proteinase K was added to a final concentration of 200 μg/ml. After incubation again at 37°C overnight, *M. bovis* BCG genomic DNA was extracted with the phenol/chloroform method and precipitated with ethanol. Human genomic DNA was purchased from Clontech Laboratories Inc. (Mountain View, California).

Low molecular weight RNA-enriched fraction (hereafter abbreviated as LMW RNA fraction) was prepared from the leaves of chrysanthemum infected with chrysanthemum stunt viroid (CSVd) according to the method described by Li *et al*. (*14*). One microlitre of 10-fold diluted LMW RNA fraction, 50 pmol of random hexamer DNA primer, 10 nmol of each dNTP were mixed to make up to 10 μl, incubated at 65°C for 5 min, and then placed on ice. The mixture, 20 units of ribonuclease inhibitor (Takara Bio Inc., Otsu, Japan), and 100 units of PrimeScript Reverse Transcriptase (Takara Bio Inc.) were mixed to make-up a 20 μl of reaction mixture. The final composition of the mixture is 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 0.5 mM of each dNTP. The reaction mixture was kept at 30°C for 10 min and at 42°C for 30 min to synthesize the first strand of complementary DNA. This cDNA/RNA hybrid preparation and its 10-fold serially diluted preparations with water were supplied as template for ICAN reaction.

*Enzymes—Bca*BEST DNA polymerase is a component of Cycleave ICAN human ALDH2 Typing Kit and also a product of Takara Bio Inc. (*15*). The gene of *Thermococcus litoralis* (DSM 5473) RNaseH (Tli RNaseH) was cloned and expressed in *Escherichia coli* and the RNaseH was purified from the culture according to procedures described in our US patent application publication (*16*). Briefly, *T. litoralis* DSM 5,473 cells, purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, were collected from its culture, and treated successively with lysozyme, proteinase K and sodium lauryl sulfate. After the treatment, *T. litoralis* genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. For the cloning of its RNaseH gene, degenerate primers (RN-F1 and RN-R0) were designed based on the amino acid sequences conserved among various thermostable RNaseHs and a partial gene sequence was obtained from *T. litoralis* genomic DNA by PCR. Upstream and downstream sequences were obtained by one-sided PCR with use of primers that were synthesized based on the partial gene sequence. Nucleotide sequence analysis of cloned fragments revealed a presumed open reading frame of RNaseH coding for 224 amino acids. The ORF sequence was inserted downstream of the lac promoter in frame in plasmid pTV119Nd, a derivative of pTV119N (Takara Bio Inc.), and an expression plasmid pTLI204 was obtained. To obtain the enzyme, *E. coli* harbouring pTLI204 was cultured in the presence of IPTG and *E. coli* cells were collected and sonicated. The supernatant of the sonicated cells was heated and centrifuged, and the resulting supernatant was subjected successively to a RESOURSE Q column, a RESOURSE S column, a HiTrap-heparin column and gel filtration (Superose 6) (all GE Healthcare Life Sciences, Buckinghamshire, UK). RNaseH was eluted at a position corresponding to the molecular weight of 26.5 kDa.

Composition of Reaction Mixture and Reaction Conditions for ICAN—A mixture containing 50 pmol each of the forward and reverse chimeric primers, template DNA or cDNA/RNA hybrid, 2.75 units of *Bca*BEST DNA polymerase and one unit of Tli RNaseH was added and the reaction mixture was made up to a volume of 25 μl. To examine the amount of each template amplified by ICAN (Fig. 1), 10 times stepwise increasing amounts of pT7Blue-GMO plasmid DNA in the range from 10 fg to 1 ng were used as template and RR-K-F1 and RR-K-R2 were used as chimeric primers for the target soybean transgene CP4 EPSPS sequence, which is 132 bp long including primer sequences. Similarly, *Mycobacterium bovis* BCG genomic DNA (20 fg to 2 ng) was used as template, and K-F-1033(68) and K-R-1133(68) were used as chimeric primers for the target *M. bovis* BCG IS6110 sequence (106 bp); human genomic DNA (1–100 ng) was used as template, and c-Ki-ras/12 FN3 and c-Ki-ras/12 RN3 were used as chimeric primers for the target human c-Ki-ras gene sequence (107 bp); 1 μl of cDNA/RNA hybrid preparation from chrysanthemum leaves infected with CSVd (10,000-fold diluted to non-diluted preparation) was used as template, and Vd1N3 and Vd2N3 were used as chimeric primers for the target CSVd sequence (107 bp). To examine the essential components or elements or optimum reaction temperature for ICAN amplification, either 13.5 ng (Fig. 3) or 27 ng (Figs 4 and 6) of human genomic DNA was used as template for the target human c-Ki-ras gene sequence (107 bp). Chimeric primers with different numbers of RNA residues were used to examine the optimum number of RNA residues for the target human c-Ki-ras gene sequence (107 bp) (Fig. 4). The final composition of the reaction mixture is 32 mM Hepes–KOH buffer (pH 7.8), 100 mM KOAc, 0.11% BSA, 1% DMSO, 0.5 mM of each dNTP and 4 mM $Mg(OAc)_2$. The reaction mixture was kept at 55°C, if not otherwise specified, for 60 min (soybean transgene CP4 EPSPS and *M. bovis* IS6110) or 90 min (human c-Ki-ras gene and CSVd cDNA/RNA hybrid).

PCR—In PCR with chimeric primers (lane 5 in Fig. 3), the reaction was performed with the same reaction mixture as ICAN containing a primer pair of c-Ki-ras/12FN3 and c-Ki-ras/12RN3 except that *Bca*BEST DNA polymerase was replaced with 0.6 units of *Taq* DNA polymerase (Takara Bio Inc.), and RNaseH was excluded. The thermal cycling condition was 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s. In the experiments that examined the yield against increasing primer concentration (Fig. 7 and Fig. S1) for comparison with that achieved by ICAN, seven PCR conditions for the CSVd target were adopted using a primer pair of d-Vd1 and d-Vd2 with *Ex Taq* HS (Takara Bio Inc.). PCR conditions were as described later:

No.1. 35 cycles at 98°C for 10 s, 55°C for 30 s, 68°C for 10 s,

No.2. 35 cycles at 98°C for 10 s, 55°C for 30 s, 72°C for 10 s,

No.3. 35 cycles at 98°C for 10 s, 55°C for 30 s, 66°C for 10 s,

No.4. 50 cycles at 98°C for 10 s, 55°C for 30 s, 68°C for 10 s,

No.5. 35 cycles at 98°C for 10 s, 55°C for 15 s, 68°C for 10 s,

No.6. 35 cycles at 98°C for 10 s, 55°C for 60 s, 68°C for 10 s,

No.7. 35 cycles at 98°C for 10 s, 55°C for 30 s, 68°C for 30 s

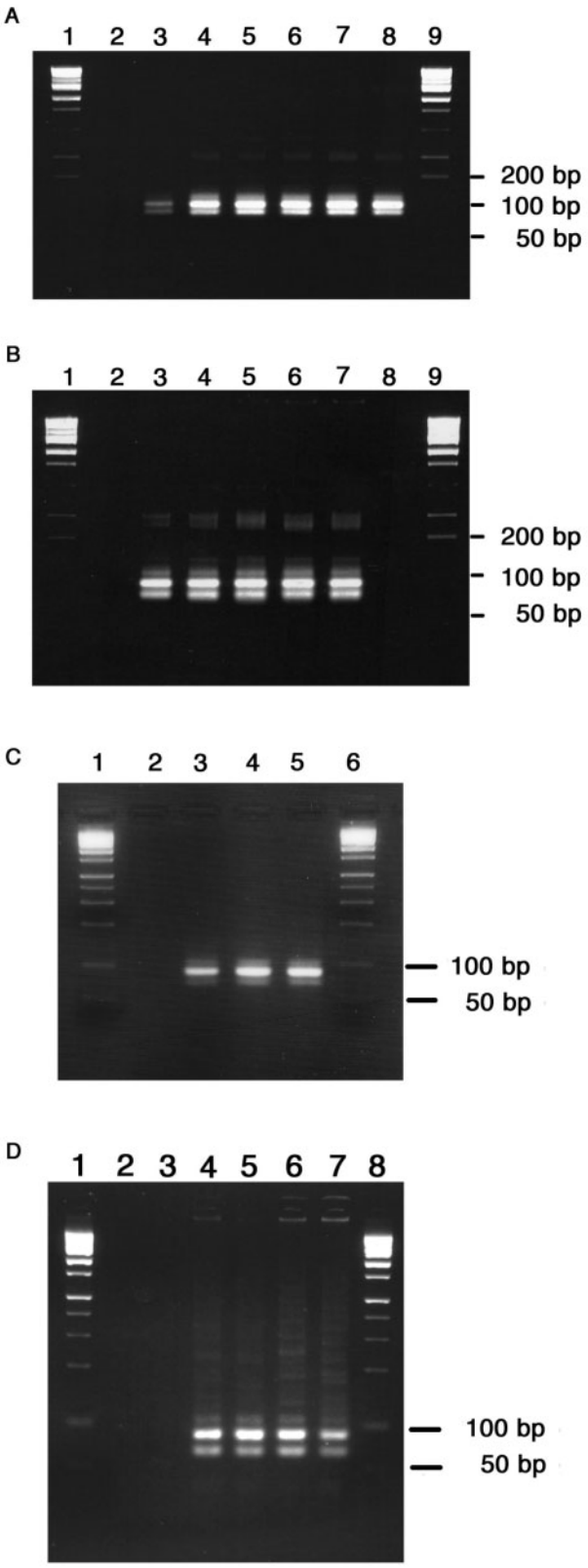

Fig. 1. **Analysis of amplified products by electrophoresis.** ICAN was carried out to amplify a sequence of (A) transgenic CP4 EPSPS gene, (B) *M. bovis* BCG IS6110, (C) human c-Ki-ras gene or (D) cDNA/RNA hybrid of CSVd. After the reactions had finished, aliquots of the reaction mixtures were applied to 4% Nusieve 3:1 agarose gel (Cambrex) for electrophoresis (A to D). (A) The following amount of plasmid pT7Blue-GMO was used as template: no template (lane 2); 10 fg (lane 3); 100 fg (lane 4); 1 pg (lane 5); 10 pg (lane 6); 100 pg (lane 7); 1 ng (lane 8). One μl of the reaction mixtures was applied for electrophoresis. Lanes 1 and 9: Wide-Range DNA Ladder marker (Takara Bio Inc.). (B) The following amount of *M. bovis* BCG genomic DNA was used as template: 20 fg (lane 2); 200 fg (lane 3); 2 pg (lane 4); 20 pg (lane 5); 200 pg (lane 6) or 2 ng (lane 7); no template (lane 8). One microlitre of the reaction mixtures was applied for electrophoresis. Lanes 1 and 9: Wide-Range DNA Ladder marker. (C) The following amount of human genomic DNA was used as template: no template (lane 2); 1 ng (lane 3), 10 ng (lane 4) or 100 ng (lane 5). And 0.4 μl of the reaction mixtures was applied for electrophoresis. Lanes 1 and 6: Wide-Range DNA Ladder marker. (D) One microlitre of original cDNA/RNA hybrid preparation (lane 7) or the following fold-dilution of the preparation was used as template: 10-fold (lane 6), 100-fold (lane 5), 1000-fold (lane 4) or 10,000-fold (lane 3). Lane 2: ICAN reaction without template DNA 0.3 μl of the reaction mixtures was applied for electrophoresis. Lanes 1 and 8: Wide-Range DNA Ladder marker.

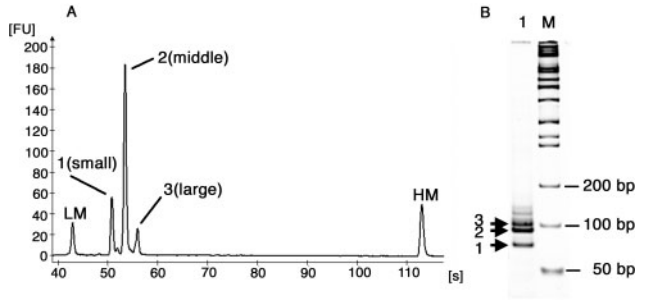

Fig. 2. **Analysis of amplified products by the Bioanalyzer and PAGE.** (A) LM: lower-size marker (15 bp); HM: higher-size marker (1500 bp). (B) Arrows indicate three distinct fragments. Lane M: Wide-Range DNA Ladder marker.

Analysis of Amplified Products—After the amplification reaction had finished, aliquots of the reaction mixtures were applied to either a 4% Nusieve 3:1 agarose gel (Figs. 1A–D and 3) or a 4% Metaphor^{R} agarose gel (Figs. 4 and 6) (Cambrex, New Jersey) containing ethidium bromide for electrophoresis. ICAN products of the human c-Ki-ras sequence (107 bp) were also analysed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California) and with 10% native PAGE. Polyacrylamide gel was stained with SYBR Green I solution after electrophoresis. 0.1 μl (Fig. 2A) or 0.25 μl (Fig. 2B) of the products were applied to the Bioanalyzer or PAGE, respectively.

Quantification of Amplified Products—The amount of amplified products of either ICAN or PCR was measured by scanning the region from 60 to 120 bp with the Agilent 2100 Bioanalyzer. A DNA LabChip kit was used according to its instruction manual. In the experiment showing the optimum number of RNA residues of a chimeric primer, the amount of amplified c-Ki-ras gene fragments in 25 μl of a reaction mixture was calculated and is shown for each primer (Fig. 4). In the experiment showing the dependence of yield on primer concentration, the amount of amplified CSVd fragments (either with ICAN or PCR) from 1 μl of 100-fold diluted cDNA/RNA

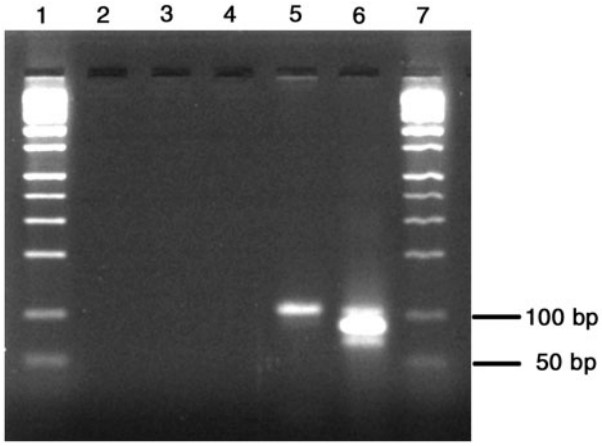

Fig. 3. **Essential components for ICAN amplification.** Both ICAN and PCR were carried out to amplify the human c-Ki-ras gene sequence from human genomic DNA. After the reactions had finished, 0.6 μl (ICAN) or 4 μl (PCR) of the reaction mixtures were applied to 4% NuSieve 3:1 agarose gel (Cambrex) for electrophoresis. Lanes 1 and 7: Wide-Range DNA Ladder marker; lane 2: lacking RNaseH (ICAN); lane 3: with DNA primers (ICAN); lane 4: with *Taq* DNA polymerase (ICAN); lane 5: PCR with chimeric primers and lane 6: original condition (ICAN).

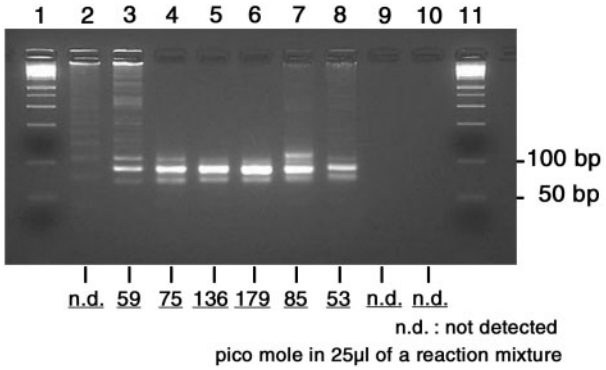

Fig. 4. **Optimum number of RNA residues of a chimeric primer.** ICAN reaction was carried out to amplify the human c-Ki-ras gene sequence from human genomic DNA with chimeric primers having a number of RNA residue(s) of either no (lane 2), one (lane 3), two (lane 4), three (lane 5), four (lane 6), five (lane 7), six (lane 8), seven (lane 9) or ten (lane 10) at the 3′ end. After the reactions had finished, 0.5 μl of the reaction mixtures were applied to 4% Metaphor^{R} agarose gel (Cambrex) for electrophoresis. Lanes 1 and 11: Wide-Range DNA Ladder marker.

hybrid preparation is shown against four different concentrations of a primer (0.2, 0.4, 1 and 2 μM) (Fig. 7 and Fig. S1).

Analysis of Amplification Pattern of ICAN—The CSVd target sequence was amplified by ICAN from 1 μl of the cDNA/RNA preparation and its 10 times serially diluted preparations (in the range of 10- to 1000-fold) of CSVd-infected chrysanthemum. Reactions were carried out and monitored in real time on the Thermal Cycler Dice^{TM} Real Time System (Takara Bio Inc.) with SYBR Green1 (Fig. 5).

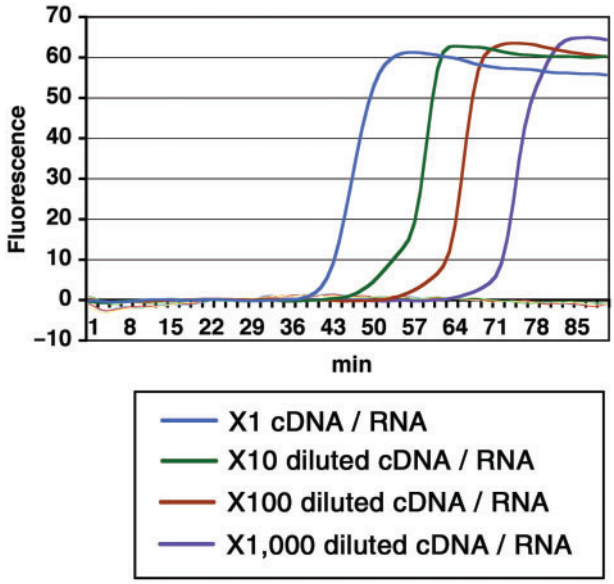

Fig. 5. **Real-time monitor of ICAN amplification**. The CSVd target sequence was amplified by ICAN from the cDNA/RNA preparation and its 10-fold serially diluted cDNA/RNA preparations of CSVd-infected chrysanthemum. Reactions were carried out and monitored in real time on the Thermal Cycler Dice^{TM} Real Time System (Takara Bio Inc.) with SYBR Green1.

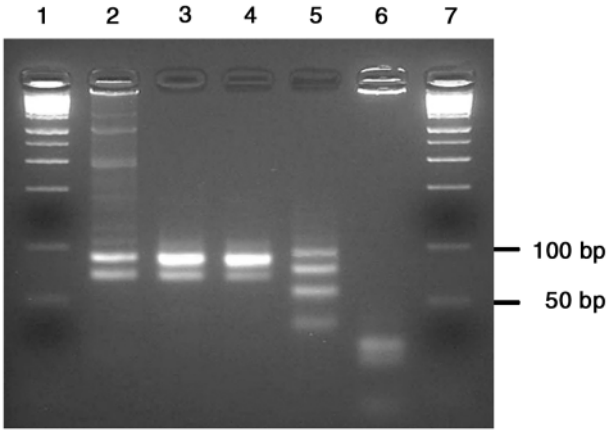

Fig. 6. **Optimum temperature for ICAN amplification**. ICAN reaction was carried out to amplify the human c-Ki-ras gene sequence from human genomic DNA at 50°C (lane 2), 53°C (lane 3), 55°C (lane 4), 58°C (lane 5) or 60°C (lane 6). After the reactions had finished, 0.5 μl of the reaction mixtures was applied to 4% Metaphor^{R} agarose gel (Cambrex) for electrophoresis. Lanes 1 and 7: Wide-Range DNA Ladder marker.

Subcloning and Sequence Analysis of ICAN Products—The ICAN reaction mixture was separated on agarose gel. ICAN products extracted from agarose gel were subjected to blunting/kination reaction and subcloned into *Hinc*II site of pUC118 using Mighty Cloning Kit (Blunt End) (Takara Bio Inc). Subclones were sequenced with a DNA sequencer [ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California].

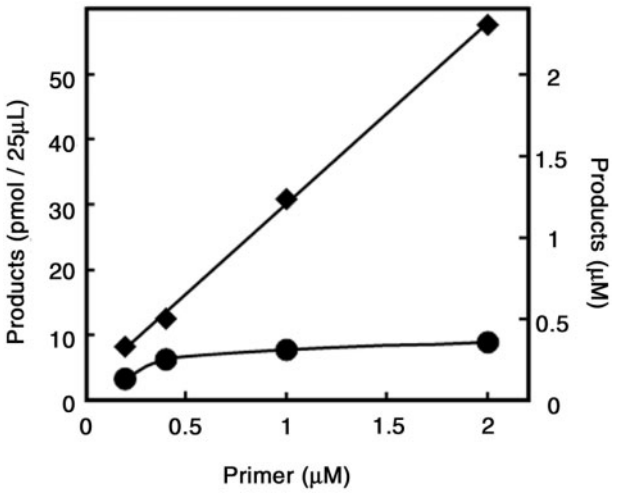

Fig. 7. **Yield of amplified products by ICAN or PCR against primer concentration.** The amount of CSVd fragments amplified with ICAN (shown by closed square) or with PCR (shown by closed circle) from cDNA/RNA hybrid preparations is shown as a function of primer concentrations. Only one experiment that exhibited the highest yield among seven trials of PCR amplification is shown. Profiles of all of the seven PCR trials, which are plotted in the same manner as this figure, are shown in the supplementary data S1.

RESULTS AND DISCUSSION

Amplification by ICAN Method—The method requires a pair of 5′-DNA-RNA-3′chimeric primers, which possess a few RNA residues at the 3′ side, RNaseH, which can cleave the RNA portion of the elongated strand, and a DNA polymerase with strand-displacing activity. We hypothesized that if RNaseH can introduce a nick at the 5′-RNA and DNA-3′ junction of an elongated strand synthesized from the chimeric primer, the polymerase could amplify the region encompassed by both primers. *Bca*BEST DNA polymerase is an N-terminal deletion derivative of a polymerase of a thermophilic bacterium, *Bacillus caldotenax*, and lacks 5′-3′-exonuclease activity (*15*). Because *Bca*BEST DNA polymerase has strong strand-displacing activity (Uemori, unpublished data), it could start DNA elongation with concomitant strand displacement from a nicked site at every cleavage of the 3′ side of the RNA portion of the elongated strand. Then the complementary strand could be synthesized starting from another chimeric primer on the displaced single-stranded DNA. This displaced strand could in turn serve as a template. We call this idea 'nick-and-run' repetition. We chose RNaseH derived from a hyperthermophilic archaeon as a source of thermostable enzyme, expecting that it can cooperatively work without loosing its activity with *Bca*BEST DNA polymerase at around 55°C, the optimum temperature of the polymerase.

We first attempted the amplification of a target sequence cloned in a plasmid vector with ICAN. pT7Blue-GMO, which has soybean transgenic CP4 EPSPS gene cloned in a plasmid vector, was used as template. As shown in Fig. 1A, a set of chimeric primers, RR-K-F1 and RR-K-R2, gave amplified fragments with expected sizes (132 bp, including primer sequences). The amplified fragments shown in lane 8 of Fig. 1A were subcloned into an appropriate plasmid vector and sequenced. The sequence encompassed by both of the chimeric primers was identified in all of the 23 subclones analysed with the exception that three sequences had a single substitution (either A to G or T to C). This suggests that ICAN amplified the intended primer-restricted region. To see whether ICAN allows specific amplification from more complicated DNA, we attempted the amplification of a sequence of *Mycobacterium bovis* BCG IS6110 using its genomic DNA as template. Since the genome size of a closely related strain of *M. bovis*, strain AF2122/97, is 4.3×10^6 bp as reported by Garnier *et al*. (*17*), the complexity of template increases three orders of magnitude compared to the case where the template is a plasmid. As shown in Fig. 1B, DNA fragments ranging from 70 to 110 bp were identified. The amplified fragments shown in lane 7 of Fig. 1B were subcloned and sequenced. The sequence existing between both of the chimeric primers was identified in all of the 19 subclones analysed. Thirteen of 19 clones contained the full sequence encompassed by both primers. This demonstrates that ICAN allows specific amplification of a target sequence when the template is a bacterial genome. The remaining six clones contained short sequences lacking 21 to 32 bases adjacent to the primer K-F-1033(68). It is not clear at this moment whether the appearance of the short sequences at a higher frequency than expected from agarose gel electrophoresis reflects the occurrence of some unidentified secondary reaction or is merely an artefact of subcloning.

Next, we tested whether ICAN could specifically amplify a target sequence from the template of human genomic DNA, the complexity of which is three orders of magnitude higher than that of *M. bovis*. The size of human genome is 3.2×10^9 bp (*18*). A sequence of human c-Ki-ras gene, 107 bp long including primer sequences, was chosen as a target and amplification was attempted using human genomic DNA as template. DNA was amplified from as little as 1 ng of template DNA with ICAN and amplified fragments ranging from 70 to 110 bp were identified (Fig. 1C). ICAN products prepared from the template of 50 ng of human genomic DNA were subcloned and sequenced as described earlier. DNA sequences obtained from all of the 24 subclones were identical to the target sequence reported (*12*). This demonstrates that the target sequence is specifically amplified with ICAN even if the template is complicated human genomic DNA. Finally we tested whether ICAN allowed specific amplification from cDNA/RNA hybrid. The cDNA/RNA hybrid preparation generated from CSVd-infected chrysanthemum leaves was used as template for ICAN reaction. Chimeric primers were designed to amplify 107 bp sequences (including primer sequences). As shown in Fig. 1D, expected size fragments were identified to the point where the hybrid fraction was diluted up to 1000-fold. (Original LMW RNA fraction is diluted 2×10^5 fold in this preparation). The amplified fragments shown in lane 5 of Fig. 1D were cloned and sequenced as described earlier. The sequence encompassed by both of the chimeric primers was identified in all 23 subclones analysed with the exception that two sequences had a single substitution of T to A,

which could occur during either cDNA synthesis from RNA or the reaction of ICAN. This demonstrates that the specific amplification of ICAN occurred starting from cDNA/RNA hybrid.

The target size of above four ICAN amplifications was around 100 bp, although DNA fragments larger than 100 bp can be amplified by ICAN. We used ICAN to amplify 21,000 cDNA sequences of Arabidopsis thaliana of 250 to 300 bp from PCR-prepared fragments by use of common chimeric primers (data not shown), and obtained large amounts of each cDNA fragment.

In most cases of amplification by ICAN, three bands were observed in agarose gel electrophoresis. When ICAN products of c-Ki-ras target sequence were analysed by either Agilent 2100 Bioanalyzer or PAGE, three distinct bands were observed (Fig. 2A and B). Sequence analysis of subcloned amplified fragments revealed that the distinct three bands consists of three fragments: one (large) having both of the primer sequences (forward and reverse), another (middle) having only one (either forward or reverse) primer sequence and the other (small) having no primer sequence. Suppose DNA is amplified based on the scenario 'nick-and-run' repetition in ICAN, most of the amplified fragment should not have primer sequences. However, two of the three fragments contained primer sequences. The discrepancy suggests that a different amplification mechanism other than 'nick-and-run' repetition exists in ICAN. We elucidated the mechanism of ICAN and found that two mechanisms, which we call multi-priming and template-switching, underlie ICAN. The details of the mechanisms are described in the accompanying paper entitled 'Investigation of the molecular mechanism of ICAN, a novel gene amplification method'.

Essential Components/Elements for ICAN Amplification—We examined whether the combination of a pair of chimeric primers and RNaseH was essential for ICAN reaction using the reaction mixture for the c-Ki-ras target sequence. As shown in Fig. 3, when either RNaseH was not included or a pair of chimeric primers was replaced with that of DNA primers, no amplified fragment with an expected size (around 100 bp for the target sequence of c-Ki-ras) was observed (lanes 2 and 3). We then examined whether the strand-displacing activity of *Bca*BEST DNA polymerase was essential for ICAN. We used *Taq* DNA polymerase, which has 5′-3′-exonuclease activity and has little, if any, strand-displacing activity (*19*), instead of *Bca*BEST DNA polymerase. No amplified fragment was seen when polymerase was replaced (Fig. 3, lane 4). To exclude the possibility that the failure of amplification is merely due to insufficient initiation of DNA synthesis by *Taq* DNA polymerase from the chimeric primers, we performed PCR with *Taq* DNA polymerase and the ICAN reaction mixture but not including RNaseH. Lane 5 in Fig. 3 showed an amplified fragment with around 100 bp indicating that the chimeric primers worked well as primers for *Taq* DNA polymerase in a thermal cycling condition, where strand-displacing activity is not necessarily required for DNA extension. Above experiments demonstrated that a pair of chimeric primers, RNaseH, and *Bca*BEST DNA polymerase, or more generally, strand-displacing activity of the polymerase was essential for ICAN.

Next, we tested the number of RNA residues at the 3′ end of chimeric primers required for ICAN. A pair of chimeric primers having an increasing number of RNA residues in the range from 0 to 10 was chosen to prepare an ICAN reaction mixture and the human c-Ki-ras target sequence was allowed to amplify. As shown in Fig. 4, amplified fragments with expected sizes were obtained when the number of RNA residues was one to six (lanes 2 to 7). When the number of RNA residues was one, a ladder of unexpected larger fragments was observed in addition to expected size fragments. Taking into account the amount of amplified fragments with expected sizes and reproducibility of the ICAN reaction, three RNA residues gave the best performance of ICAN amplification.

Amplification Pattern of ICAN—It is useful to measure the amount of RNA in isothermal amplification system taking into account a variety of RNA target sequences including HIV. As the first step to see whether ICAN can be used for this purpose, we monitored the amount of amplified DNA against reaction time and defined the pattern of amplification by ICAN. The CSVd sequence was amplified from a 10-fold serially diluted cDNA/RNA preparation of CSVd-infected chrysanthemum. As shown in Fig. 5, sigmoid-shaped curve was observed in the ICAN amplification from either amount of template DNA and the time required to reach a certain amount of DNA was a function of the dilution fold of template.

Influence of Reaction Temperature on the Specificity of Amplification—We tested whether the ICAN reaction can be optimized by adjusting reaction temperature as was achieved by changing annealing temperatures in the case of PCR. We hypothesized that in ICAN the reaction temperature could highly influence specific annealing between a chimeric primer and template, which is the most critical factor leading to specific amplification of a target sequence. As shown in Fig. 6, when the human c-Ki-ras gene sequence was amplified at five different temperatures ranging from 50 to 60°C, it was most specifically amplified at the temperature of either 53 or 55°C (lanes 3 and 4). This demonstrates that the specificity of amplification of a target sequence by ICAN can be optimized by adjusting the reaction temperature.

Yields of ICAN Against Primer Concentration—To see the dependence of the yield of ICAN product on chimeric primer concentration, we measured the yield against increasing concentration of chimeric primers. As shown in Fig. 7, the amount of amplified fragments of a CSVd target sequence increased up to 7-fold in proportion to primer concentration in its range of 0.2–2 μM. On the contrary, when the same target sequence was amplified by PCR using DNA primers (d-Vd1 and d-Vd2), the fold of increase was at most 2.8, and the yield was less than half of that of ICAN through the whole range tested. The yield obtained by PCR could vary by changing its conditions; we tried seven different combinations of parameters including extension temperature, cycle number, annealing temperature and extension time; however, neither the fold of increase nor yield exceeded the above values (supplementary data S1). When another

target sequence, the c-Ki-ras sequence was amplified, the amount obtained in the reaction mixture by ICAN (125 pmol) was 77 times higher than that obtained by PCR (1.61 pmol) using chimeric primers in the ICAN-optimized reaction solution (Fig. 3, lanes 5 and 6).

Thermostable RNaseHs Available for ICAN—Here we arbitrarily selected RNaseH derived from *T. litoralis* to make-up the ICAN reaction mixture and to intensively see the performance of ICAN. RNaseH genes were identified in thermophilic archaea or bacteria and some of them were cloned and expressed in *E. coli* as thermostable enzymes (*20–26*). In a separate line of study, we found that thermostable RNaseHs other than *T. litoralis* RNaseH can be used to specifically amplify a target sequence in combination with *Bca*BEST DNA polymerase and chimeric primers (*27*, *28*).

Lowest Amount of Template DNA Detected by ICAN—In this study the lowest amount of genomic DNA required to detect amplified target sequences of IS6110 and c-Ki-ras gene with ICAN was 100 fg and 1 ng, respectively. These correspond to a few hundred copies, taking into account the genome size (shown in 'Amplification by ICAN method') and the copy number of each target sequence on its genome. The copy number of IS6110 that exists on the genome of *M. bovis* BCG strain can be speculated to be <10 based on the study of Viana-Niero, C. *et al*. (*29*). So far as the c-Ki-ras target sequence was searched on the human genome database, one copy of the relevant sequence was found on the genome (*18*). This is consistent with the report that the human genome contains one functional c-Ki-ras gene and one c-Ki-ras pseudogene (*12*). At present we have not optimized the ICAN reaction focusing on sensitivity and in this study we used conventional agarose-gel-based assay to detect amplified fragments. If we increase the sensitivity of the ICAN reaction one order of magnitude higher than the present one and use a detection system one order of magnitude higher than the agarose-gel-based assay, detection of a single copy of a target sequence with ICAN could be realized.

Application of ICAN for SNP Detection—Allele-specific PCR (ASPCR) allows discrimination of a point mutation from a normal sequence with a primer having a 3′-end residue that can form a base pair with either the mutation or normal sequence (*30*). It would be interesting to see whether ICAN can allow discrimination of a point mutation in the same manner as ASPCR by designing a chimeric primer with altered sequence only at the 3′-end RNA residue. In our preliminary experiments, we found discrimination was also possible with ICAN with appropriate conditions including the reaction temperature (*27*). There was concern that ICAN would not allow discrimination as ASPCR because an RNA residue at the 3′-end was removed by RNaseH every time a chimeric primer was extended from its 3′-end. Once a shortened primer lacking the 3′-end RNA residue(s) was generated, whichever template sequence, either mutant or normal type, could be amplified. We can interpret the success of mutation discrimination in the following way. In ICAN the most critical step that determines either success or failure of amplification is the specific hybridization of the original, 'intact' primer to template followed by primer extension in the early stage of reaction as is the case for PCR. If one can set an appropriate condition including reaction temperature that allows DNA synthesis only from a primer having a matched 3′-end residue, it would be possible to discriminate point mutation also by ICAN.

ICAN was further developed with use of a chimeric primer, 5′-DNA-RNA-DNA-3′ having a 3′-blocked DNA residue, to use specifically for SNP detection as described by Sagawa *et al*. (*31*). In this system, which is called UCAN, a point mutation or SNP was discriminated based on the property of the RNaseH that cleaved a diester bond in the chimeric primer only when an RNA residue formed a base pair with an examining DNA residue on the opposite strand.

Combination with Cycling Probe for On-site and Real-time Detection—ICAN can be applied to on-site DNA detection including gene diagnosis, and would also be suitable for a 'real-time' detection system when combined with a cycling probe (*32*). The cycling probe is composed of DNA residues with a few RNA residues in the middle, and is labelled with a fluorescent molecule and a quencher molecule at each end. When this probe is added to the ICAN reaction mixture, it emits a fluorescent signal that intensifies as the amount of amplified fragment increases and one can monitor the DNA amplification in real time by measuring the intensity of the signal. To make a real-time detection system, both a cycling probe and thermostable RNaseH have to be added in the case of PCR, but ICAN needs only a cycling probe because RNaseH is already present. In the real-time system of ICAN, RNaseH has two roles. It splits a hybridized probe molecule to emit a fluorescent signal as well as introduces a nick to chimeric primers to drive the amplification reaction. This system can be also used to strictly distinguish single nucleotide polymorphisms (SNPs) in amplified fragments.

Supplementary data are available at JB online.

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