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Evolution

Strand displacement amplification as an *in vitro* model for rolling-circle replication: Deletion formation and evolution during serial transfer

(error rate/fluorescent detection/quasi-species/single-stranded phage/thiazole orange)

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ABSTRACT **Strand displacement amplification is an isothermal DNA amplification reaction based on a restriction endonuclease nicking its recognition site and a polymerase extending the nick at its 3′ end, displacing the downstream strand. The reaction resembles rolling-circle replication of single-stranded phages and small plasmids. The displaced sense strand serves as target for an antisense reaction and vice versa, resulting in exponential growth and the autocatalytic nature of this *in vitro* reaction as long as the template is the limiting agent. We describe the optimization of strand displacement amplification for *in vitro* evolution experiments under serial transfer conditions. The reaction was followed and controlled by use of the fluorescent dye thiazole orange binding to the amplified DNA. We were able to maintain exponential growth conditions with a doubling time of 3.0 min throughout 100 transfers or ≈350 molecular generations by using an automatic handling device. Homology of *in vitro* amplification with rolling-circle replication was mirrored by the occurring evolutionary processes. Deletion events most likely caused by a slipped mispairing mechanism as postulated for *in vivo* replication took place. Under our conditions, the mutation rate was high and a molecular quasi-species formed with a mutant lacking internal hairpin formation ability and thus outgrowing all other species under dGTP/dCTP deficiency.**

Molecular *in vitro* evolution has classically been observed by using the RNA-dependent RNA polymerase of Qβ phage (1–5). This enzyme is able to isothermally replicate RNA molecules of different lengths and certain structural constraints for a large number of generations when applying the method of serial transfer (6–8). Under these conditions, jumpwise changes of sequence length of the evolving RNA species have been observed.

Recently, an additional isothermal replication system for DNA sequences has been described, the strand displacement amplification (SDA) reaction (9, 10). It shows exponential enrichment of template sequences, which can easily be defined by flanking primer binding sites. Until now, SDA has been used to detect genomic DNA of mycobacteria either isotopically or with chemiluminescence (11, 12).

Here we describe the adaption of SDA for *in vitro* evolution experiments under serial transfer conditions (1). The reaction is followed and can be controlled by the use of fluorescent dyes binding to the amplified DNA. Similarity of *in vitro* replication to rolling-circle replication (RCR) of single-stranded phages is mirrored by the occurring evolutionary processes. Deletion events similar to those postulated for RCR take place. By analysis of large numbers of individual sequences, the evolutionary changes are monitored in detail.

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MATERIALS AND METHODS

Materials. Exonuclease-deficient large fragment of *Escherichia coli* DNA polymerase I (exo$^-$ Klenow polymerase) (13) at a concentration of 5–10 units/μl was purchased from United States Biochemical. *Hin*cII at a concentration of 50–75 units/μl was obtained from New England Biolabs, and *Taq* DNA polymerase was from Perkin–Elmer. 2′-Deoxyadenosine 5′-[α-thio]triphosphate (dATP[αS]; diastereomer mixture) and all other nucleotides were purchased from Pharmacia. Ethidium bromide (EtdBr) was from Serva and thiazole orange (TO) was from Molecular Probes. All oligodeoxynucleotides were synthesized by standard phosphoramidite chemistry on a gene assembler (Pharmacia) and were purified by either HPLC or denaturing polyacrylamide gel electrophoresis. Aerosol-resistant tips (Biozym, Hameln, Germany) were routinely used to avoid contamination of SDA reaction mixtures with previously amplified products.

Optimized SDA Reaction for Fluorescent On-Line Detection. SDA was usually performed starting from the 59-mer oligodeoxynucleotide TRIIS1 as template: 5′-d(ACTCGACCT-GAAAAGAAAAGGGGGGGACGTTATCCACCATACG-GATAGGGGATCTCAGT-3′). SDA buffer contained 50 mM K_iPO_4 (pH 7.4), 3 mM $MgCl_2$, 4 mM dithiothreitol, 0.01% Triton X-100, and 3% (vol/vol) 1-methyl-2-pyrrolidinone. Standard conditions were 0.25 mM each dGTP, dCTP, dTTP, dATP[αS] and 0.5 μM each primers SDARSAP1 [5′-d(TT-GAATAGTCGGTTACTTgttgacACTCGACCTGAAA)-3′] and SDARSAP3 [5′-d(GCATTATGAATCCTGTCTgttga-cACTGAGATCCCCT)-3′] (the *Hin*cII recognition sites are in lowercase letters), either 2 μM TO or 3 μM EtdBr, 56 μg of bovine serum albumin (BSA) per ml, 1.67 units of *Hin*cII per μl, and 0.056 unit of exo$^-$ Klenow polymerase per μl. Addition of the fluorescent dyes TO (λ_{Ex} = 488 nm, λ_{Em} = 530 nm) and EtdBr (λ_{Ex} = 514 nm, λ_{Em} = 600 nm), which bind to double-stranded nucleic acids under increase of fluorescence quantum yield, allows following the amplification reaction on-line. After addition of typically 2 × 10^{10} template strands in a final vol of 60 μl and before addition of any proteins, the reaction sample was incubated for 2 min at 95°C, followed by 1 min at 37°C (or 39°C). Upon addition of BSA and enzymes, the amplification mixture was incubated 7 h at 37°C (or 39°C) in a thermostated fluorometer cuvette. The increase in fluorescence was monitored by using a Perkin–Elmer LS5B fluorometer. The reaction was stopped by heating at 95°C for 2 min and the mixture was stored at −20°C.

Serial Transfer of SDA Reaction Products. In a first serial transfer, 1.25 μl of a 1:10 dilution of one of the fluorometer reaction samples described above after 7 h of incubation at 39°C was added to a final vol of 25 μl of amplification mixture

Abbreviations: SDA, strand displacement amplification; RCR, rolling-circle replication; dATP[αS], 2′-deoxyadenosine 5′-[α-thio]triphosphate; TO, thiazole orange; EtdBr, ethidium bromide.
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(standard conditions with TO, excluding template), incubated 2 min at 95°C, and then 1 min at 30°C. BSA and enzymes were added and the sample was incubated at 30°C (transfer 0). Increase of TO fluorescence signal was followed by a fiber optic fluorometer device featuring argon laser excitation (488 nm) and photodiode emission detection. After reaching a threshold of twice the starting fluorescence intensity, 2 μl of the reaction sample was transferred to a sample carrier with 22 μl of fresh amplification mixture without template. This and the following reaction samples were incubated at 30°C as before without preceding denaturation, and further serial transfers were performed as described above. Reactions were stopped by shock freezing at −70°C. The concentrations of dTTP and dATP[αS] were held constant at 0.25 mM each, while those of dGTP and dCTP were subsequently lowered from 100% to 50% (beginning in transfer 23), 20% (transfer 33), 4% (transfer 39), and 1% (transfer 50) of dTTP and dATP[αS] concentrations. Sample handling, incubation, serial transfer, and on-line monitoring of fluorescent signal were performed with an automatic machine device described elsewhere (7, 14, 15).

Analysis of Reaction Products by PCR Amplification and Cloning. After termination, each 1 μl of selected transfer solution was carried over into a final 100-μl vol of standard PCR mixture (Perkin–Elmer) containing 0.5 μM each primers SDAPCRP1 [5′-d(TTGAATAGTCGGTTACTTaagcttACTCGACCTGAAA)-3′] and SDAPCRP3 [5′-d(GCATTATGAATCCTGTCTggattcACTGAGATCCCCT)-3′] (with *Hin*dIII and *Bam*HI restriction sites in lowercase letters, respectively). Thermocycling was performed in a Biometra (Göttingen, Germany) TRIO-Thermoblock cycler for a total of 19 cycles with a temperature program of 94°C for 30 s, 30°C (in the first 2 cycles) or 68°C (in the following 17 cycles) for 30 s, and 72°C for 30 s or 7 min (in the final cycle). The products were purified by nondenaturing polyacrylamide gel electrophoresis, digested with *Hin*dIII and *Bam*HI, and cloned into pUC18 vector.

Sequencing. Double-stranded plasmid DNA from single clones was prepared by the MAGIC miniprep protocol (Promega) according to the manufacturer's instructions. The DNA was directly sequenced by the dideoxynucleotide chain-termination method using a 24-mer reverse sequencing primer and the *Taq* cycle sequencing protocol with dye terminators (Applied Biosystems) according to the instructions of the supplier (but with 40 standard thermal cycles) on a model 373A DNA sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

SDA Reaction and Fluorescent Detection. Walker and coworkers (9, 10) developed the SDA reaction as an isothermal *in vitro* DNA amplification method for detection of genomic DNA of pathogens like mycobacteria. It is based on the ability of a restriction enzyme to nick the unmodified strand of a hemiphosphorothioate form of its recognition site. A DNA polymerase subsequently initiates replication at the nick, displacing the downstream nontemplate strand, and inserting 1-thiotriphosphate nucleosides into the newly synthesized strand, thereby creating further hemiphosphorothioate recognition sites. The reaction resembles the mechanism of RCR (Fig. 1).

To compare our results with those previously described, we started using the same template (47-bp fragment positions 977–1023 of insertion element IS*6110*) and primers as in ref. 9 with buffer conditions (including enzyme concentrations) as described (10). EtdBr (3 μM) was added to the buffer and the reaction was followed on-line at 37°C in a fluorometer. Fig. 2*a* shows the increase in EtdBr fluorescence during reaction due to increasing concentrations of double-stranded nucleic acids: first a lag time can be observed until the fluorescence

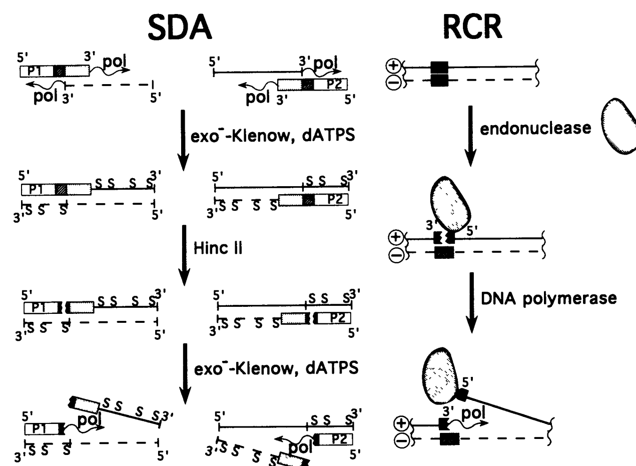

FIG. 1. Comparison of SDA and RCR mechanisms. SDA: Primers P1 and P2 both contain a 5′ overhang (open bars), a *Hin*cII restriction enzyme recognition site (hatched bars), and a 3′ annealing site (shaded bars). After annealing to sense (solid line) and antisense (dashed line) strands of the target, all priming 3′ ends are elongated by exo⁻ Klenow polymerase (pol). The enzyme inserts dATP[αS] (S in the strands), creating a hemiphosphorothioate *Hin*cII site. This restriction site is nicked by the endonuclease. Exo⁻ Klenow polymerase finally extends the nick, displacing the downstream nontemplate strand. With two primers used, both strands of a double-stranded DNA target are copied and amplified exponentially. RCR: A specific endonuclease recognizes a region of twofold symmetry (hatched bars) on the double-stranded circular genome or plasmid and selectively cuts the (+)-strand. The endonuclease becomes bound to the 5′ phosphate produced. The 3′ OH binds DNA polymerase (pol) and is extended, while the downstream nontemplate strand is displaced.

detection limit for amplified nucleic acids is reached, then exponential growth of product strands is observed, finally turning to a linear growth phase. Similar reaction profiles under application of fluorescent intercalating dyes have been described for other *in vitro* amplification techniques (14, 16). Doubling time of the fluorescent signal during exponential amplification in this SDA reaction was 5.5 min, which is slightly higher than the 5.3 min observed for DNA production by Walker *et al.* (9) in the absence of EtdBr. Fig. 2*b* shows the corresponding denaturing polyacrylamide gel with samples taken at the indicated time intervals from the SDA reaction mixture. Specificity of reaction products was confirmed by sequencing.

Optimization of SDA Reaction for Fluorescent On-Line Detection. For further experiments, TRIIS1 was chosen as the template together with primers SDARSAP1 and -P3, which show few secondary structure elements. They weakly bind fluorescent ligands, resulting in a low initial fluorescence signal.

Moreover, since we were expecting to need large amounts of reaction mixture for serial transfer, we reduced the concentrations of primers, dNTPs, and those of *Hin*cII and exo⁻ Klenow polymerase without severe loss of amplification efficiency.

EtdBr was found to inhibit the reaction. Increasing concentrations of dye result in decreasing amounts of product with concentrations of >5 μM nearly terminating the reaction. In contrast to these findings, the fluorescent dye TO clearly enhanced SDA reaction up to a concentration of 2 μM, inhibiting it at higher concentrations. TO is known to form readily dissociable complexes with double-stranded DNA accompanied by large fluorescence enhancement on binding (17). The reason for its positive interference with SDA reaction is not known, but DNA binding dyes can, on the one hand, inhibit the rate of chain elongation by poly-

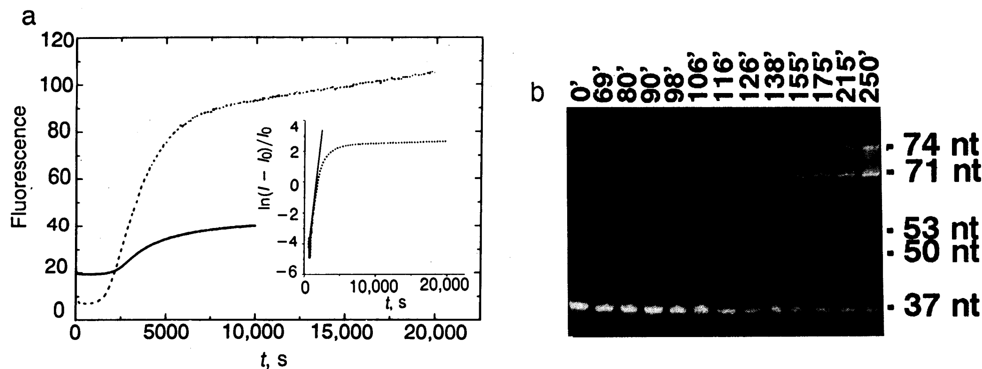

FIG. 2. On-line fluorescence monitoring of SDA reaction. (*a*) Fluorescence intensity I increases characteristically in the course of SDA. For 3 μM EtdBr with 2×10^{10} 47-mer initial template molecules and 1 μM primers (9), the overall increase in signal is quite low with high initial fluorescence (solid line) in comparison to the signal obtained under optimized conditions with 2 μM TO as dye, 2×10^{10} 59-mer template molecules TRIIS1, and 0.5 μM primers as described (dashed line). (*Inset*) Semilogarithmic plot of I versus time for the TO curve, giving a linear regression line (solid line) from which a doubling time of 2.9 min during exponential growth could be calculated. (*b*) Reaction samples of 4 μl each were taken from an EtdBr on-line monitored SDA reaction mixture (similar to the solid line plot in *a*, but with 2 μM primers) at the indicated times (min) after enzyme addition. The reaction was stopped by addition of formamide-containing loading buffer and heating to 95°C. The samples were loaded on a 16% 8.3 M urea denaturing polyacrylamide gel, electrophoresis was performed at 15 V/cm for 1 h, and the gel was stained with EtdBr. In the course of the reaction, the primers of 37 nt are consumed, while the concentrations of the longer products (50 and 53 nt, single strands with cut *Hin*cII site; 71 and 74 nt, single strands with uncut phosphorothioate *Hin*cII site; see ref. 9) increase, resulting in an increased on-line fluorescence signal.

merases (3) and, on the other hand, enhance the strand-specific nicking efficiency of restriction enzymes on hemiphosphorothiophosphate forms of their recognition sites (18). Both reactions are essential for SDA (see Fig. 1), possibly leading to a dye concentration dependence with an optimum.

Finally, different buffer conditions were tested to get high specific product yields and a long exponential growth phase. Triton X-100 and dithiothreitol were found to increase product yields (data not shown), most probably because they stabilize the SDA enzymes. The Mg^{2+} concentration was adjusted to compensate the lowered dNTP concentrations.

Under these optimized conditions (see *Materials and Methods*), the proceeding amplification was followed at 39°C by fluorescent detection. Fig. 2*a* shows that a fast exponential growth phase was typically observed with an average doubling time of 2.9 min and, subsequently, a long-lasting linear amplification.

Serial Transfer of SDA Reaction Products. Serial transfer was performed under optimized reaction conditions for fluorescent detection with TO. Observing the fluorescent signal enabled us to keep amplification in the exponential phase by transferring reaction products to a fresh reaction mixture before reaching the linear phase. Doubling times of fluorescent signals were calculated by linear regression of semilogarithmic signal-versus-time plots and gave an average of 3.0 min for the first 14 transfers (Fig. 3*a*). The concentrations of dGTP and dCTP were subsequently lowered, resulting in an increase in average doubling times.

By transfer of reaction products of the previous mixture to 11 times the volume of fresh mixture, a serial dilution of 1:12 was performed by each serial transfer. Assuming pure exponential amplification of target during each incubation and a constant product concentration at the end of each, this equals 3.5 doublings or generations of target strands between two transfers. The experiment was performed up to transfer 100, which corresponds to ≈350 generations.

***In Vitro* Evolution.** The large number of generations accompanying controlled serial transfer conditions forms the basis for observation of *in vitro* evolution with the polymerase introducing mutations into the amplified target (1, 3, 6). The mutants can then be copied more or less efficiently depending on their reproduction rates under the actual buffer conditions. Lowering the dGTP/dCTP concentrations in this sense imposes a selection pressure.

Until now, not much is known about the fidelity of exo$^-$ Klenow polymerase under SDA reaction conditions (9). The

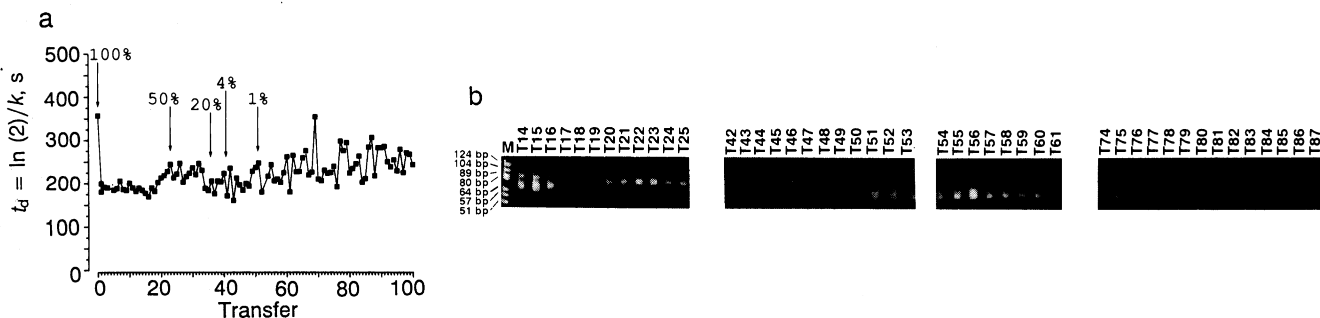

FIG. 3. Serial transfer of SDA reaction products. (*a*) Plot of doubling time t_d (calculated from the time constant k) versus transfer number illustrates the ongoing experiment under exponential growth conditions. Since transfer 0 was initiated with a differently handled template, it exhibits a higher doubling time. Beginning from the indicated transfers (arrows), the dGTP and dCTP concentrations in the fresh reaction mixtures were subsequently lowered in relation to those of TTP and dATP[αS] (both held constant at 0.25 mM). (*b*) Various stopped transfer reaction mixtures (5 μl) were electrophoresed under native conditions on an 11% polyacrylamide gel at 15 V/cm for 1 h and stained with EtdBr. The double-stranded products jumpwise decrease in their molecular weights (compared to a marker in lane M) with intermediate lanes showing two or more bands. While the initial template TRIIS1 results in an 86-bp full-length SDA product, the finally observed 31 deletions in the target core (see Fig. 4) cause a 55-bp product (see ref. 9).

enzyme is a DNA polymerase with one of the highest error rates characterized so far since it is depleted of all exonucleolytic proofreading activities (13) {dATP[αS] as α-thio-substituted nucleotide triphosphate would most likely inhibit any 3′–5′ exonuclease proofreading activity (19)}. Under standard conditions, it exhibits an error rate of $\approx 10^{-4}$, which is 4- to 7-fold less accurate than Klenow polymerase (exo$^-$ Klenow polymerase is identical with the double-mutant D355A/E357A of Klenow fragment; see ref. 20). Moreover, DNA binding dyes like TO can decrease the fidelity of polymerases by interference with the template, although error rates have not been determined yet (3). Finally, error-prone replication results in mutants that are themselves copied with limited fidelity resulting in error accumulation and formation of a quasi-species distribution of sequences (7, 21). The developing quasi-species is the target for appropriate selection pressures leading to *in vitro* evolution.

Analysis of serial transferred SDA reaction products by native polyacrylamide gel electrophoresis with EtdBr staining showed clearly visible, distinct bands (Fig. 3*b*). The molecular weight of product bands decreased step by step during continual serial transfer, a fact that is also known from the Qβ system under serial transfer conditions (1) and that can be explained by subsequent deletion events in the target core.

To directly monitor changes in the amplified products, a preparative PCR was performed to transform them into easily clonable unmodified double strands with *Bam*HI and *Hin*dIII sites. The fidelity of this procedure with low chosen PCR cycle number should be high enough to determine errors due only to SDA reaction (22). The results of sequence analysis of a number of clones from different transfers are shown in Fig. 4: Of six sequenced clones of transfer 0 only three are zero-error, one is a one-error, and two are two-error mutants (including point deletions). Thus, the error frequency is already quite high with five mutations in 198 analyzed nt (nearly 2.5% detected errors; three transversions, one transition, one point deletion).

Point mutations are also found in later transfers, but soon large deletions occur and become predominant. In clone T14/1 a certain G to T base substitution (in position 13) is found, which occurs later on more often, accompanied by large deletions. Eight of a total of 77 independent sequences show this mutation, 6 of which also show the same deletion pattern. All other base substitutions (eight altogether) are found only in single clones.

Point deletions are found nearly exclusively in clusters of at least 2 identical bases (deletion of the 3′ T also belongs to this category, since the primer binding site here continues with TTT . . .; the only exception to this rule is the deleted 5′ T of sequence T32/4). Twice (clones T27/2 and T32/2) an insertion also occurs in the C_7 cluster, giving C_8.

As long as the dGTP/dCTP content is 20% or more of TTP/dATP[αS] (up to transfer 38; see Fig. 3*a*), the C_7 cluster of the target remains mainly intact. After lowering dGTP/dCTP concentrations further, the cluster is rapidly lost within a couple of serial transfers, indicating that unbalanced dNTP concentrations in fact act as selection pressure. This pressure lets the sequence exemplified in clone T37/2 outgrow all competitors after a small number of transfers. The system seems to show real evolutionary behavior.

Sequence	Clone
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	**consensus**
ATCCGTATGGTGGATAACGTCCCCCCCTTTTC***G***	T0/1
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T0/2
ATCCGTATGGTGGATAA***A***GT-CCCCCCTTTTCT	T0/3
ATC***A***GTATGGTGGATAACGTCCCCCCC***C***TTTCT	T0/4
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T0/5
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T0/6
ATCCGTATGGTGGATAACGTCCCC***A***CCTTTTCT	T1-3/1
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T1-3/2
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T1-3/4
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T1-3/5
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T5-7/1
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T5-7/2
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T5-7/3
ATCCGTATGGTGGATAACGTC-------------	T5-7/4
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T5-7/5
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T8-10/1
AT-----------------------CCTTTTCT	T8-10/2
ATCCGTATG***T***TGGATAACGTCCCCCCC-TT***C***CT	T8-10/3
ATCCG-ATGGTGGATAACGTCCCCCCCTTTTCT	T8-10/4
ATCCGTATGGTGGATAACGTCCCCCCCTTTTCT	T8-10/5
ATCCGTATGGTG***T***ATAACGTCCCCCCCTTTTC-	T14/1
-----TATGGTGGATAACGTCCCCCCCTTTTCT	T14/3
AT---------------------CCCCTTTTCT	T14/5
AT-------------------CCCCCCTTTTCT	T14/6
AT-----------------TCCCCCCCTTTTCT	T14/7
ATCCGTATGGT***T***GATAACGTCCCCCCCTTTTC-	T14/8
AT---------------------CCCCTTTTCT	T18/1
AT------------------CCCCCCCTTTTCT	T18/2
AT------------------CCCCCCCTTTTCT	T18/3
AT-----------------TCCCCCCC------	T22A/1
---------------------CCCCCCTTTTCT	T22A/2
AT------------------------CTTTTCT	T22A/4
AT-------------------CCCCCCTTTTCT	T22A/5
ATCCGTATGGTG***T***ATAACGTCCCCCC-------	T22B/2
ATC----------------TCCCCCCCTTTTC-	T27/1
ATC----------------TCCCCCCC**C**TTTTC***G***	T27/2
AT-------------------CCCCCCTTTTCT	T27/3
ATC---------------GTCCCCCC-TTTTCT	T27/4
AT------------------CCCCCCCTTTTCT	T27/5
AT-----------------------CCTT--CT	T32/1
AT------------------CCCCCCC**C**TTTTCT	T32/2
AT-----------------TCCCCCCCTTTTC-	T32/3
A-CCGT--------------CCCCCCCTTT-C-	T32/4
AT--------------------CCCCCTTTTCT	T37/1
ATC-GTATGGTG***T***ATA------------TTTC-	T37/2
ATCCGTATG------------------TTTTCT	T37/4
ATC----------------TCCCCCCCTTTTCT	T37/5
AT------------------CCCCCCCTTTTC-	T40/1
AT-------------------CCCCCCTTTTC-	T40/2
AT-------------------CCCCCCTTTTC-	T40/5
ATC-GTATGGTG***T***ATA------------TTTC-	T43/1
AT--------------------CCCCCTTTTC-	T43/2
ATC-GTATGGTG***T***ATA------------TTTC-	T43/3
A--------------------------------	T43/4
ATCCGTATG------------------TTTTCT	T43/5
ATC-GTATGGTG***T***ATA------------TTTC-	T47/1
ATC-GTATGGTG***T***ATA------------TTTC-	T47/2
A--------------------------------	T47/3
A--------------------------------	T47/4
ATC-GTATGGTG***T***ATA------------TTTC-	T47/5
A--------------------------------	T52/1
----------------------------TTTC-	T52/2
A--------------------------------	T52/3
-------------------------------CT	T52/4
-------------------------------CT	T52/5
A--------------------------------	T56/2
A--------------------------------	T56/4
A--------------------------------	T56/5
AT-------------------------------	T76/1
AT----A--------------------------	T76/2
AT-------------------------------	T76/3
AT-------------------------------	T76/4
AT-------------------------------	T100/1
AT-------------------------------	T100/2
-------------------------------CT	T100/3
AT-------------------------------	T100/4
AT-------------------------------	T100/5

FIG. 4. Individual sequences cloned from various SDA transfer mixtures. Transfers 1–3, 5–7, and 8–10 were combined before analysis. The sequences are aligned in the 5′–3′ direction to the starting or consensus sequence of the complementary strand of target molecule TRIIS1 (excluding constant primer binding sites). Deletions are marked by hyphens, point mutations are underlined and boldface, and insertions are large and boldface.

Similarity to RCR. The SDA reaction can be regarded as an *in vitro* analogue of RCR (Fig. 1). Filamentous single-stranded DNA phages and small (<10 kb) plasmids from Gram-positive bacteria generate their single-stranded DNA intermediates by RCR (23, 24). For both species, deletions are known to form during replication (25, 26), imposing severe constraints on recombinant DNA technology (27). These deletions are typically induced by the formation of a hairpin structure in the single-stranded template through annealing of adjacent inverted repeats. The hairpin arrests DNA synthesis since it has to be melted by the polymerase. Short direct repeats (≥3 nt) flanking the stem can now cause deletion formation by slipped mispairing (28–30).

The sequence element GGTGGATAACGTCCCC of the complementary strand of starting SDA template TRIIS1 (Fig. 4) can form such a hairpin with a 6-bp G+C-rich stem. It is the most stable stem–loop structure of the DNA core of TRIIS1 (calculated with the Genetics Computer Group program package; see ref. 31) and is flanked by a short direct

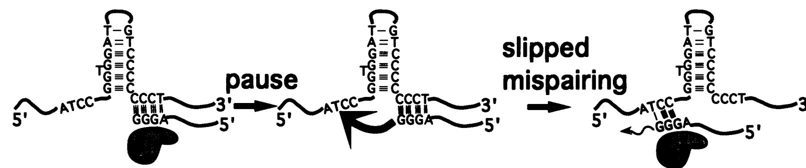

FIG. 5. Suggested deletion formation mechanism. Polymerase (shaded area) elongates the primer up to a hairpin with strong G·C base pairs. Upon reaching the stem, it pauses and eventually leaves the template. The nascent DNA strand can now slip from one site of the template to another homologous one. Finally, the polymerase continues elongation, thereby deleting the hairpin structure in the copied strand.

repeat sequence, TCC or CCC (both base pairing with the nascent 3′ GGG of the elongated complementary strand). These elements fulfill the requirements for deletion formation. DNA synthesis is arrested near the base of the hairpin structure with the nascent strand slipping from one direct repeat to the next, deleting the hairpin (Fig. 5). This deletion formation mechanism can act on both strands of the double-stranded SDA target, which share a homologous secondary structure. Depending on the exact pause site, the resulting copied strand will show one of the sequences with . . . AT-- . . . --CCC . . . in Fig. 4. Further deletions can be formed by slippage of the nascent strand within a cluster of at least 2 identical nt. Exponential growth conditions impose an internal selection pressure, which promotes all kinds of deletions since shorter targets are generally copied more rapidly as long as the deletion is not spreading into one of the primer binding sites.

Thus, a reason for the sequence exemplified in clone T37/2 being the only one after transfer 30 to carry at least part of the described hairpin sequence becomes plausible. It probably survived in earlier stages of the *in vitro* evolution process because of its G to T base transversion GGTG*T*AT, resulting in a strong destabilization of the stem, so the hairpin could not form and deletion did not take place. Since this sequence also lacks the C_7 cluster, it could outgrow all competitors with C_7 clusters after lowering the concentrations of dGTP and dCTP to 10 μM each in transfer 39. Finally, with a dGTP and dCTP concentration of 2.5 μM remaining after transfer 49, only extremely shortened SDA products can be found in the sequence analysis (Fig. 4).

In conclusion, our data suggest that *in vitro* evolution can indeed take place in exponential enrichment techniques of nucleic acids other than the Qβ system. SDA of DNA based on exo$^-$ Klenow polymerase in particular yields predominantly deletion mutants dependent on internal structural constraints and external selection pressures like unbalanced dNTP concentrations. We propose that SDA under serial transfer conditions may serve as an *in vitro* model for RCR—e.g., to test different templates on their ability to form deletions after a number of subsequent copying events or generations. This may be of interest for recombinant DNA technology in single-stranded phages and plasmids. Although fluorescent dyes added to SDA reaction mixtures considerably facilitate on-line monitoring of the ongoing experiment and most probably also influence mutation rates during polymerization, they can be omitted. Other polymerases with different processivities and fidelities could be used to model *in vivo* conditions in more detail.

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