

Template-switching during DNA synthesis by *Thermus aquaticus* DNA polymerase I

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Received December 22, 1994; Revised and Accepted April 18, 1995

ABSTRACT

Recombinant DNA molecules are often generated during the polymerase chain reaction (PCR) when partially homologous templates are available [e.g., see Pääbo *et al.* (1990) *J. Biol. Chem.* 265, 4718–4721]. It has been suggested that these recombinant molecules are a consequence of truncated extension products annealing to partially homologous templates on subsequent PCR cycles. However, we demonstrate here that recombinants can be generated during a single round of primer extension in the absence of subsequent heat denaturation, indicating that template-switching produces some of these recombinant molecules. Two types of template-switches were observed: (i) switches to pre-existing templates and (ii) switches to the complementary nascent strand. Recombination is reduced several fold when the complementary template strands are physically separated by attachment to streptavidin magnetic beads. This result supports the hypothesis that either the polymerase or at least one of the two extending strands switches templates during DNA synthesis and that interaction between the complementary template strands is necessary for efficient template-switching.

INTRODUCTION

Recombination between partially homologous templates can occur during amplification by the polymerase chain reaction (PCR) (1–5). It has been suggested that these recombinational events are a consequence of prematurely terminated products acting as primers by annealing to partially homologous templates during subsequent PCR cycles. Although this process may occur during the thermal cycling reactions, it is not the only plausible mechanism for the generation of recombinant molecules. An alternative explanation involves a template-switching process in which the polymerase or the nascent strand switches from the original template to a secondary template during DNA synthesis.

Template-switching, also referred to as copy-choice replication, has been a postulated mechanism for various recombinational events. It is thought to play a role in recombination of both

positive and negative single-stranded RNA viruses. Evidence strongly suggests that the positive-stranded picornaviruses (e.g., poliovirus) and the mouse hepatitis virus recombine by copy-choice replication (6–12). This mechanism is also implicated in the formation of defective interfering (DI) particles, which are commonly found among negative-stranded RNA viruses, e.g., Sendai virus, vesicular stomatitis virus and measles virus (13–16). Likewise, plant ssRNA viruses may recombine by a similar mechanism (17,18). Recombination between retroviruses appears to be mediated, in part, by a template-switching process during reverse transcription (19–23). Reverse transcriptases can also exhibit strand-transfer synthesis, a process in which the polymerase processively switches to successive templates, thereby producing DNA strands that are much longer than the original template (19,24). Furthermore, evidence suggests that during strand-displacement synthesis, several polymerases including *Escherichia coli* DNA polymerase I (25,26), T5 DNA polymerase (27) and T7 DNA polymerase (28) may occasionally switch from their original template to the complementary displaced strand and continue synthesis to the end of this secondary template. Template-switching has also been invoked to explain the acquisition of chromosomal markers by plasmids in *Haemophilus influenzae* (29), repair of DNA lesions in bacteriophage T4 (30), plasmid deletions in *Bacillus subtilis* (31), duplication of genetic elements via formation of a stem-loop structure during replication of the ColE1 plasmid (32) and reversion of Q β RNA phage mutants (33).

We demonstrate here that template-switching can also occur during *in vitro* DNA synthesis by *Thermus aquaticus* (*Taq*) DNA polymerase I (34). This switching process requires the presence of double-stranded templates and appears to depend upon interaction between the two complementary template strands. We show that the secondary templates utilized in these switches can be either pre-existing homologous templates or complementary nascent strands.

MATERIALS AND METHODS

Plasmid constructs

The recombinant plasmids used in this study were constructed by cloning various DNA segments from the 5'-end of the lipoprotein

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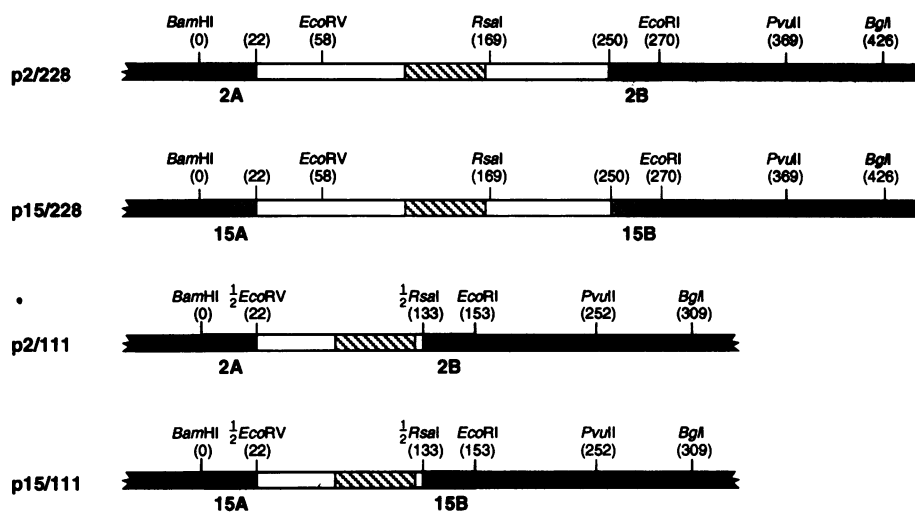


Figure 1. The recombinant plasmids constructed for this study p2/228 and p15/228 contain a 228 bp insert flanked by the 20 bp unique identifier sequences 2A and 2B and 15A and 15B, respectively. p2/111 and p15/111 contain a 111 bp insert flanked by the indicated identifier sequences. The restriction sites pertinent to this study are shown. The numbers in parentheses indicate the distance in base pairs from the 5'-end of primer pr2A or pr15A. The insert is shown as a white box, while the light hatched region represents the d(CA)-repeat within the insert. The identifier sequences are represented by either the black boxes (2A and 2B) or the dark hatched boxes (15A and 15B). The remaining portion of the vector is stippled.

lipase gene that contained a d(CA)₂₅-repeat into the unique *DraI* cloning site of either the pKZ2 or pKZ15 vector (hereafter referred to as p2 and p15, respectively). p2 and p15 contain unique 20 bp identifier sequences that flank the *DraI* cloning site. The identifier sequences for p2 are 2A (5'-AACCATCCTACCC-ACACCT-3') and 2B (5'-CCAACTAACCTCACTCCCTC-3') and those for p15 are 15A (5'-ACAAAACCATTTCCCAACA-3') and 15B (5'-ACTCCTCACCCCTCTATT-3').

Figure 1 shows the four plasmid constructs used in this study. Plasmids p2/228 and p15/228 were generated by cloning a 209 bp *PstI*-*XbaI* fragment containing the dinucleotide repeat into the pBluescript II SK+ plasmid (Stratagene, La Jolla, CA). The insert was sequenced and a 228 bp *HaeIII* fragment containing the d(CA)₂₅ repeat and part of the polylinker region of the plasmid was subcloned into the *DraI* site of the p2 and p15 vectors to generate the recombinant plasmids p2/228 and p15/228. The orientation of the inserts within these new recombinant plasmids was determined by restriction mapping and sequencing using a Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical Corp., Cleveland, OH). Plasmids p2/111 and p15/111 were constructed by subcloning a 111 bp *EcoRV*-*RsaI* fragment from p2/228 into p2 and p15, respectively.

Assays using a single thermal cycle

The clone p2/228 was linearized by digestion with *BglI*. Primer pr2A (5'-CCAACCATCCTACCCACACCT-3') was end-labelled with ³²P by the following procedure. Ten pmol of pr2A was incubated for 30 min at 37°C in a 10 µl solution containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 10 mM MgCl₂, 50 µCi [γ -³²P]ATP (10 pmol) and 4.2 U T4 polynucleotide kinase. A 10 µl reaction mixture containing 125 ng (0.065 pmol) of p2/228-*BglI*, 1 × buffer [10 mM Tris-HCl (pH 8.8), 40 mM NaCl and 1.5 mM MgCl₂], 200 µM of each dNTP, 1 pmol of end-labelled pr2A and 1.25 U (~0.065 pmol) of recombinant *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus, Norwalk,

CT) was prepared. Fifteen microliters of light mineral oil was layered onto the mixture. A single thermal cycle with the following profile was performed: (i) denaturing at 94°C for 5 min and (ii) primer-annealing and extension at 60°C for 2 min. A second reaction, run in parallel to the first, included the above reagents plus primer pr2B (5'-TTCGAGGGAGTGAGGT-TAGTTGG-3'). Five microliters of stop dye (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) was added and the DNA was denatured at >90°C and fractionated on a 6% denaturing polyacrylamide gel containing 8.3 M urea (made from a SequaGel kit, National Diagnostics, Manville, NJ). Autoradiography was performed at -70°C overnight.

In a second assay, the plasmids p2/228 and p15/228 were each digested with *BglI*. A single thermal cycle was performed in a 10 µl solution containing 125 ng (0.065 pmol) of each of the appropriate *BglI*-digested recombinant plasmids, 1 × buffer [10 mM Tris-HCl (pH 8.8), 40 mM NaCl, either 1 or 1.5 mM MgCl₂], 1 pmol of each of the appropriate primers, 200 µM of each dNTP and 1.25 U (~0.065 pmol) of AmpliTaq. Fifteen microliters of light mineral oil was loaded onto each sample. Cycling conditions were the same as those above. Immediately following the single thermal cycle, the samples were placed on ice and 5 µl of stop-dye was added. The samples were stored at -20°C until needed for the next stage of analysis (see the following section).

In a third assay, the p2 vector was linearized by digestion with *BamHI* and the following primers were constructed: p2BamA (5'-CTGGTATCTTTATAGTCTCTGTCGG-3') and p2BamB (5'-GGGCGCTCTTCGCTTCCTCG-3'). p2BamA was end-labelled with ³²P and 1 pmol of this primer was added to a reaction mixture containing 125 ng (0.065 pmol) of p2-*BamHI*, 1 × buffer [10 mM Tris-HCl (pH 8.8), 40 mM NaCl and 1.5 mM MgCl₂], 200 µM of each dNTP and 1.25 U of AmpliTaq (10 µl total volume). In a parallel experiment, 1 pmol of unlabelled p2BamB was also added to the reaction cocktail. Fifteen microliters of light mineral oil was layered over the cocktail and a single round of primer extension was performed with the same

thermal cycling profile as described above. Five microliters of stop dye was added to each reaction and the DNA was denatured at $>90^{\circ}\text{C}$ and fractionated on a 6% denaturing polyacrylamide gel containing 8.3 M urea. Autoradiography was performed at -70°C overnight.

Polyacrylamide gel electrophoresis, electroblotting and hybridization

The unlabelled products obtained from the single round of primer extension in the presence of p2/228-*Bgl*II and p15/228-*Bgl*II were denatured for 5 min at $>90^{\circ}\text{C}$ and plunged on ice. They were then loaded onto a 6% denaturing polyacrylamide gel (SequaGel) and electrophoresis was carried out in $1 \times \text{TBE}$ at 40 W for 4.5 h. The fractionated DNA fragments were transferred to GeneScreen Hybridization Transfer Membrane (DuPont NEN, Boston, MA) by electroblotting in $0.5 \times \text{TBE}$ at 1.5 A for 35 min. The DNA was UV crosslinked by applying 180 000 μJ using a UV Stratalinker 2400 (Stratagene, La Jolla, CA); the blot was then rinsed in 1% sodium dodecyl sulfate (SDS)/50 mM sodium phosphate/1 mM Na_2EDTA .

Four oligodeoxynucleotide probes, c2A (5'-AGGTGTGGGGT-AGGATGGTT-3'), pr15B (5'-CCAATAGGAGGGGGTGAAGGAGT-3'), c15B (5'-ACTCCTCACCCCTCCTATT-3') and pr2A (5'-CCAACCATCCTACCCACACCT-3') were end-labelled as described above, except that following incubation at 37°C , T4 polynucleotide kinase was inactivated by adding 2 μl of 0.5 M Na_2EDTA . The probes were precipitated with ethanol in the presence of 2.5 M ammonium acetate and 120 μg of carrier salmon sperm DNA. Prehybridization was carried out at room temperature for 5 min in a solution containing 5% w/v SDS, 50 mM sodium phosphate and 1 mM Na_2EDTA . The end-labelled probe was added to the prehybridization solution ($>1 \times 10^6$ c.p.m./ml), and the hybridization mixture was incubated overnight with shaking at 37°C . The membranes were washed three times at room temperature with the prehybridization solution and once with 1% SDS/50 mM sodium phosphate/1 mM Na_2EDTA . Autoradiography was carried out at -70°C for 24–50 h with two intensifying screens.

Quantitation of recombinant and non-recombinant products by competitive PCR

To confirm the presence of recombinant molecules among products generated from a single round of primer extension, we excised the apparent recombinant fragments (Fig. 2a), eluted them from the gel, and subjected them to competitive PCR (35) using sets of primers that would preferentially amplify either recombinant molecules or non-recombinant molecules. The competitor template contained the 228 bp *Hae*III fragment flanked by either identifier sequences 2A and 2B (non-recombinant) or 2A and 15B (recombinant) (Fig. 2b). The test template used in the single round of primer extension contained a smaller 111 bp *Rsa*I-*Eco*RV fragment inserted into the *Dra*I cloning site of vectors p2 and p15. The complete sequence of the 111 bp insert is contained within the 228 bp *Hae*III fragment but the smaller insert lacks sequences present at both ends of the larger 228 bp fragment (see Fig. 1). Primer pr2A was end-labelled with ^{32}P and a single round of primer extension was performed in the presence of either: (i) just the p2/111-*Bgl*II template or (ii) both the p2/111-*Bgl*II and p15/111-*Pvu*II templates (Fig. 2a). The 252 nt extension product, apparently generated by a template-switch,

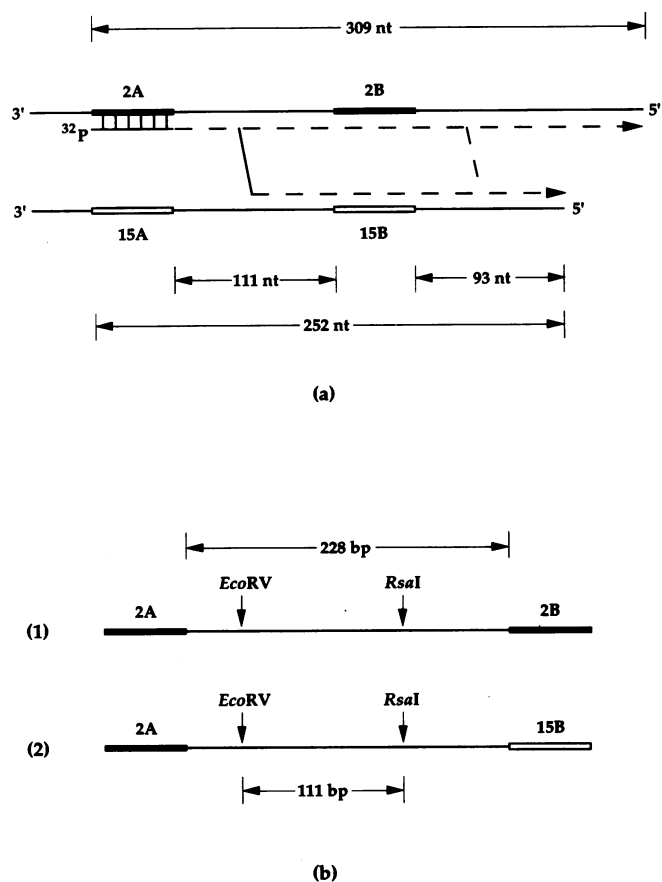


Figure 2. Schematic diagram of the competitive PCR assay. (a) Potential extension products following a single round of primer extension. End-labelled primer pr2A, which anneals to 2A, is extended by *Taq* DNA polymerase in the presence of both p2/111 and p15/111. The expected full-length extension product is 309 nt; however, a template-switch would be characterized by a shorter, 252 nt fragment. Template-switches that occur between the identifier sequences (diagonal solid line) would generate recombinant molecules, while those occurring after the synthesis of the 2B segment (diagonal dashed line) would be non-recombinant. (b) Competitor DNA templates used in the competitive PCR assay. (1) The non-recombinant competitor DNA template. (2) The recombinant competitor DNA template.

was excised, eluted and subjected to competitive PCR using varying amounts of competitor templates and the appropriate primers. The 10 μl reaction mixtures contained the 252 nt eluate, 0.04–10 pg of competitor template, $1 \times$ rapid thermal cycling buffer (50 mM Tris-HCl, pH 8.4, 20 mM NaCl, 3 mM MgCl_2 , 500 $\mu\text{g/ml}$ BSA), 500 μM of each dNTP, 0.1 pmol of ^{32}P -end-labelled pr2A or pr15A (5'-GATCCACAAAACCATTTCCCAACA-3'), 4.9 pmol of unlabelled pr2A or pr15A, 5 pmol of pr2B or pr15B and 0.4 U of AmpliTaq. Amplification by the PCR was performed in microcapillary tubes on a Idaho Technology Inc. 1605 Air Thermo-Cycler. Thirty thermal cycles were carried out using the following profile: initial denaturation, 94°C for 15 s; subsequent denaturation, 94°C for 0 s (temperature spike); primer annealing, 55°C for 0 s; primer extension, 74°C for 5 s. The PCR products were separated on 7% denaturing polyacrylamide gels (5.6 M urea, 32% v/v formamide) and the relative intensities of the amplification products of the competitor DNA and the eluate DNA were determined on the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analysis of primer extension products generated from complementary template strands that have been physically separated by attachment to magnetic beads

Biotinylated complementary template strands were physically separated from each other by attachment to different sets of streptavidin magnetic beads (Dynabeads M-280, Dynal, Inc., Great Neck, NY). This was accomplished by digesting p15/228 with *Bam*HI and *Pvu*II and then gel purifying the 371 bp fragment that contained both the 15A and 15B identifier sequences. This gel purified product was used as template in a single round of primer extension using either primer pr15A-bio (5'-biotin-CACAAAACCATTTCCCAACATTTTC-3') or primer prPvu-bio (5'-biotin-CTGGCGAAAGGGGGATGTGCTGC-3'). The latter primer anneals to the 3'-end of the 371 bp template and will generate the complementary template strand. The biotinylated products were then allowed to bind to different sets of streptavidin magnetic beads for 15 min and the supernatant was removed using a magnetic particle concentrator (MPC-E, Dynal, Inc.) according to the manufacturer's instructions. The bound double-stranded products were denatured in 0.15 M NaOH for 5 min and again the supernatant was removed. The beads were washed once in STE [100 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM Na₂EDTA] and resuspended in 20 μ l of TE⁻⁴. Primer extensions using ³²P-end-labelled pr15A and AmpliTaq were performed in the presence of either one or both of the template strands and in either the presence or absence of primer pr15B. A 24-fold excess of beads with no attached DNA template was added to each primer extension reaction to help ensure that the complementary template strands remained physically separated during the reaction. The extension products were fractionated on 6% denaturing polyacrylamide gels (Sequagel) and autoradiography was carried out at -70°C.

RESULTS

An extra band is observed following a single thermal cycle

A template-switching model predicts that recombinant molecules will be generated following a single round of primer extension; this is in contrast to alternative models, which require truncated products to anneal to partially homologous templates on subsequent PCR cycles. Recombination during the primer extension phase of a single PCR cycle could occur via two different routes: (i) an extending strand could switch from its original template to a pre-existing, partially homologous template or (ii) a nascent strand could switch from its original template to the complementary nascent strand being extended from the other PCR primer. In either scenario, continued synthesis using the secondary template would produce a recombinant. To screen for possible template-switching events of the latter variety, we performed parallel extensions of a *Bgl*II-digested p2/228 construct in the presence of just the labelled primer (pr2A) in one reaction and both the labelled (pr2A) and unlabelled (pr2B) primers in a second reaction (Fig. 3). The expected extension product of 426 nt was the predominant band in both sets of reactions; however, in the reaction that contained both primers (lane 2), an extra band of ~279 nt was observed. This extra product is approximately equal in length to the distance between and including both primers and is the expected size of the PCR product following multiple cycles. However, we observed this product after a single thermal cycle. Furthermore, this extra band was observed even when *Taq* DNA

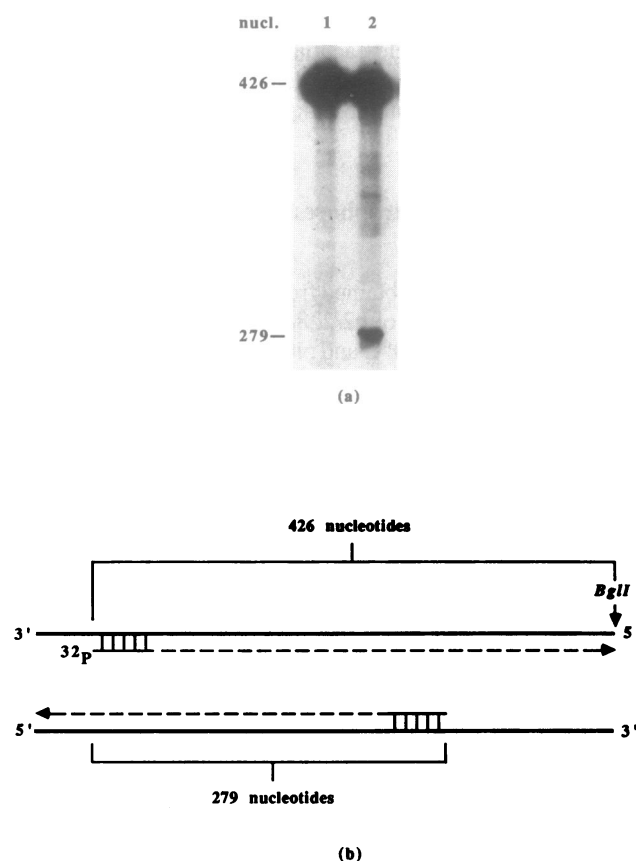


Figure 3. An extra band is present following a single thermal cycle. p2/228 was linearized with *Bgl*II and subjected to one thermal cycle using a single ³²P-end-labelled primer (pr2A). (a) Lane 1, only the end-labelled primer pr2A was added to the reaction and the expected 426 nt fragment is observed. Lane 2, both the end-labelled primer pr2A and the unlabelled primer pr2B were added to the reaction. The expected 426 nt fragment is present but, in addition, a 279 nt fragment is observed. This additional fragment is the size of the PCR product expected after multiple cycles and is defined by the distance between and including the two primers. (b) A schematic diagram representing the observations in (a).

polymerase was added after the primer-annealing temperature had been reached (data not shown), indicating that the anomalous product was generated during a single primer-extension phase. The molar ratio of the expected 426 nt fragment to the extra 279 nt fragment varied from ~5:1 to 50:1, as determined by quantitation on the PhosphorImager.

These results are consistent with a template-switching mechanism in which the polymerase or 3'-end of the nascent strand switches from the original template to the complementary nascent strand that is being generated from the other PCR primer. However, they do not rigorously establish that such a mechanism has occurred; this would require a more extensive analysis designed to detect the presence of recombinants, as discussed in the following section.

Test for recombination during a single round of primer extension

Template-switching during DNA synthesis could account for the extra band observed following a single round of primer extension. Therefore, to directly test this hypothesis, we mixed *Bgl*II-digested

recombinant plasmids p2/228 and p15/228, added primers pr2A (specifically recognizes identifier sequence 2A in the p2 vector) and pr15B (specifically recognizes identifier sequence 15B in the p15 vector) and subjected the mixture to one thermal cycle. After electrophoresis through a denaturing polyacrylamide gel, the extension products were transferred onto a nylon membrane by electroblotting and then hybridized successively with ^{32}P -end-labelled probes pr15B and c2A (the complement of primer pr2A). Due to the orientation of the insert within the vectors, hybridization with the above probes allowed us to determine whether some of the nascent strands containing the d(TG)-repeats were recombinants. Figure 4 shows an example of the results obtained from these experiments.

No primer extension products should hybridize with probe pr15B, unless a recombinational event has occurred. However, Figure 4a (lane 3) demonstrates that two extension products that do not appear in one of the control lanes do hybridize to pr15B. The predominant fragment is 426 nt. This is the size expected if partially extended products of primer pr2A switched, in register, to the complementary p15/228 template and were then extended to the *Bgl*I site by *Taq* DNA polymerase. A second, less intense band of ~274 nt is also present. This is approximately the size expected if partially extended products of primer pr2A had switched to the nascent extension products of primer pr15B and then used these strands as templates for DNA synthesis. In several independent experiments, the ratios of the 426 nt fragment to the ~274 nt fragment ranged from 5:1 to 14:1, as determined by the PhosphorImager; we conclude that, given these experimental conditions, the switch to the partially homologous template is favored over a switch to the other nascent strand.

The 426 nt fragment is also present in lane 4 of Figure 4a (this reaction contained p2/228, p15/228 and primer pr2A), while the ~274 nt fragment is absent. This result is consistent with the template-switching hypothesis. Because both p2/228 and p15/228 were present in the reaction, the nascent strand extending from primer pr2A could switch from the p2/228 template to the p15/228 template and extend to the cut *Bgl*I site on p15/228, thereby producing a 426 nt fragment that would hybridize to probe pr15B. However, a ~274 nt fragment would not be expected because the absence of primer pr15B would prevent the synthesis of any complementary nascent strands and preclude this type of template-switch.

To provide further evidence that these extension products were recombinants, we stripped the membranes and hybridized with the oligodeoxynucleotide c2A. This probe is complementary to the pr2A primer and therefore hybridizes to all pr2A extension products; if the 426 and ~274 nt fragments in Figure 4a are recombinants, they would also hybridize to c2A. Figure 4b demonstrates that this is the case for the ~274 nt fragment (lane 3). However, hybridization with c2A does not produce corroborative evidence that the 426 nt fragment is a recombinant because c2A is expected to hybridize to a fragment this size regardless of whether a template-switch has occurred.

No extension products are observed in lane 7, which is a control designed to determine whether the stop-dye used in these studies actually prevents DNA synthesis. This control is necessary because the extra band could otherwise be explained by postulating a second round of primer extension during the preparation of the sample for gel loading. The absence of any extension products in lane 7 precludes this possibility.

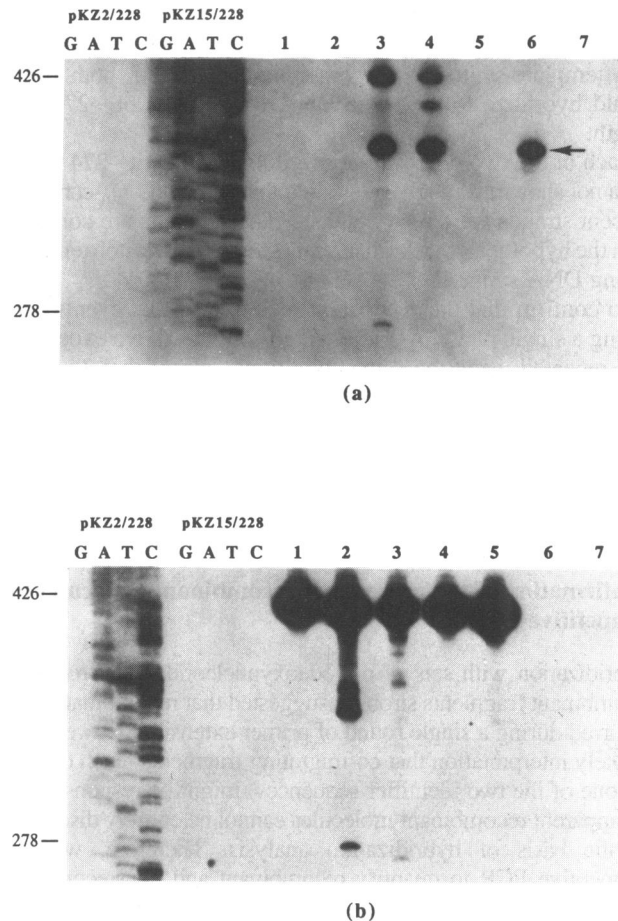


Figure 4. Recombination occurs during a single thermal cycle. Products of a single thermal cycle were denatured and loaded onto a 6% denaturing polyacrylamide gel. Following electrophoresis, the DNA fragments were transferred to a nylon membrane by electroblotting and were hybridized with various probes. Lane 1: p2/228-*Bgl*I and primer pr2A. Lane 2: p2/228-*Bgl*I and primers pr2A and pr2B. Lane 3: recombination test lane; p2/228-*Bgl*I and p15/228-*Bgl*I and primers pr2A and pr15B. Lane 4: p2/228-*Bgl*I and p15/228-*Bgl*I and primer pr2A. Lane 5: p2/228-*Bgl*I and primers pr2A and pr15B. Lane 6: p2/228-*Bgl*I and p15/228-*Bgl*I and no primers. Lane 7: control to test for second thermal cycle during denaturing process prior to loading the sample onto the gel; p2/228-*Bgl*I and primer pr2A + stop-dye—this sample was not subjected to any prior thermal cycle. Sequencing ladders for the pKZ2/228 (p2/228) and pKZ15/228 (p15/228) plasmids were used to size the fragments found in lanes 1–7. (a) Electrophoretogram hybridized with probe pr15B. The arrow indicates a DNA fragment presumably caused by a specific nick in the p15/228-*Bgl*I template DNA. This interpretation is based on the fact that it is observed in the control lane containing only the two template DNAs (lane 6). (b) Electrophoretogram hybridized with probe c2A. The extra bands in lane 2 that lie between the 426 and 278 nt fragment are of unknown origin but are often observed (see Fig. 3).

The above results present evidence that strongly suggests recombination can occur when the extending strand contains d(TG)-repeats. To test whether the other nascent strand, which contains d(CA)-repeats, is also involved in recombinational events, we hybridized the same membranes with probes pr2A and c15B (the complement of pr15B). If a recombinational event had not occurred, we would expect to observe only very large run-on extension products of various sizes when hybridizing with c15B.

Likewise, probe pr2A would hybridize only to template DNA. However, if the $d(CA)_n$ -containing nascent strands were involved in a template-switch to the other nascent strand, both probes would hybridize to the same DNA fragment(s) of ~278 nt in length.

Each of the two probes hybridized to a fragment ~274 nt long (data not shown), strongly suggesting that some $d(CA)_n$ -containing nascent strands were recombinants. These results are consistent with the hypothesis that both nascent strands can switch templates during DNA synthesis.

To confirm that these apparent recombinational events occur during a single phase of primer extension, the above experiment was repeated using the same conditions, except that *Taq* DNA polymerase was added after the annealing temperature (60°C) was reached. The extra ~274 nt fragment was observed only in the recombination test lane (analogous to lane 3 in the above experiment), thus confirming that this fragment is generated during a single phase of primer extension (data not shown).

Confirmation of the presence of recombinant molecules by competitive PCR

Hybridization with sets of oligodeoxynucleotides that recognize recombinant fragments strongly suggested that recombination had occurred during a single round of primer extension. However, the unlikely interpretation that co-migrating fragments (each containing one of the two identifier sequences) might be responsible for the apparent recombinant molecules cannot be entirely discounted on the basis of hybridization analysis. Therefore, we used competitive PCR to quantify recombinant and non-recombinant molecules contained within a 252 nt fragment, which we hypothesized was generated by a switch from the original template to a pre-existing secondary template (see Fig. 2a).

Two types of template-switches are possible: (i) a switch that occurs during DNA synthesis of the region between the identifier sequences and (ii) a switch that occurs following the synthesis of the 2B identifier sequence. The first type of switch will generate a recombinant molecule with 2A on the 5'-end and 15B toward the 3'-end, while the second type of switch will generate a non-recombinant molecule containing identifier sequences 2A and 2B. If template-switching does not exhibit a sequence preference, one would expect approximately equal amounts of recombinant and non-recombinant products within the 252 nt fragment, because the number of nucleotides that lie between the identifier sequences is approximately equal to the number of homologous nucleotides downstream of 2B and 15B (111 nt versus 93 nt). However, if the 252 bp fragment did not result from a template-switch, very few recombinant molecules would be expected. If both of the original templates were present in the eluate, some recombinant molecules would be generated during the PCR; however, this process alone would generate very few recombinants and the amplified non-recombinant molecules would greatly outnumber the amplified recombinants.

By employing a competitive PCR analysis, we were able to compare the relative number of recombinant and non-recombinant molecules contained within the 252 nt fragment. Competitor DNA templates of known concentration were added to the PCR cocktails. These competitor templates compete with the target DNA for the available primers and therefore the ratio of amplified competitor products to amplified target DNA can be used to quantitate the original amount of target DNA (see Fig. 5). The

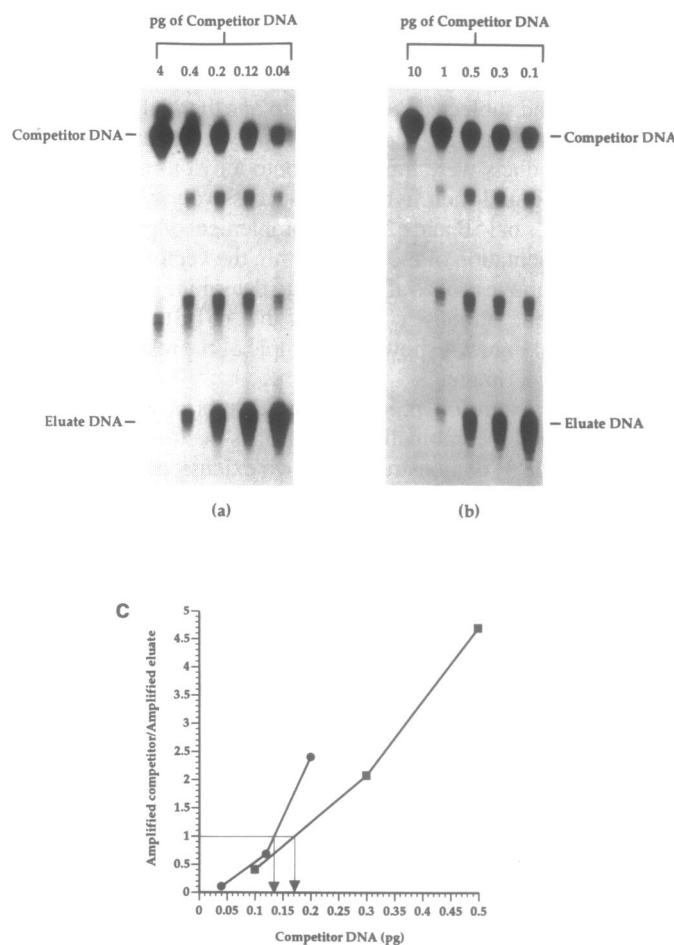


Figure 5. Competitive PCR quantitation of recombinant and non-recombinant products generated in a single round of primer extension. (a) Products generated during a competitive amplification assay of recombinant molecules. (b) Products generated during a competitive amplification assay of non-recombinant molecules. The amount of competitor DNA added before amplification is indicated above each lane in (a) and (b). The intermediate-sized bands are the size expected from recombinational events between competitor and eluate DNA. (c) Plot of the ratio of competitor DNA/252-nt eluate following amplification versus competitor DNA added to the reaction mixture before amplification. ● represents data generated from amplification of recombinant molecules; ■ denotes data generated from amplification of non-recombinant molecules. The concentration of equivalence for recombinants and non-recombinants is denoted by their respective vertical arrows.

252 nt fragment, suspected to be generated by template-switching, was excised, eluted and subjected to two sets of competitive PCR reactions, each set containing one of the two different competitor DNA templates shown in Figure 2b. The primers used in these analyses were those which would amplify both the competitor DNA and any target DNA molecules that contain the appropriate set of identifier sequences, e.g., primers pr2A and pr15B were used to amplify competitor DNA #2 and any recombinants present in the eluate. The target DNA and competitor DNA could be distinguished because of their differences in size. The competitor DNA generates a larger product than the target eluate DNA. Figure 5 demonstrates that the number of recombinant and non-recombinant molecules present in the 252 nt fragment was approximately equal, thus confirming the results of the hybridization analysis. We conclude that template-switching is occurring during DNA synthesis by

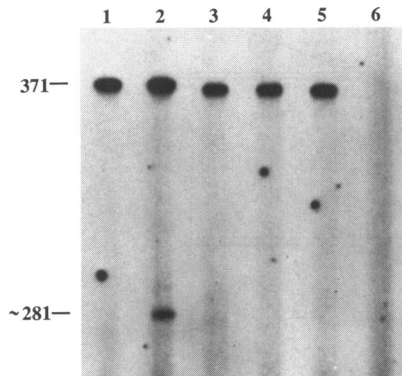


Figure 6. Recombinational events are reduced when the complementary template strands are physically separated from each other. Complementary template strands from the 371 bp gel purified fragment were physically separated from each other by attachment to different sets of streptavidin magnetic beads and an analysis designed to detect recombinants was performed. Lane 1: control, 371 bp fragment (template not attached to beads) and ^{32}P -end-labelled primer pr15A. Lane 2: control, unattached 371 bp fragment, end-labelled primer pr15A, and unlabelled primer pr15B. Lane 3: recombination test lane, both template strands present but separated from each other by attachment to magnetic streptavidin beads, plus end-labelled primer pr15A and unlabelled primer pr15B. Lane 4: control, both template strands present but separated from each other, plus end-labelled primer pr15A. Lane 5: control, template strand for primer pr15A (attached to magnetic beads) plus end-labelled pr15A and unlabelled primer pr15B. Lane 6: stop-dye control, unattached 371 bp fragment and end-labelled pr15A in the presence of stop-dye.

Taq DNA polymerase and that these switches can generate recombinant molecules during a single round of primer extension in the presence of partially homologous templates.

Interaction between the complementary template strands is required for efficient template-switching between nascent strands

The proportion of recombinants to full-length extension products was reduced ~4-fold when the template strands were physically separated from each other by attachment to different sets of streptavidin magnetic beads (Fig. 6). These tests were done in the presence of excess beads having no DNA attached to their surfaces so that interaction between template strands would be minimized. The reduction in recombinant molecules suggests that interaction between the complementary template strands plays a significant role in the recombinational process.

Screening for template-switches during DNA synthesis of random sequences

We also wanted to determine whether template-switching is a general phenomenon of *Taq* DNA polymerase I-catalyzed DNA synthesis or whether it is induced by d(CA)-repeats. Therefore, we screened a sequence within the p2 vector that lacked dinucleotide repeats to determine whether the presence of two primers, as opposed to one primer, produced an extra DNA fragment of the appropriate size following a single thermal cycle. The p2 vector was linearized by digestion with *Bam*HI and a unique set of primers was constructed for the linearized vectors so that the expected PCR product following multiple cycles would be 278 bp and the extension product from the 5'-end of the ^{32}P -labelled

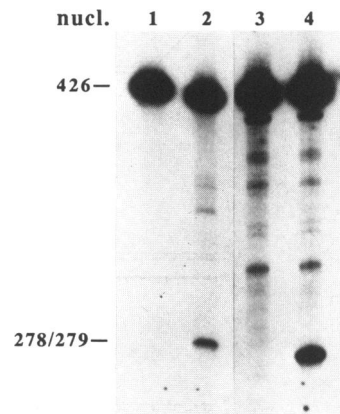


Figure 7. Recombination during DNA synthesis may be a general phenomenon in PCR amplifications. An extra band, apparently resulting from a template-switch, was observed following a single thermal cycle using two different sequences that do not contain long stretches of dinucleotide repeats. Lane 1: control; p2/228-*Bgl*II and end-labelled primer pr2A. Lane 2: control; p2/228-*Bgl*II, end-labelled primer pr2A and primer pr2B. Lane 3: p2-*Bam*HI and end-labelled primer p2*Bam*A. Lane 4: p2-*Bam*HI, end-labelled primer p2*Bam*A and unlabelled primer p2*Bam*B. Note the extra ~278/279 nt fragment in each of the lanes that contains both primers.

primer to the restriction site would be 426 nt in length. A single thermal cycle was performed either in the presence of the labelled primer alone or in the presence of both primers. An additional fragment of ~278 nt was observed when both primers were present in the reaction but was absent when only the end-labelled primer was added (Fig. 7). This additional fragment is presumably a result of a template-switch to the nascent strand and accounts for 5–12.5% of the extension products (as determined using a PhosphorImager). These results suggest that template-switching during primer extension may be a general phenomenon of the PCR.

DISCUSSION

For several decades, template-switching has been postulated as a mechanism for various recombinational events. We present evidence that indicates this mechanism is also involved in the generation of recombinants during *in vitro* DNA synthesis by *Taq* DNA polymerase I. However, to our knowledge, this is the first demonstration that a template-switch can occur between two nascent extending strands. These results suggest that many of the recombinants produced during the PCR probably arise via a template-switching process.

These experiments were conducted using relatively high template concentrations (6.5 nM), simulating those commonly found in the last few cycles of the PCR. Under these conditions, repeated experiments using various templates indicated that 1–17% of the extension products involve template-switches between the nascent strands, while switches to the pre-existing partially homologous template are more frequent by a factor of 5–14. These two observations suggest that under the relatively high template concentrations commonly present in the last few cycles of the PCR, switches may be quite common, consistently accounting for >5% of the products generated during each cycle.

The proportion of recombinant molecules to full-length extension products was reduced ~4-fold when the complementary

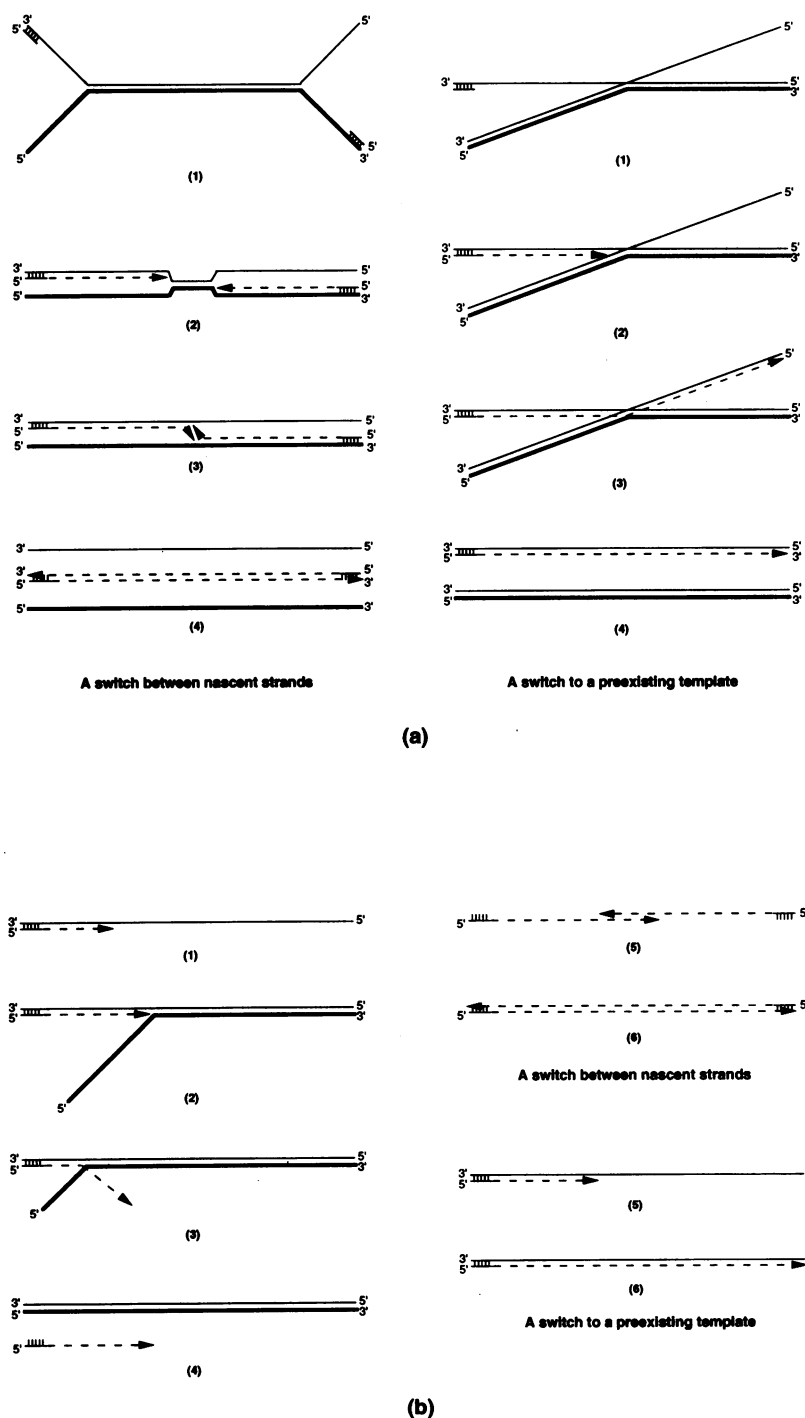


Figure 8. Two models for the template-switching process. (a) Partial displacement of the nascent strands. A switch between nascent strands. (1) Partial hybridization occurs between the complementary template strands and the primers anneal to their respective targets. (2) Strand-displacement synthesis (or, more likely, nick-translation) begins. For illustrative purposes, we only show strand-displacement synthesis. (3) As the two extending strands meet, a template-switch occurs. Note: This diagram depicts a reciprocal switch; however, we have no evidence that suggests the template-switch must be a reciprocal event. The switch may involve just one of the two extending strands. (4) Continued strand-displacement synthesis/nick-translation generates the recombinant molecule. A switch to a pre-existing template. (1) A single template strand partially hybridizes to two different complementary template strands. The primer anneals to its target sequence. (2) Primer extension begins. (3) When the extending nascent strand encounters the branch it switches from the original template to a secondary homologous template and DNA synthesis continues. (4) Branch migration could resolve the branched structure. Note: partial displacement of the nascent extension product by the strand that is complementary to the target template could facilitate both types of template-switches. (b) Complete displacement of the nascent strand(s) (1) A primer anneals to its target sequence and DNA synthesis begins. (2) Before DNA synthesis is complete, a complementary strand begins to reanneal to the template strand. (3) Continued annealing begins to displace the nascent extending strand. (4) Complete annealing of the two complementary strands completely displaces the nascent strand. A switch between nascent strands. (5) Two completely displaced nascent strands anneal to each other in their complementary region. (6) Primer extension generates the recombinant molecule. A switch to the pre-existing template. (5) A completely displaced nascent strand anneals to the secondary template. (6) Continued DNA synthesis generates the recombinant molecule.

template strands were physically separated from each other by attachment to different sets of streptavidin magnetic beads. This result suggests that efficient template-switching involves a process in which interaction between the complementary template strands plays an important role.

There are at least two possible template-switching models that are consistent with this result (Fig. 8). Both models require that the two complementary templates begin to reanneal. In the first model (Fig. 8a), the two template strands only partially reanneal, allowing the secondary template to be in proximity to the extending nascent strands and thereby facilitating the template switch. Partial displacement of the nascent extension product by the strand that is complementary to the target template could assist in the template-switching process. In the second model (Fig. 8b), reannealing of the complementary templates completely displaces the nascent truncated extension products and allows them to reanneal to a secondary template. We have evidence demonstrating that template-switches to the complementary nascent strand occur even in the absence of template sequences that lie upstream of the 5'-primer (data not shown). Under these conditions, the nascent strand can only be displaced by template DNA in a 3'→5' direction as illustrated in Fig 8b. *Taq* DNA polymerase has been shown to have a 5'→3' exonuclease activity that could 'nick-translate' through these structures rather than be displaced by them (36). However, *Taq* DNA polymerase has an average processivity of 50–60 nt at 70°C (37), and the approximately 1:1 molar ratio of template–primer/polymerase used in our studies would virtually ensure that some extension products would be polymerase-free at any given moment. These polymerase-free extension products would be susceptible to either partial or complete displacement by the complementary strand of the target template. Both of the models shown in Figure 8 would be dependent upon template concentration and would be expected to occur at a higher rate during the last few cycles of the PCR. The two models are not mutually exclusive; both could be playing important roles in the generation of the observed recombinants.

Lastly, it should be noted that this study, as well as others (1–5), demonstrates that caution should be exercised when interpreting results where PCR amplification and cloning are used to identify either previous recombinational events or the phase of two closely linked polymorphisms. Given the appropriate circumstances, the recombinational event or apparent phase of the polymorphisms could be a product of the polymerase chain reaction that does not accurately reflect the original structure of the target DNA. Our results indicate that these recombinational events can occur during a single round of primer extension and do not require multiple PCR cycles.

ACKNOWLEDGEMENTS

We appreciate the technical assistance of Diane Dunn, Margaret Robertson and Paige Bradley and wish to thank Ed Meenen for synthesizing the oligodeoxynucleotides used in this study. We are grateful to Tim Formosa and Aron Branscomb for many helpful

suggestions and to Ruth Foltz for editing the manuscript. This work was supported by a grant to RW (R01HG00367) from the National Institutes of Health.

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