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Investigation of the Molecular Mechanism of ICAN, a Novel Gene Amplification Method

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Isothermal and Chimeric primer-initiated Amplification of Nucleic acids (ICAN) allows the amplification of target DNA under isothermal conditions at around 55°C using only a pair of 5′-DNA-RNA-3′ chimeric primers, thermostable RNaseH and a DNA polymerase with strand-displacing activity (H. Mukai *et al. J. Biochemistry*, in the preceding paper in this issue). Here we elucidated the mechanism of ICAN by analysing the nicking site of RNaseH, behaviour of chimeric primers and extension products. We found that the ICAN reaction was composed of two unique mechanisms, multi-priming and template-switching, that were responsible for the highly efficient amplifying capability of ICAN. The simultaneous occurrence of two types of reactions, one based on multi-priming and the other based on template-switching, is likely to drive the DNA amplification in ICAN.

Key words: ICAN, multi-priming, RNaseH, strand displacement, template-switching.

Abbreviations: FITC, fluorescein isothiocianate; ICAN, isothermal and chimeric primer-initiated amplification of nucleic acids; ROX, 6-carboxy-X-rhodamine; Tli RNaseH, *Thermococcus litoralis* RNaseH; XRITC, X-rhodamine isothiocyanate.

In the preceding paper, we showed that a target sequence of DNA or cDNA was specifically amplified by ICAN from its genomic DNA or cDNA/RNA hybrid preparation and that three components comprising ICAN, *i.e.* 5′-DNA/RNA-3′ chimeric primers, RNaseH and a strand-displacing DNA polymerase were essential for amplification. Also the yield obtained by ICAN was found to be highly dependent on the concentration of chimeric primers.

We first assumed that DNA was amplified by the mechanism of 'nick-and-run' repetition in ICAN as depicted in Fig. 1. In this scenario, RNaseH introduces a nick at the 5′-RNA/DNA-3′ junction of an elongated strand keeping the incorporated RNA residues 'intact' throughout the reaction and ensuring constant priming from the same residue. However, in most cases of amplification by ICAN, three distinct bands, one (large) having both of the primer sequences, another (middle) having only one primer sequence and the other (small) having no primer sequence were generated [Fig. 2A and B, in our preceding paper (*1*)]. If DNA is amplified based on the scenario of the 'nick-and-run' repetition in ICAN, most of the amplified fragments should not have primer sequences. The fact is that two of the three fragments contain primer sequences. This discrepancy suggests that a different amplification mechanism other than 'nick-and-run' repetition exists in ICAN.

In this study, we first examined the possibility of the scenario 'nick-and-run' repetition by testing the nicking site by RNaseH and then elucidated the most probable mechanism for the amplification. On scrutinizing the ICAN reaction, we found that the two unique mechanisms, multi-priming and template-switching, were involved in the ICAN method. In this paper, we describe the amplification mechanism that underlies ICAN.

MATERIALS AND METHODS

Primer—Nucleotide sequences of the primers c-Ki-ras/12 FN3, c-Ki-ras/12 RN3, c-Ki-ras-12F and c-Ki-ras-12R are shown in the preceding paper of this issue (*1*). Nucleotide sequences of other primers used in this study are shown below. Deoxyribonucleotides and ribonucleotides are represented by capital and small letters, respectively.

MF2(30): 5′-GGATGTGCTGCAAGGCGATTAAGTTGGGTA-3′

MR1(30): 5′-TTTACACTTTATGCTTCCGGCTCGTATGTT-3′

c-Ki-ras/12 FN3-1: 5′-GACTGAATATAAACTTGug-3′

c-Ki-ras/12 FN3-2: 5′-GACTGAATATAAACTTGu-3′

M4: 5′-GTTTTCCCAGTCACGAC-3′

RV: 5′-CAGGAAACAGCTATGAC-3′

MCSF: 5′-CCATTCAGGCTGCGCAACTGTT-3′

MCSR: 5′-TGGCACGACAGGTTTCCCGACT-3′

PCR and Enzymes—DNA fragments used in this study were amplified by PCR in a volume of 100 μl

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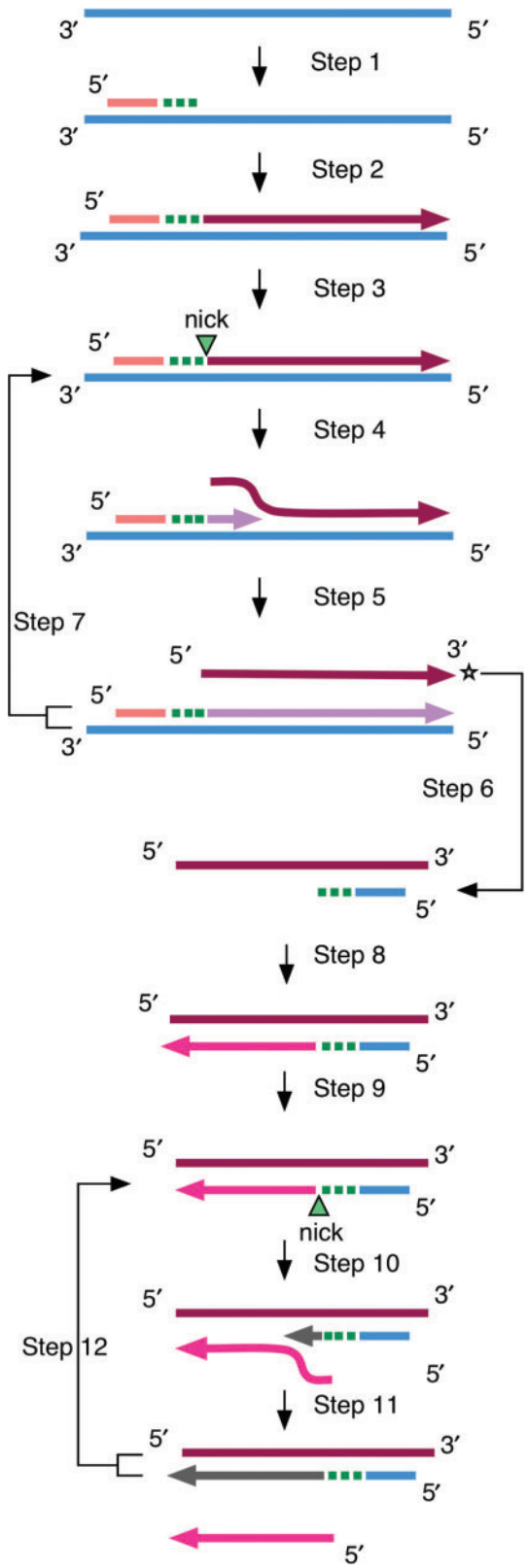

Fig. 1. **'Nick-and-run' repetition model for ICAN.** A reaction starting from only one strand of duplex DNA is depicted.

with ExTaq^{R} (Takara Bio Inc.), which is supplied with a magnesium-containing buffer and a dNTP mixture. Twenty picomoles each of primer was used in all of the reactions. For the preparation of fragments used in the three assays, *'Identification of the site cleaved by RNaseH'*, *'Analysis of the behaviour of a pre-staying chimeric primer'* and *'Measurement of the amount of an incorporated free primer'*, the following thermal profile was used: 98°C, 10 s; 55°C, 30 s; 72°C, 10 s; 30 cycles. For the preparation of templates used in the two assays, *'Analysis of initiation reaction of ICAN with DNA primers'* and *'Demonstration of the occurrence of template-switching',* the following thermal profile was used: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; 30 cycles. Enzymes used in this study other than PCR enzymes are described in the preceding paper of this issue (*1*).

Identification of the Site Cleaved by RNaseH—Three DNA fragments that contained three continuous RNA residues (5′-UGG-3′), two continuous RNA residues (5′-UG-3′) and a single RNA residue (U) in one strand of a duplex DNA were obtained by PCR with the combination of the following corresponding chimeric primer labelled with ROX at the 5′ end and DNA primer c-Ki-ras-12R, and 0.1 μg of human genomic DNA as a template. For the sequence UGG, c-Ki-ras/12 FN3 was used. For the sequence UG, c-Ki-ras/12 FN3-1 was used. For the sequence U, c-Ki-ras/12 FN3-2 was used. The DNA fragments amplified by PCR, 107-bp long, were purified with Microcon-100 (Takara Bio Inc.) and precipitated with ethanol. The fragments were dissolved in 15 μl of manufacturer-recommended buffer solution containing 17.5 unit of exonuclease I (Takara Bio Inc.), incubated at 37°C for 30 min, and at 80°C for 15 min. Obtained fragments were used as substrate for the cleavage test with Tli RNaseH. The reaction solution contained 0.3 pmol of the amplified fragment having RNA residue(s) and 1.6 units of Tli RNaseH in 40 μl (final volume) of 32 mM Hepes–KOH buffer (pH 7.8), 0.11% BSA, 1% DMSO, 4 mM $Mg(OAc)_2$ and 100 mM KOAc. The reaction solution was incubated at 55°C for an hour. After the reaction had finished, 2 μl of the reaction solution was subjected to 10% denaturing PAGE. The analysis was carried out with FMBIO Multi-View (Hitachi Soft Engineering, Tokyo, Japan). Three chimeric primers c-Ki-ras/12 FN3, c-Ki-ras/12 FN3-1 and c-Ki-ras/12 FN3-2 labelled with ROX at 5′ ends were used as size markers.

Analysis of the Behaviour of a Pre-staying Chimeric Primer—The 5′ FITC-labelled chimeric primer c-Ki-ras/12RN3 and DNA primer c-Ki-ras-12F were used along with 0.1 μg of human genomic DNA as a template for PCR. The amplified DNA fragment containing three RNA residues, 107-bp long, was treated as described in *'Identification of the site cleaved by RNaseH'* to serve as a substrate. The reaction mixture was composed of 0.65 pmol of the substrate, 32 mM Hepes (pH 7.8), 4 mM $Mg(OAc)_2$, 100 mM KOAc, 1% DMSO, 0.11% BSA, with or without 20 pmol of 5′ XRITC-labelled DNA primer c-Ki-ras-12R, 2.75 units of *Bca*BEST DNA polymerase, 0.5 mM of each dNTP and one unit of Tli RNaseH and had a volume of 25 μl. The reaction mixture was incubated at 55°C for 15 min. After the reaction mixtures were electrophoresed in 6% denaturing polyacrylamide

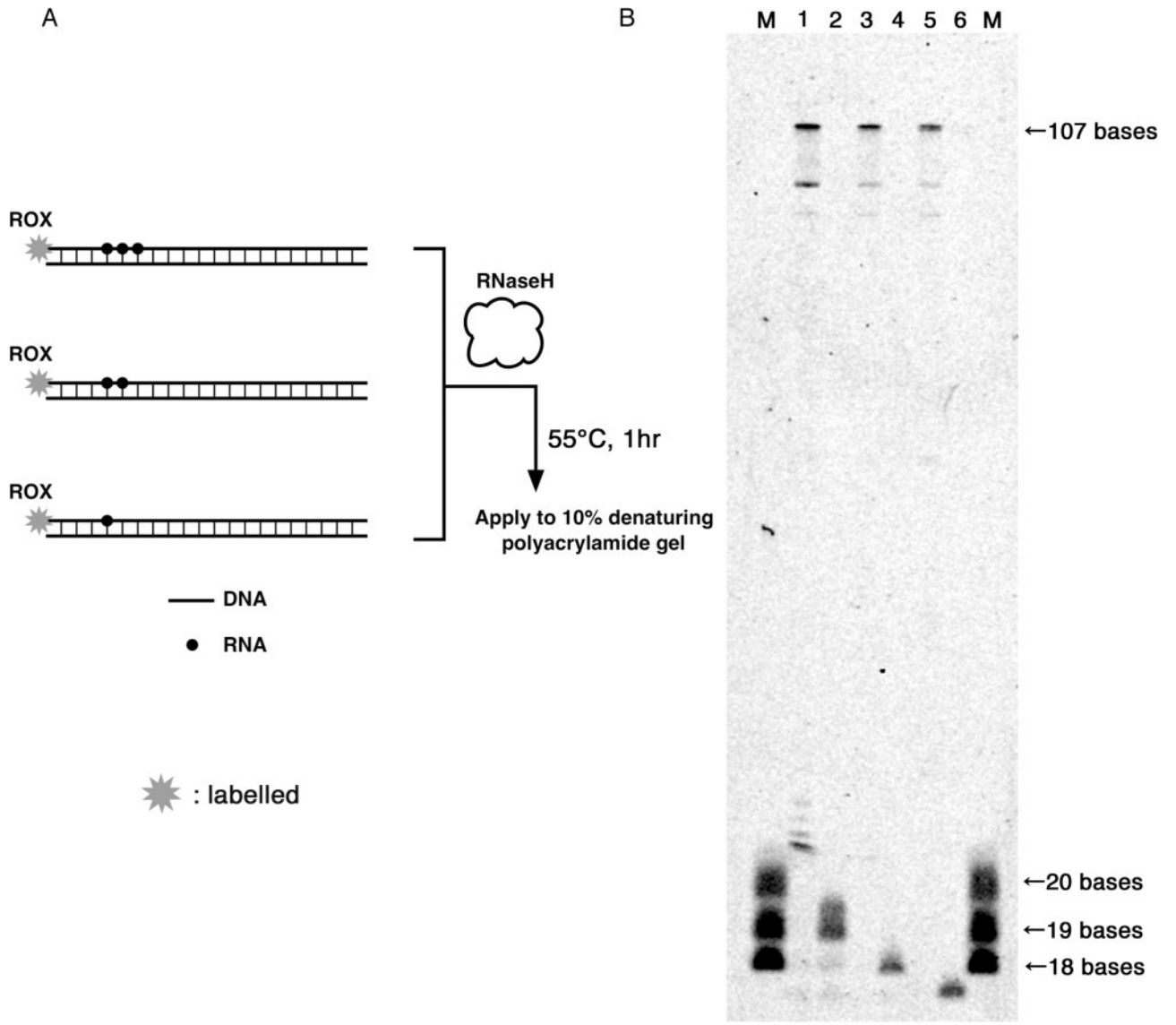

Fig. 2. **Analysis of cleaved site in RNA/DNA hybrid by RNaseH.** (A) Schematic representation of the design of the experiment. (B) Fluorogram of PAGE, lane M: mixture of three size markers, 5′-ROX-labelled chimeric primers, lanes 1 and 2: amplified fragment containing three RNA residues, lanes 3 and 4: amplified fragment containing two RNA residues, lanes 5 and 6: amplified fragment containing one RNA residue, lanes 1, 3 and 5: without treatment with Tli RNaseH and lanes 2, 4 and 6: treated with Tli RNaseH.

gel, fluorescent signals were detected with FMBIO II Multi-View.

Measurement of the Amount of an Incorporated Free Primer—To perform PCR for preparation of the fragment, 0.1 μg of human genomic DNA (template), the DNA primer c-Ki-ras-12F, and either the DNA primer c-Ki-ras-12R (A) or chimeric primer c-Ki-ras/12RN3 (B) were used. The substrate (40 ng) prepared by PCR and treated as described in *'Identification of the site cleaved by RNaseH'*, 20 pmol of XRITC-labelled c-Ki-ras-12R, and 2.75 units of *Bca*BEST DNA polymerase were incubated at 55°C for 15 min in the presence or absence of one unit of Tli RNaseH in 25 μl of reaction mixture containing 32 mM Hepes–KOH (pH 7.8), 4 mM $Mg(OAc)_2$, 100 mM KCl, 1% DMSO, 0.11% BSA and 0.5 mM of each dNTP. In another reaction a mixture having the same composition as above but no enzymes (neither RNaseH nor DNA polymerase) was treated at 98°C for 2 min for heat denaturation and incubated at 55°C for 15 min in the presence of *Bca*BEST DNA polymerase. Products were electrophoresed in 6% denaturing polyacrylamide gel and analysed using FM BIO II Multi-View. The amount of DNA extended from the XRITC-labelled DNA primer c-Ki-ras-12R was measured from the signal intensity in each lane. The amount in the case where the primer and the substrate were annealed after heat denaturation (annealing) is given as 100 and the relative amounts compared to the case of annealing were calculated for the other two cases, the reaction in the presence and absence of RNaseH.

Analysis of Initiation Reaction of ICAN with DNA Primers—A 384-bp DNA fragment, which corresponds to position 246 to 629 of pUC19, was amplified by PCR using 1 pg of pUC19 as a template and MCSF and MCSR as primers. Amplified DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNA was dissolved in water and purified with Microcon-100 to be used as a template in the following analysis. One-hundred fifty femtomoles of the template DNA and 300 fmol or 30 pmol of both of the primers MR1(30), which was ^{32}P-labelled at the 5′-end, and MF2 (30) were mixed in 5 μl of 0.01% propylene diamine. The template–primer mixture prepared above was heated at 98°C for 2 min and kept at 55°C for 1 min in the test experiment. Only one primer, ^{32}P-labelled MF2(30), was mixed with the template in the control experiment. The template–primer mixture was made up to a volume of 25 μl with solution containing one unit of *Bca*BEST DNA polymerase and adjusted to the following final composition: 42.5 mM Tricine–HCl buffer (pH 8.7), 12.5 mM KCl, 12.5 mM $(NH_4)_2SO_4$, 0.0125% BSA, 1.25% DMSO, 5 mM $Mg(OAc)_2$ and 0.625 mM each of TTP, dCTP, dGTP and dATP. The polymerase reaction mixture prepared above was incubated at 55°C for 15 min. The reaction was then terminated by adding 12.5 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.5% xylene cyanol). Next, 1.6 μl of sample was analysed by denaturing gel electrophoresis. A sequence ladder formed with M13 mp single-stranded DNA, which has the same sequence as the 384-bp template DNA, and 5′-^{32}P-labelled MF2, was used to determine the size of elongated fragments. An Imageplate (Fuji Film, Tokyo, Japan) was exposed for 30 min at room temperature and the autoradiogram obtained was analysed using the bioimaging analyzer BAS 2000 (Fuji Film).

Demonstration of the Occurrence of Template-switching—A 236-bp DNA (MCSF-RV fragment) and 271-bp DNA (M4-MCSR fragment), which correspond to position 246 to 481 and position 359 to 629 of pUC19, respectively, were prepared by PCR using 10 pg of pUC19 as a template. MCSF and RV were used as primers for the amplification of the MCSF-RV fragment, and MCSR and M4 for that of the M4-MCSR fragment. Amplified DNAs were treated as described in '*Analysis of initiation reaction of ICAN with DNA primers*' to serve as templates for the following assays. In reaction (α), 15 ng of the MCSF-RV fragment and 20 pmol of 5′-^{32}P-labelled MF2(30), and 15 ng of the M4-MCSR fragment and 20 pmol of MR1(30) were separately mixed in 2.5 μl of 0.01% propylene diamine. These two template–primer mixtures were heated separately at 98°C for 2 min and kept at 55°C for 1 min. They were then combined and made up to a volume of 25 μl with solution containing one unit of *Bca*BEST DNA polymerase and adjusted to the final composition described for the previous experiment: '*Analysis of initiation reaction of ICAN with DNA primers*'. In reaction (β), the two template–primer mixtures prepared separately in reaction (α) were combined before heat treatment at 98°C and processed in the same way as in reaction (α). Reaction mixtures were incubated at 55°C for 15 min for the polymerase reaction and reaction products were analysed in the same way as described in '*Analysis of initiation reaction of ICAN with DNA primers*'.

RESULTS

Cleavage Mode of RNaseH in ICAN—In our preceding study, sequence analyses of subcloned amplified fragments as well as precise analysis of amplified ICAN products with Agilent 2100 Bioanalyzer or PAGE revealed three fragments in the ICAN products: one (large) having both of the primer sequences, another (middle) having only one primer sequence and the other (small) having no primer sequence (*1*). The behaviour of ICAN primers described above suggested that a different mechanism from the original scenario for amplification, 'nick-and-run' repetition, existed in ICAN. Sequence analyses of cloned fragments also revealed that a large portion of the middle and small fragments contained a few residual bases at the 5′ end (data not shown), the sequence of which is the same as the RNA residues of chimeric primers, suggesting that Tli RNaseH did not always cleave between the 5′-RNA and DNA-3′ junction in the ICAN reaction.

To investigate the cleavage mode by Tli RNaseH of the RNA portion in the ICAN reaction, double-stranded DNA containing three continuous RNA residues (5′-UGG-3′) in only one strand was prepared first by PCR. It was then used as a substrate for Tli RNaseH in the reaction and the size of the digested fragment was analysed by denaturing PAGE as illustrated in Fig. 2A. It was found that the site between the sequential Gs in the RNA sequence UGG was mainly cleaved (Fig. 2B, lane 2). Next, we treated a substrate having two RNA residues (UG) in one strand of a duplex DNA with Tli RNaseH and analysed it by electrophoresis. The site between U and G of the RNA sequence UG was cleaved (lane 4). Lastly, a substrate having one RNA residue (U) was treated with Tli RNaseH and analysed. The site between the 5′-adjacent DNA residue G and the RNA residue U was cleaved (lane 6).

These results indicated that Tli RNaseH cleaved the RNA portion of an extending strand in the following manner. When two or three RNA residues existed in tandem in one strand of the double-stranded DNA, Tli RNaseH preferentially cleaved the strand at the 5′ site of an RNA residue existing at the most 3′-end. Even if only one RNA residue remained after the other 3′-RNA residues were replaced one by one with DNA residues by *Bca*BEST DNA polymerase, Tli RNaseH could efficiently cleave the 5′ site of the RNA residue. This was the case when other RNaseHs derived from thermophilic archaea, *Pyrococcus furiosus*, *Pyrococcus horikoshii* and *Archaeoglobus fulgidus* were used to cleave a different RNA sequence, 5′GUA3′, with the exception that no cleavage occurred by *A. fulgidus* RNaseH when only one RNA residue at the most 5′ end (G) remained (data not shown). Because the number of remaining RNA residues is reduced one by one at each time of strand extension, DNA can be extended from a primer staying on the template as few as four times. Together with the fact that a large proportion of ICAN products contain primer sequences, this suggests the existence of some mechanism by which the chimeric primers were

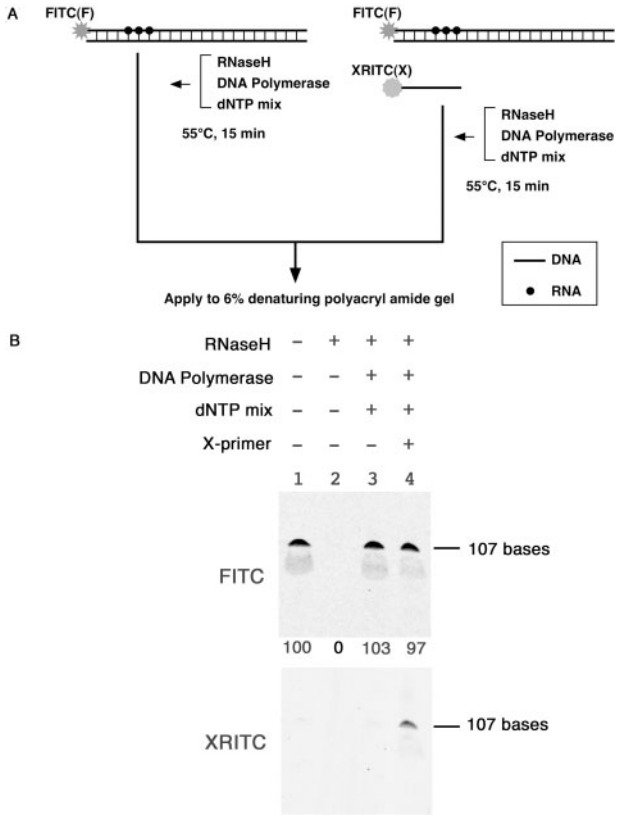

Fig. 3. **Analysis of the behaviour of pre-staying chimeric primer.** (A) Schematic representation of the design of the experiment. (B) Fluorogram of PAGE. Fluorograms of FITC and XRITC, which represent the behaviour of the pre-staying primer (F-primer) and free primer (X-primer), are shown in the upper and lower panels, respectively. The presence or absence of each of the components is shown at the top of lanes by + or−, respectively. The number below each of the lanes shows the relative amounts of FITC-labelled DNA extension fragments. The amount that existed in the substrate (lane 1) is given as 100.

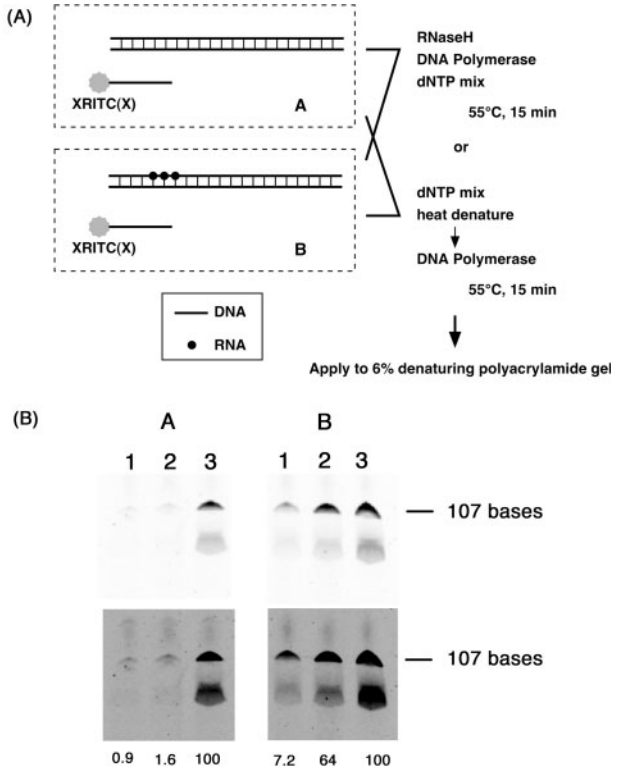

Fig. 4. **Analysis of the cooperative incorporation by a free primer with RNaseH cleavage.** (A) Schematic representation of the design of the experiment. (B) Fluorogram of PAGE. In panel A, the substrate contains RNA residues and in panel B, it does not contain RNA residues. Lane 1: reactions in the absence of RNaseH, lane 2: reactions in the presence of RNaseH, lane 3: the primer and substrate were heat-denatured and annealed. The number below each of the lanes shows the relative amount of DNA extended from the XRITC-labelled DNA primer c-Ki-ras-12R, giving 100 to the case where the primer and the template were annealed after heat denaturation. To visualize all of the six bands, data are shown in over-saturated brightness in the lower panel.

efficiently incorporated into ICAN products leading to a chain reaction.

Multi-priming—To reveal the mechanism of efficient incorporation of chimeric primers, we analysed primer extension products from a reaction intermediate containing the RNA portion as illustrated in Fig. 3A. Double-stranded DNA containing three RNA residues and having a 5′-end labelled with FITC in the upper strand was supplied as a substrate for all of the reactions in this experiment. We monitored the behaviour of the primer which had been incorporated into the substrate (F-primer) and a free DNA primer which was labelled with XRITC at the 5′ end (X-primer) in the reaction mixture by measuring the intensity of the fluorescence from FITC and XRITC, respectively. We incubated this substrate with four components in the following order and combination as shown in Fig. 3B: Tli RNase H (lanes 2 to 4), *Bca*BEST DNA polymerase (lanes 3 and 4), dNTPs (lanes 3 and 4), X-primer (lane 4).

A nick was completely introduced by RNaseH into the RNA portion of the substrate (Fig. 3B, lane 2). The presence of *Bca*BEST DNA polymerase and dNTPs in addition to RNaseH in the reaction mixture caused extension of the upper strand from all of the F-primer to the 5′ end of the complementary (lower) strand (Fig. 3B, lane 3). The amount of DNA synthesized from F-primer was not influenced by the addition of X-primer to the reaction mixture (Fig. 3B, lanes 3 and 4, upper panel) and was the same as had existed in the substrate before the nick was introduced by RNaseH (Fig. 3B, lanes 1, 3 and 4, upper panel). The addition of X-primer to the reaction mixture also caused extension of the upper strand from X-primer (Fig. 3B, lane 4 lower panel).

Next, we tested whether nicking by RNaseH in the RNA portion of the ICAN products facilitated incorporation of a free primer into the products as illustrated in Fig. 4A. Double-stranded DNA containing three RNA residues in the 5′ part in one (upper) strand served as a substrate. We incubated this substrate with a 5′ XRITC-labelled c-Ki-ras-12R (DNA primer), *Bca*BEST DNA polymerase, and dNTP mixtures in the presence or absence of Tli RNaseH. The DNA primer c-Ki-ras-12R

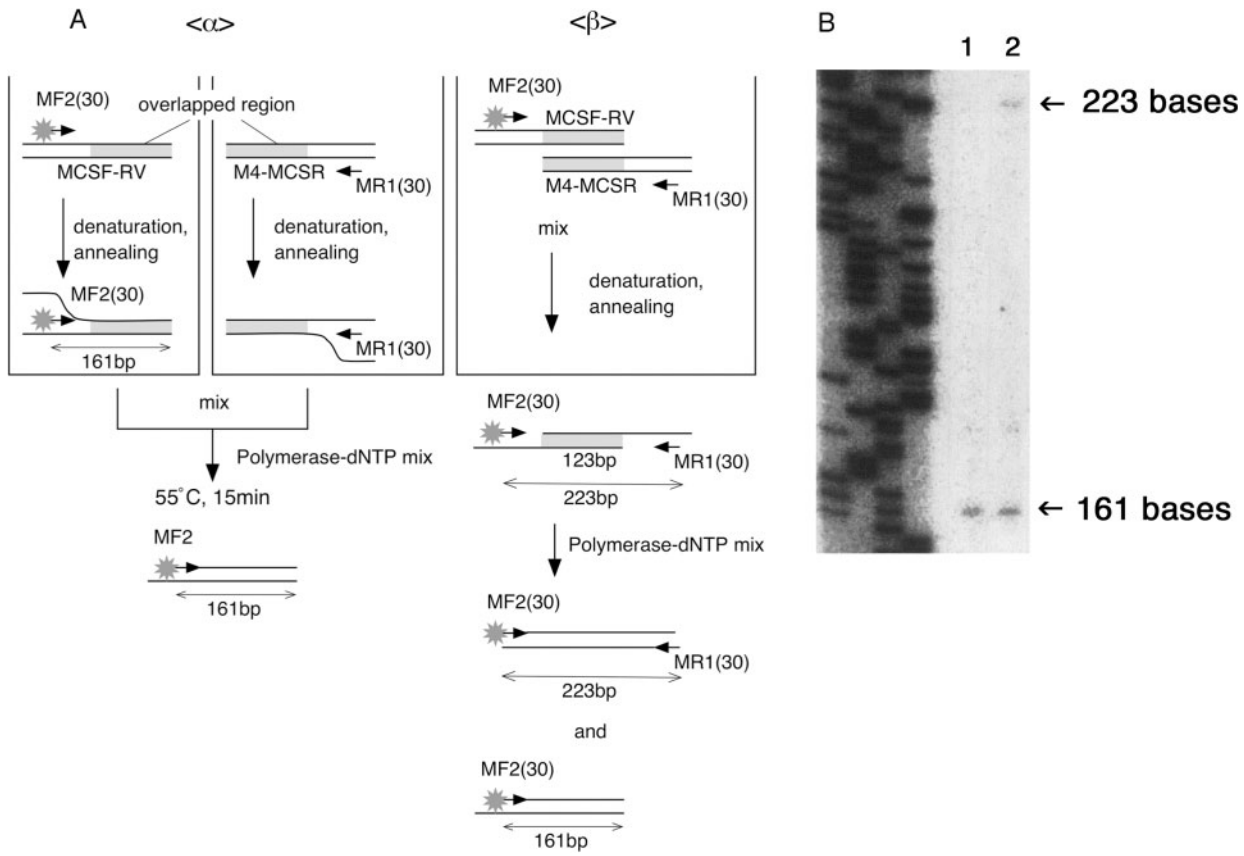

Fig. 5. **Analysis of the occurrence of template-switching.** (A) Schematic representation of the design of the experiment. The common 123-bp sequence shared by the MCSF-RV fragment and M4-MCSR fragment (overlapped region) is shaded. (B) Autoradiogram of PAGE. Fragments detected on the autoradiogram are indicated by arrows with their size at the right of the sequencing gel.

has a 5′-end sequence of the upper strand including the RNA sequence of the substrate. It was found that when RNaseH was present in the reaction mixture, the amount of DNA strand extended from the labelled DNA primer was increased 9-fold (Fig. 4B part B, lanes 1 and 2) and to 64% of the case in which the primer was annealed to the substrate after heat treatment (annealing) (Fig. 4B part B, lanes 2 and 3). If double-stranded DNA did not contain RNA residues, no extension occurred from the labelled primer without annealing (Fig. 4B part A, lane 2).

The results obtained in the above two experiments showed that (i) pre-staying primer (F-primer) can initiate DNA synthesis at an efficiency of almost 100% at each of three nickings by RNaseH and subsequent priming by DNA polymerase from the 5′ site of the most 3′-end RNA residue and (ii) at the same time a free primer (X-primer) in the reaction mixture is also used to initiate strand extension with an efficiency of about two-third (64%) of the annealing case. This leads to multiple primers that work simultaneously on the same template. We refer to this mechanism as the 'multi-priming' mechanism.

Magnesium ion included in the reaction mixture can catalyse the hydrolysis of RNA. The activity increases as the temperature becomes 55°C or higher and the pH becomes 7.5 or higher as reported by AbouHaidar and Ivanov (*2*). The higher band intensity of lane 1 of panel B in Fig. 4B than that of panel A could be due to the non-enzymatic hydrolysis by magnesium ion.

Template-switching Reaction—It is reported that template-switching can occur between two nascent extending strands during a single round of primer extension by Taq DNA polymerase in the absence of subsequent heat denaturation (*3*). If template-switching occurs between two nascent extending strands by *Bca*BEST DNA polymerase as well, chimeric primers can be incorporated into partially double-stranded DNA without competing with the complementary template in ICAN.

To explore the occurrence of template-switching by *Bca*BEST DNA polymerase in ICAN, we carried out experiments as illustrated in Fig. 5A. The 236-bp template DNA (MCSF-RV) and ^{32}P-labelled MF2(30) primer, which can hybridize to MCSF-RV, and the 271-bp template DNA (M4-MCSR) and MR1(30) primer, which can hybridize to M4-MCSR, were separately mixed in two tubes. After each of the templates was annealed to the primer in two tubes, the two mixtures were combined and incubated with *Bca*BEST DNA polymerase to extend the primers. Hereafter, this reaction is described as reaction (α). Two fragments, MCSF-RV and M4-MCSR, share a common 123-bp sequence as shown in Fig. 5A. The reaction products were separated by denaturing

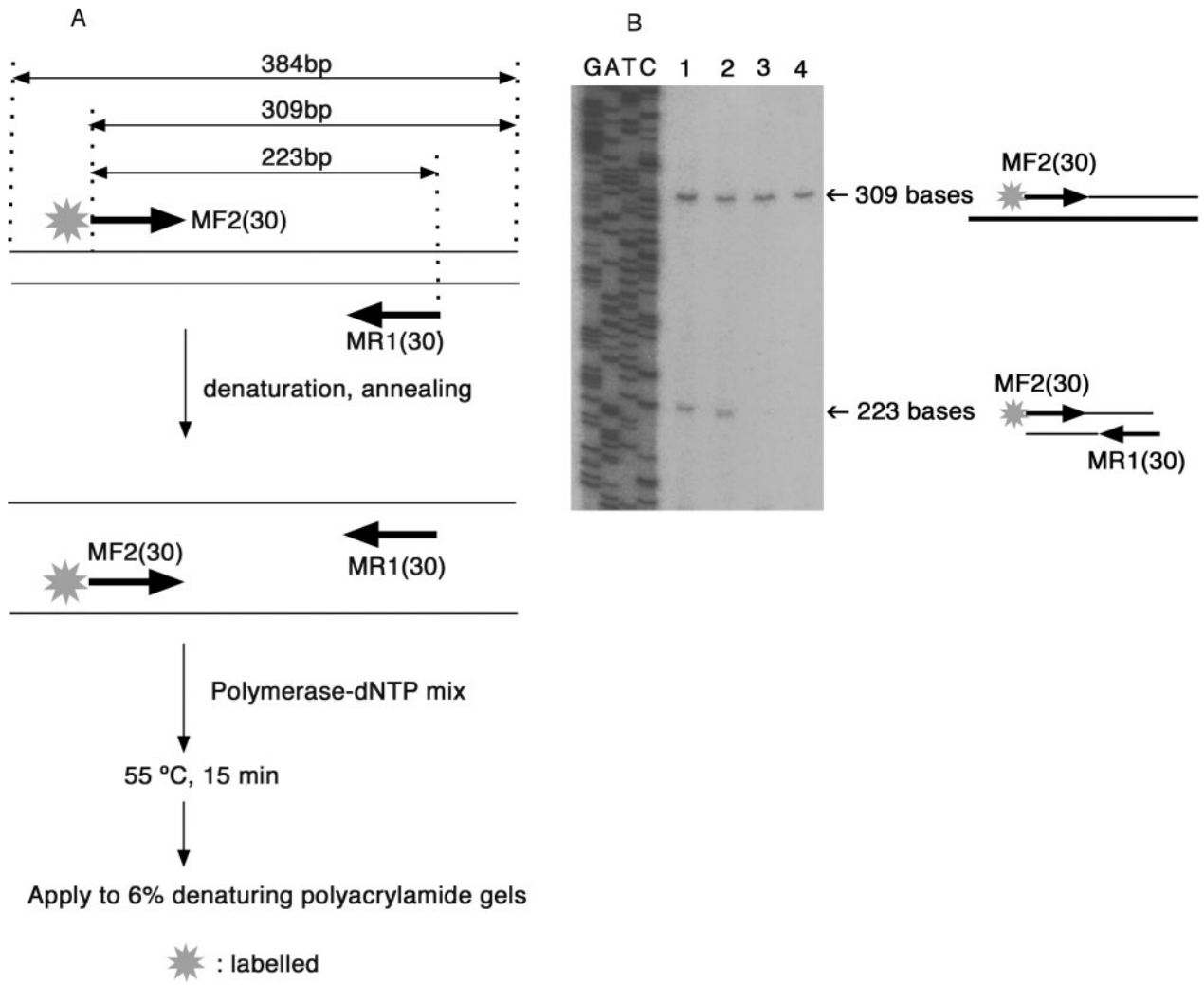

Fig. 6. **Analysis of initiation reaction of ICAN by DNA primer.** (A) Schematic representation of the design of the experiment. (B) Autoradiogram of PAGE. Lane 1: test experiment 30 pmol of both MR1(30) primer and ^{32}P-labelled MF2(30) primer was used. Lane 2: test experiment 300 fmol of both MR1(30) primer and ^{32}P-labelled MF2(30) primer was used. Lane 3: control experiment 30 pmol of only ^{32}P-labelled MF2(30) primer was used. Lane 4: control experiment 300 fmol of only ^{32}P-labelled MF2(30) primer was used.

PAGE. An autoradiogram of the gel showed, as we expected, only one band of 161 bases, which corresponds to the length from the 5′ end of MF2(30) to the 5′ end of the lower strand of MCSF-RV (Fig. 5B lane 1). In another reaction [reaction (β)], the two reaction mixtures prepared separately in reaction (α) were combined. Then annealing of the two templates with the two primers, which were conducted in separate tubes in reaction (α), was carried out in one tube and the primers were extended by *Bca*BEST DNA polymerase. The reaction product was analysed as in reaction (α). An autoradiogram of the gel showed a band of 223 bases, which corresponds to the length from the 5′ end of MF2(30) to 5′ end of MR1(30), in addition to the product of 161 bases (Fig. 5B, lane 2). Generation of the 223-base band in reaction (β), but not in reaction (α) demonstrates the occurrence of template-switching between nascent extending strands with *Bca*BEST DNA polymerase. Two strands were extended from two primers on the template hybrid molecule to the region where the two template strands were hybridized, and then template-switching occurred. If the primer extension strands were displaced from two different template DNAs by multi-priming instead, they would have hybridized to each other and the extension product of 223 bases should have been detected in reaction (α). The fact that the 223-base band was not detected in reaction (α) shows that it was not produced by the multi-priming mechanism.

DISCUSSION

A Hint that Led us to Find the Occurrence of Template-switching in ICAN—We attempted the analysis of initiation reaction of ICAN by tracing it after annealing both the forward and reverse DNA primers to a template. This analysis eventually gave us a hint for finding the occurrence of template-switching in ICAN. As shown in Fig. 6A, a mixture of template DNA, ^{32}P-labelled

MR1(30) (forward primer), and MF2(30) (reverse primer) was annealed to generate duplex DNA with both of the primers hybridized to the complementary strands (dDNA–primers complex). *Bca*BEST DNA polymerase and dNTPs were then added to the solution containing the dDNA–primers complex, the reaction mixture was kept at 55°C, and the products were separated by denaturing PAGE. An autoradiogram of the gel revealed a band of 223 bases in addition to that of 309 bases (Fig. 6B, lane 1). The 223 bases and 309 bases each correspond to the length from the 5′ end of primer MF2(30) to the 5′ end of primer MR1(30) and 5′ end of the template DNA, respectively (Fig. 6A). The primer concentration used in this experiment, 1.2 μM, is in the same order as the standard concentration in ICAN, 2 μM. We first interpreted that the 223-bp fragment was produced by multi-priming, generating displaced single-stranded DNA. However, it was obtained without a big difference in the ratio to the amount of 309-base fragment, when the above experiment was conducted with 100 times lower concentrations of both MR1(30) primer and ^{32}P-labelled MF2(30) primer (12 nM, Fig. 6B, lane 2). If it had been produced by multi-priming, the amount of the 223-base fragment should have been reduced more drastically than it was when the experiment was performed with a 100 times lower concentration of primers. The data obtained from the experiment indicated that the product of the size encompassed by the two primers, *i.e.* 223-base fragment, may be formed by a mechanism other than multi-priming, leading us to explore the occurrence of template-switching in ICAN. The result obtained in *'Template-switching reaction'* (Fig. 5) shows that the fragment of 223 bases encompassed by primers MF2(30) and MR1(30) (Fig. 6) results mostly from template switching, if not exclusively, and not from multi-priming.

Comparison of the Frequencies of Template-switching—The ratio of the signal intensity from the primer-encompassed fragment to that from the fragment extended from a labelled primer to the 5′ end of an original template strand reflects the frequency of template-switching. The ratio was measured in a similar assay using Taq DNA polymerase by Odelberg *et al.* (*3*). Comparison of the ratio obtained in our above assay with *Bca*BEST DNA polymerase to that obtained in their assay with Taq DNA polymerase showed that the former was higher than the latter, suggesting that *Bca*BEST DNA polymerase causes template-switching at higher frequency than Taq DNA polymerase. Because the ratios described above were obtained in different assays including template and primer, it was possible that the difference in the ratio merely reflected the difference of the assays. We compared the frequency of template-switching caused by two DNA polymerases by measuring the ratio of signal intensities from two kinds of fragment as described above using common primers and a template. A fragment having the size encompassed by two primers was detected when *Bca*BEST DNA polymerase was used, but was not detected with Taq DNA polymerase, showing that *Bca*BEST DNA polymerase causes template-switching at higher frequency than Taq DNA polymerase (data not shown).

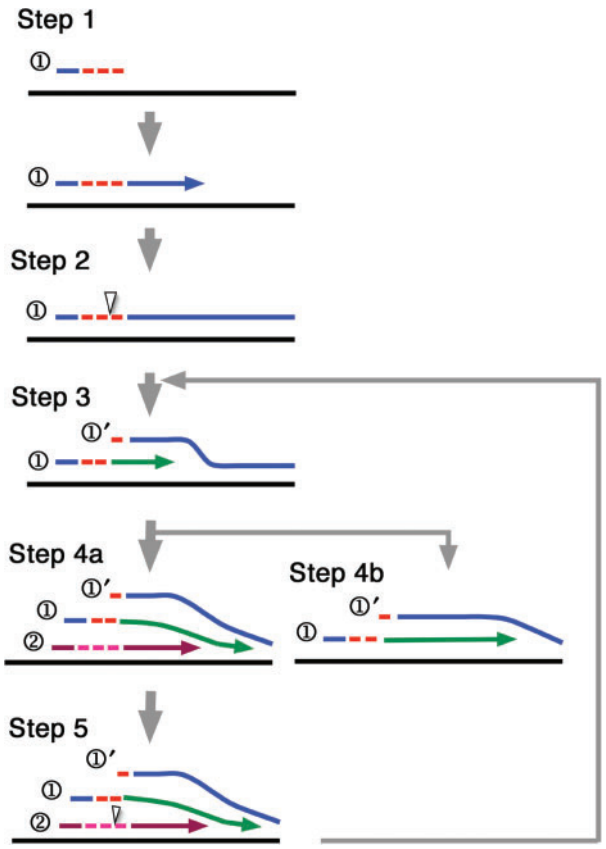

Fig. 7. **Multi-priming model for ICAN.** Only the reaction starting from one strand of duplex DNA is depicted and only the phase in which nicking occurs when three RNA residues remain intact is drawn.

Amplification Scheme by Multi-priming—A scheme for the mechanism of ICAN based on 'multi-priming' is shown in Fig. 7.

Step 1: A chimeric primer hybridizes to the template, DNA extension occurs from the 3′ end of the primer (1#).

Step 2: RNaseH introduces a nick in the RNA portion.

Step 3: Strand-displacing DNA extension occurs from the nicked site.

Step 4a: Soon after a new strand (1#) starts extending from the nicked site displacing the pre-existing strand (1′#), a chimeric primer (2#) in the reaction solution hybridizes to the template.

Step 4b: A new strand primed at the nicked site (1#) keeps extending displacing the pre-existing strand (1′#), but not being displaced by a free primer.

Step 5: Strand-displacing DNA extension from the second chimeric primer (2#) occurs without being displaced by another free primer. RNaseH then introduces a nick in the RNA portion and the resultant product returns to step 3 to be used again as a substrate for strand-displacing DNA extension.

Displaced strands in steps 4a and 4b (1# and 1′#) were used as templates to synthesize the complementary strands.

Amplification Scheme by Template-switching—A scheme for the mechanism of ICAN based on 'template-switching' is shown in Fig. 8.

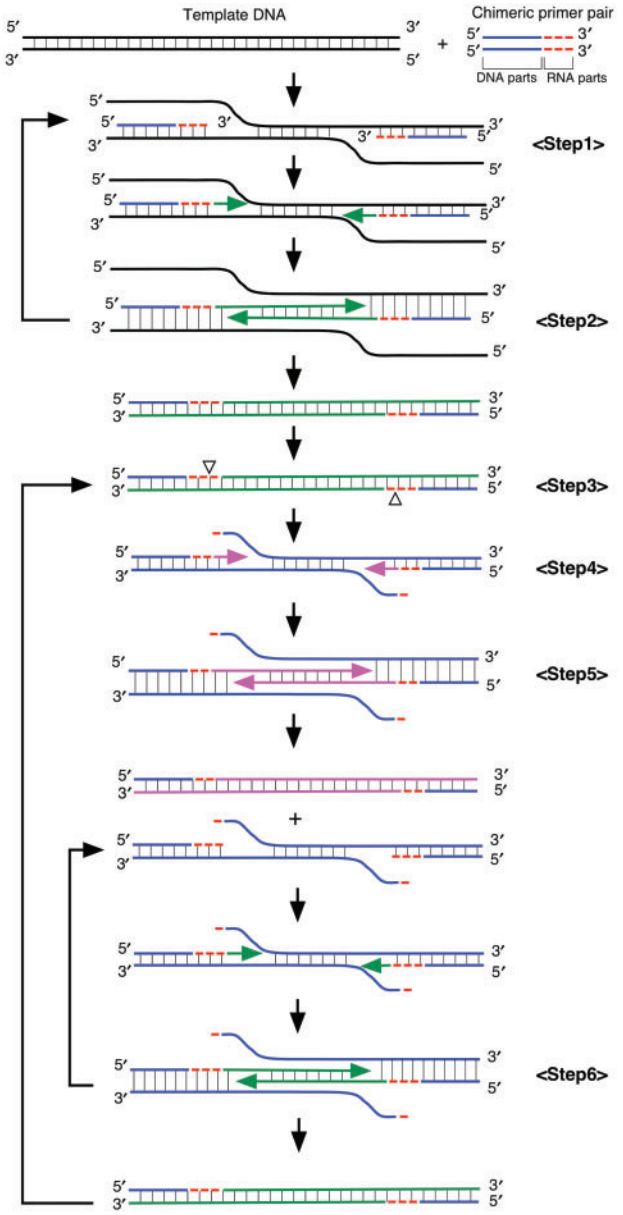

Fig. 8. **Template-switching model for ICAN.** In this figure is drawn only the phase in which nicking occurs when three RNA residues remain intact in tandem in one strand.

Step 1: A pair of primers hybridizes to a duplex template and DNA strands are extended from the 3′ end of the primers with concomitant strand displacement.

Step 2: The two strands extended from forward and reverse primers separate from the original templates in some ratio and switch templates to strands extended from opposite primers.

Step 3: RNaseH introduces a nick at the RNA portion in one RNA-containing strand.

Step 4: A strand-displacing reaction is initiated from the nicked site.

Step 5: A template-switching reaction occurs in some ratio in the same way as in step 2.

Step 6: A template-switching reaction occurs in some ratio in the same way as in step 2 and step 5. A chain reaction occurs in which the generation of both of the reaction intermediates is repeated, leading to specific amplification of the region encompassed by two chimeric primers.

Speculation of Real Reaction in ICAN—The real reaction in ICAN may not be as simple as depicted in the figures (Figs. 7 and 8) in at least two ways. First, both multi-priming and template-switching can occur simultaneously, so the kinetics of the amplification would be more complicated than those deduced from each of the figures. Second, in both of the schemes the overall efficiency of amplification is significantly influenced by the rate and times at which nicks are introduced by RNaseH. If a strand extended by the DNA polymerase from a newly generated 3′ RNA end can be nicked again (at the 5′ site of an RNA residue) by RNaseH, it follows that the duplex DNA molecule is still active as a reaction intermediate in both of the schemes. This is supported by the fact that DNA can be amplified in substantial level with ICAN even if the number of RNA residues at the 3′ end of chimeric primers is two or one [Fig. 4 in the preceding paper (*1*)]. Tli RNaseH preferentially cleaves a strand at the 5′ site of an RNA residue existing at the most 3′-end, if two or more RNA residues exist in tandem in double-stranded DNA. Furthermore, it can efficiently cleave the 5′ site of an RNA residue even if only one RNA residue exists in double-stranded DNAs (Fig. 2).

Unique Features of ICAN—We point out three key factors responsible for the high yield achieved by ICAN, which is shown in the preceding paper (*1*). The first factor is that free chimeric primers can efficiently hybridize to the template DNA to form the dDNA–primers complex. This is because, as shown in Fig. 8, primers do not have to compete with a complementary template strand to associate with a target template strand, unlike in PCR. In the case of PCR, the competition with a complementary template strand can be an inhibitory factor for DNA amplification, especially at the late stage of amplification (*4*). The phenomenon whereby more than half of the chimeric primers were exhausted in the ICAN reaction (data not shown) would reflect mainly this efficient mechanism of primer–template association. The second factor is that in the system with Tli RNaseH, the same primer can initiate priming two or more times staying on the original template as described above. The third factor is the occurrence of multi-priming caused by *Bca*BEST DNA polymerase in concert with RNaseH and a chimeric primer.

The high yield means that ICAN could be utilized in areas where DNA of a defined sequence has to be supplied on a large scale as exemplified with the preparation of large amount of *Arabidopsis thaliana* cDNA fragments described in our preceding paper (*1*). This is a major advantage over other methods of amplification including PCR. One can prepare a DNA fragment in a volume at most one-fifth of that required for PCR. Also it is quite easy to increase the volume of a reaction mixture without changing the reaction conditions because of the isothermal amplification system. So far we have confirmed that the same yield per volume of the reaction mixture was achieved without changing the reaction conditions when the reaction volume was increased from 50 μl to 5 ml

(data not shown). It would be very difficult to achieve this by PCR because of the necessity of the precise temperature-cycling. We expect this feature of ICAN can open up a new field of 'DNA industry'.

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