Modelling Viral Evolution in Vitro Using exo− Klenow Polymerase: Continuous Selection of Strand Displacement Amplified DNA that Binds an Oligodeoxynucleotide to Form a Triple-helix

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Evolution comprises cycles of amplification, mutagenesis and selection. To study evolutionary phenomena, isothermal strand displacement amplification (SDA) of double-stranded DNA as an in vitro model for rolling-circle replication of viruses has been coupled to a positive selection procedure. First, two subsequent amplification reactions utilizing exo− Klenow polymerase were performed under direct observation using the fluorescent dye thiazole orange. Under the chosen conditions, the mutation rate was $1.5 \times 10^{-3}$ and $0.4 \times 10^{-3}$ for base substitutions and deletions, respectively. Then, a 16mer oligodeoxynucleotide with an acridine moiety coupled to its 5' end was used to select for double strands that retained their ability to form a triple-helix with the oligodeoxynucleotide. Conditions for triple-helix formation were chosen such that only 10 to 40% of the SDA products were allowed to bind the third strand. Non-denaturing polyacrylamide gel electrophoresis was used to separate triple-helices from unmodified double strands, and only triplex strands were used to initiate a new round of error-prone amplification and selection. Nine such rounds with about 270 molecular generations were performed. The final mutant spectrum was characterized and compared with those of amplification reactions without additional selection pressure. While without selection pressure base substitutions and deletions throughout the initial wild-type rapidly produce a diverse mutant distribution, the consensus after nine selection rounds clearly shows two mutational hotspot positions. Using gel shift assays and a newly developed non-radioactive DNase I footprinting technique, it could be shown that both the initial wild-type and the final consensus do not differ significantly in their triplex formation ability. As opposed to this, they do show different amplification efficiencies. The final consensus sequence is amplified with the highest rate in the exponential reaction phase, while the most abundant clone, which is characterized by two additional point deletions, is the sequence with the highest amplification rate in the linear growth phase.

Keywords: DNA virus; quasispecies; rolling-circle replication; selection value; triple-helix

Introduction

Strand displacement amplification (SDA) is a novel isothermal amplification technique for ds-DNA that resembles rolling-circle replication (Walker et al., 1992; Walter & Strunk, 1994). It comprises cycles of primer annealing, elongation, nicking and strand displacement (Figure 1). As in rolling-circle replication of, for example, single-stranded DNA viruses and small (<10 kb) bacterial plasmids (Gilbert & Dressler, 1968), the crucial
reaction steps of SDA are cleavage of only one strand of a DNA double strand and displacement of the downstream copy during elongation of the obtained 3’ end. By using two primers complementary to both 3’ ends of the double-stranded target molecule, the in vitro amplification reaction defines the sequence in between to be enriched exponentially under consumption of the primers. After the concentration of target has reached levels in the range of the primer concentrations, the reaction turns into a linear enrichment phase, where elongation and displacement of strands dominates over primer annealing (Walker et al., 1992; Walter & Strunk, 1994).

In contrast to the polymerase chain reaction, which requires temperature cycling for annealing and elongation of primers and separation of double strands (Saiki et al., 1988), all reaction steps in the isothermal SDA protocol occur simultaneously. As in other isothermal amplification techniques like the Qβ-reaction (Spiegelman, 1971) or the self-sustained sequence replication (Breaker & Joyce, 1994), this gives rise to competition among different target molecules for resources like primers and enzymes. The faster a specific sequence is copied, the higher its proportion in the final mutant distribution will be. This phenomenon has been termed in vitro evolution (Mills et al., 1967; Eigen, 1971).

In the case of SDA with exo-Klenow polymerase, prolonged exponential amplification of DNA by continuous serial transfer of products into fresh reaction mixture leads to base substitutions and deletion formation and thereby loss of sequence information in the developing quasispecies of replicated molecules (Walter & Strunk, 1994). Similar deletion events have long been known from the in vivo replication of single-stranded DNA phages and bacterial plasmids that occurs via a rolling-circle mechanism (Horiuchi, 1983; Vilette et al., 1992). Thus, SDA resembles rolling-circle replication in its reaction mechanism, and in its evolution products. This led to the question of whether SDA could also be an in vitro model for further aspects of evolutionary processes in vivo.

One question was, whether SDA could be coupled to a positive selection scheme that requires specific sequence information in the double-stranded DNA products.

A basic feature of double-stranded DNA is that specific nucleic acid sequences can be recognized by either proteins or other nucleic acids. For example, ligand binding to genomic sequences plays a major role in the regulation of gene expression. Since oligonucleotides can bind to the major groove of double-helical DNA via specific Hoogsteen or reverse Hoogsteen hydrogen bonds and are easily

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**Figure 1.** Reaction scheme of strand displacement amplification. Both primers, P1 and P2, contain 18 nt 5’ overhangs (open bars), 6 nt of *HincII* restriction enzyme recognition sites (hatched bars) and 13 nt of 3’ annealing sites (shaded bars). After annealing to sense (continuous line) and antisense (broken line) strands of the target, the priming 3’ ends are elongated by exo-Klenow polymerase (pol). The enzyme incorporates dATP αS (S in the strands), creating a hemiphosphorothioate *HincII* site, which is subsequently nicked by the endonuclease. The exo-Klenow polymerase then extends the nick, displacing the downstream non-template strand. The displaced strands can hybridize the second primer and be copied likewise, resulting in an exponential enrichment of target sequences (pathway E). Linear amplification (pathway L) occurs with the formerly nicked, now restored double strand, that again contains a hemiphosphorothioate *HincII* site and can repeatedly be nicked and copied independent of primer binding.
available by synthesis, they are ideal model ligands to study DNA recognition. The obtained triple-helices are even discussed as a basis for an “antigene” approach of artificial gene control (Cooney et al., 1988; for a recent review, see Thuong & Hélène, 1993).

In the present work, strand displacement amplification utilizing exo^- Klenow polymerase has been coupled to selection of double strands with ability to bind a 16mer 5'-acridine-modified oligodeoxynucleotide in a triple-helical form. Starting from a defined wild-type sequence, amplification was initiated, followed by incubation with the oligodeoxynucleotide and an electrophoretic mobility shift assay as the selection step for the formed triple-helices. Nine rounds of coupled amplification and selection with altogether 270 molecular generations were performed. Comparison of replicated molecule populations under selection for triple-helix formation with amplified populations without selection show different mutant distributions. The chosen system resembles the in vivo situation, where selection for a fast replication of the genome and for distinct features like specific recognition of genomic sequences by ligands are always coupled.

**Results**

**Coupled amplification and selection system**

Amplification and mutation under the use of exo^- Klenow polymerase and selection for triple-helices were coupled by alternating two subsequent SDA reactions including a serial transfer and a separate selection step for oligodeoxynucleotide-binding dsDNA products as shown in Figure 2(a). A target molecule named TRIIS1 was used to inoculate the very first amplification reaction (Table 1). The amplification reactions were followed in a fluorimeter by addition of the dye thiazole orange, that binds to the dsDNA products under increase of fluorescence intensity (Lee et al., 1986; Zhu et al., 1994; Walter & Strunk, 1994). Under the conditions chosen, both the exponential (identified in the fluorescence curve by an increasing slope after a lag-time) as well as the linear reaction phase (steep linear slope) of SDA were utilized to generate new molecule generations. Afterwards, the dsDNA products were selected for double strands binding the 16mer ACR-coupled oligodeoxynucleotide TRIPEL1 (Table 1, sequence with the three bases thymine, cytosine and guanine in analogy to Giovannangéli et al., 1992) by incubation in binding buffer and a subsequent mobility shift assay in a Mg^2+ -containing electrophoresis buffer. The initial target molecule TRIIS1 (Table 1) contains a homopurine-tract that can form such a triple-helix with TRIPEL1 via Hogsteen base-pairs in its major groove, provided the cytosine residues of TRIPEL1 are protonated (Thuong & Hélène, 1993; Figure 2(b) and (c)). To increase the stringency of selection in the course of the performed nine selection rounds, the pH of the incubation buffer was increased and concentration of the oligodeoxynucleotide was lowered as depicted in the legend to Figure 3. Under all of these conditions, the corresponding oligodeoxynucleotide without the ACR-moiety did not bind to the TRIIS1 sequence (results not shown), for a 5'-acridine strongly stabilizes the triplex (Sun et al., 1989; Stonehouse & Fox, 1994).

Figure 3(a) shows a native gel with samples as used to select for triple-helices in each selection round. As expected (Giovannangéli et al., 1992; Thuong & Hélène, 1993), the yield of triple-helix decreases with decreasing concentration of oligodeoxynucleotide TRIPEL1 (from selection round 5) and increasing pH (from selection round 6; at a pH of 7.5 or above, triple-helix-formation could no longer be detected). In every case, the triplex band with lower electrophoretic mobility than the dsDNA was precisely cut out, diffusion eluted and the derived DNA used to initiate a subsequent amplification and selection round. Thus, only about 10 to 40% of the SDA products passed this selection criterion, as judged by band intensities in the ethidium bromide-stained gel.

In each round, the complexity of the product mixture was monitored using single-strand conformational polymorphism (SSCP) analysis (Orita et al., 1989; Hayashi & Yandell, 1993), showing that a diverse mutant distribution or quasispecies (Eigen et al., 1989) with broadly distributed electrophoretic mobilities of single strands was already obtained in the first four rounds (Figure 3(b)). The same can be seen in Figure 3(a), where the dsDNA bands up to selection round 4 become increasingly diffuse. After changing the stopping procedure for SDA from heating to addition of EDTA in selection round 5, the electrophoretic smears due to heteroduplex formation between different sequences of the quasispecies disappear.

Generation numbers of strand displacement amplified molecules could be estimated under the assumption of roughly exponential growth by determination of initial and final template concentrations. In the first amplification reaction, about 13 molecule generations were passed through (amplification factor 10^5), while all further reactions covered about 16 generations each (amplification factor 6 × 10^5). This led to about 270 molecular generations after altogether 17 subsequent amplification reactions (in nine selection rounds, one of them without serial transfer).

**Sequence analysis of selected SDA products**

To obtain appropriate amounts of clonable DNA, the SDA products and the purified triplex DNA, respectively, were amplified by PCR with low cycle number. The PCR product was cloned into pUC18 vector and sequenced with a reverse sequencing primer. Figure 4 shows the sequences of SDA products after one (clones A1S0), two (A2S0) or five (A5S0) subsequent SDA reactions without selection for triplex formation and after nine selection rounds.
In Vitro Evolution of Strand Displacement Amplified DNA

Figure 2. Experimental design for coupled amplification and selection.
(a) A selection round consists of two subsequent SDA reactions under fluorescent control with the dsDNA reaction products of the first amplification being serially transferred into the second. Then, a 2-methoxy-6-chlor-9-aminoacridine (ACR)-coupled oligodeoxynucleotide is added in an appropriate buffer, incubated and the products are separated on a non-denaturing polyacrylamide gel. The obtained triplex in lane T is identified in comparison with a reference in lane R, excised, eluted and used to initiate the next selection round.
(b) The ACR-coupled oligodeoxynucleotide TRIPEL1 can bind to the major groove of a homopurine-homopyrimidine tract, formed by Watson-Crick base-pairs between starting molecule TRIIS1 and its complement cTRIIS1. Provided the cytosine residues of TRIPEL1 are protonated (+), each base of the oligodeoxynucleotide can form two Hogsteen hydrogen bonds with the GC and AT base-pairs. The ACR moiety further stabilizes the triplex by intercalation into the double strand two base-pairs upstream (arrow).
(c) Utilized base triplets $T \times A T$, $C^+ \times G C$ and $G \times G C$ of the triplex (after Thuong & Hélène, 1993).

(with 17 amplification reactions and nine triplex selections, A17S9). When the same analysis procedure was initiated with 0.38 pmol of target molecule TRIIS1 (Table 1), no mutation was found in eight sequenced clones, indicating a low error frequency of PCR amplification and cloning. Thus, the observed sequence heterogeneity of analyzed clones should be mostly due to strand displacement amplification by $exo^-$ Klenow polymerase.

After the first amplification reaction with about 13 molecule generations, among the 22 clones of sequence length 33 (excluding constant primer binding sites), eight base substitutions and two deletions (one point deletion and an eight base deletion) are found (clones A1S0, ... in Figure 4): 14 clones (64%) still have the original sequence of TRIIS1, the initial target. The error rate $r$ (defined as errors per base per replication cycle) of exo$^-$ Klenow
polymerase under the described SDA conditions can then be estimated as
\[ r \approx 2(p/(nd)) - r' \]

(Saiki et al., 1988), where \( p \) is the number of mutated positions among \( n \) sequenced bases after \( d \) cycles of replication. \( r' \) is the error rate for the background amplification process (PCR and cloning), which is dominated by the error-prone in vitro amplification by Taq DNA polymerase in PCR. \( r' \) can thus be identified as the error rate of \( \Delta r' \) determined to be \( 2 \times 10^{-4} \) (Saiki et al., 1988; Tindall & Kunkel, 1988). These values lead to an error rate of exo−Klenow polymerase during SDA of 1.5 \( \times 10^{-3} \) for base substitutions and 0.4 \( \times 10^{-3} \) for deletions, which is about one order of magnitude higher than the error rate determined under standard conditions (Bebene et al., 1990).

After two subsequent amplification reactions or about 29 molecular generations beyond initiation with TRIISI, among nine analyzed clones (A2S0... in Figure 4) only three (33%) still exhibit the correct TRIISI sequence. The other six mutants carry altogether seven base substitutions and three large deletions, indicating a broadened quasispecies. In accordance with the above calculations, the error rate of exo−Klenow polymerase estimated from these values would be 1.7 \( \times 10^{-3} \) for base substitutions and 0.4 \( \times 10^{-3} \) for deletions, respectively. After five subsequent SDA reactions or 77 molecule generations without selection for triplex formation (clones A2S0... in Figure 4), only one of ten analyzed clones (10%) is still identical with TRIISI. The other clones carry mainly large deletions, indicating that the latter become dominating in the quasispecies during prolonged amplification, since shorter templates with less information content are copied more rapidly in SDA (Walker & Strunk, 1994).

Analysis of the products after nine selection rounds (clones A17S9... in Figure 4) shows a dramatically different quasispecies of replicated molecules. Though amplification was performed here for 17 SDA reactions or about 270 molecule generations, the sequence information of replicated templates is strongly retained. Selection for triplex-formation tolerated only single base exchanges at the end of the triplex-forming region in TRIISI (underlined in the complementary strand cTRIISI in Figure 4). Ten of altogether 33 positions (1, 2, 24, and 26 to 32 in Figure 4) are completely conserved, while five positions (4, 10 and 12 to 14) are changed in ten or more of the analyzed 30 clones. The final majority consensus after nine selection rounds carries a G-to-T base transversion in position 13, a point deletion of an adenosine in position 14 (Figure 4) and is unchanged in its triplex-forming region. Thus, molecular in vitro evolution from an initial wild-type sequence to a slightly changed final consensus did occur.

**Characterization of selected sequence variants**

The final majority consensus sequence after nine selection rounds (FC, Table 1) and its complementary strand (cFC) were synthesized to compare them with the initiating template TRIISI (Table 1) and its complement cTRIISI. First, the ability of the formed double strands dsTRIIS1 and dsFC to bind the oligodeoxynucleotide TRIPEL1 used for the selection step were studied. Figure 5(a) shows that both double strands form a triplex in the presence of TRIPEL1 with about the same yield.

Second, the regions of dsTRIIS1 and dsFC binding TRIPEL1 were characterized. A novel fluorescent footprinting assay was established, with the dsDNA being labeled with tetramethylrhodamine (TMR) in a PCR using the 5' modified primer SDARHOP3 (Table 1), then incubated with TRIPEL1 and digested with DNase I. The nuclease is known to bind to the minor groove of dsDNA and to cleave the phosphodiester backbone in a random fashion in the presence of Mg²⁺. Cleavage is inhibited by the alteration of duplex structure induced by a triplex-forming oligodeoxynucleotide binding to the major groove of the DNA (Stonehouse & Fox, 1994).

The fluorescent label on the homopyrimidine-carrying

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**Table 1. Oligodeoxynucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>SDAR5FP1</td>
<td>TTAATAGCTGGTTACTTgacACTGACTGCTAGAAAA</td>
</tr>
<tr>
<td>SDAR5FP3</td>
<td>GCATTAGTTAAGCTCGTAgtgacACTGAGATCCCCCT</td>
</tr>
<tr>
<td>SDARHOP3</td>
<td>TTTATAAGCCTCGTACTAagctgACGTTAGATCCCCCT</td>
</tr>
<tr>
<td>SDAPCRP1</td>
<td>TTAATAGCTGGTTACTTgacACTGACTGCTAGAAAA</td>
</tr>
<tr>
<td>SDAPCRP3</td>
<td>GCATTAGTTAAGCTCGTACTAagctgACGTTAGATCCCCCT</td>
</tr>
<tr>
<td>TRIPEL1</td>
<td>ACR-CTTTTCCTTTGGGGGG</td>
</tr>
<tr>
<td>TSDAB1</td>
<td>CGATCAGCGACCA</td>
</tr>
<tr>
<td>TRIISI</td>
<td>ACTGACCTGATTAAAAGAAAAGGGGGGAGCTTATCCCATACCATACGATAGGGGATCTCAGT</td>
</tr>
<tr>
<td>cTRIISI</td>
<td>ACTGACCTGATTAAAAGAAAAGGGGGGAGCTTATCCCATACCATACGATAGGGGATCTCAGT</td>
</tr>
<tr>
<td>FC</td>
<td>ACTGACCTGATTAAAAGAAAAGGGGGGAGCTTATCCCATACCATACGATAGGGGATCTCAGT</td>
</tr>
<tr>
<td>cFC</td>
<td>ACTGACCTGATTAAAAGAAAAGGGGGGAGCTTATCCCATACCATACGATAGGGGATCTCAGT</td>
</tr>
<tr>
<td>MC</td>
<td>ACTGACCTGATTAAAAGAAAAGGGGGGAGCTTATCCCATACCATACGATAGGGGATCTCAGT</td>
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The sequence of TRIISI is derived from IS6110, an insertion element of *Mycobacteria* already used by Walker et al. (1992) as target for SDA, with addition of a potential triplex-forming region; underlined, potential triplex-forming region; lower-case letters, HinClI, HinDIII and BamHI recognition sites; italics and bold, in TRIISI and its complement cTRIISI the two bases are marked that are exchanged or deleted in MC; TMR and ACR are tetramethylrhodamine and acridine labels, respectively.
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Figure 3. Gel analysis of the evolution experiment. (a) Stopped SDA reaction mixtures were incubated with the triplex-forming oligodeoxynucleotide TRIPEL1 and loaded onto a nondenaturing polyacrylamide gel in 50 mM Tris-borate, 1 mM MgCl₂, as described in Materials and Methods. Lanes 1 to 9 correspond to the incubation mixtures that were used for triplex selection in rounds 1 to 9. Conditions were as in Materials and Methods with the following details: selection rounds 1 to 3, serial transfer dilution 1:60,000; selection round 4, no serial transfer; selection rounds 5 to 9, serial transfer dilution 1:15,000; selection rounds 1 to 4, SDA stopped by two minutes at 95°C; selection rounds 5 to 9, SDA stopped by addition of 10 mM EDTA; selection rounds 1 to 4, 5 µM TRIPEL1, selection rounds 5 to 9, 2 µM TRIPEL1; selection rounds 1 to 3, sodium cacodylate buffer; selection rounds 4 to 9, potassium phosphate buffer; selection rounds 1 to 4, pH 6.5; selection rounds 5 to 9, pH 7.0. Lane 10 shows the same SDA reaction mixture as in lane 9 without addition of TRIPEL1. The obtained triplex exhibits a lower electrophoretic mobility than the unmodified dsDNA. (b) A volume of 2 µl each of terminated SDA reactions A1S0 to A17S8 (named after the number of subsequent amplification reaction and triplex selection cycles they had undergone) were subjected to SSCP analysis as described by Orita et al. (1989). In principle, double strands were melted in a formamide and EDTA-containing buffer and the obtained single strands subjected to non-denaturing gel electrophoresis in a high-resolution polyacrylamide gel. Arrows indicate triplex selection. In the fourth selection round, A7S3 was used (instead of A8S3) for triplex selection. Lane P was loaded with 2 µl of A17S8 without preceding denaturation.

Third, the putative secondary structures of TRIIS1 and FC were computationally analyzed using the algorithms of Hofacker et al. (1994). Figure 5(c) shows that the G-to-T base transversion and the point deletion in FC (Table 1) strongly destabilize a stem-loop-structure of TRIIS1. The resulting less compactly folded structure is accompanied by a slightly lower electrophoretic mobility of FC and its complement cFC compared with TRIIS1 and cTRIIS1 on a native gel (Figure 5(a)). Since secondary structure of single strands plays an important role in strand displacement amplification (Walter & Strunk, 1994), it seemed likely that the amplification efficiencies of the different targets might differ. This difference could then account for the selection of the consensus sequence FC in the course of the evolution experiment.

To test this hypothesis, the standard SDA reaction mixture was initiated with either 1 nM template TRIIS1 or FC and the amplification kinetics were followed by fluorescence detection. The difference between both samples was quite significant, with TRIIS1 being characterized by a faster increase in fluorescence intensity (Figure 6(a)). When 1 nM TRIIS1 and 1 nM FC were used to initiate the same SDA sample, always TRIIS1 grew out of the mixture (results not shown). To exclude differences due to unequal concentrations of template stock solutions, concentrations were controlled independently by absorbance measurement at 260 nm and gel electrophoresis followed by ethidium bromide staining (Sambrook et al., 1989). Moreover, the number of temperature cycles necessary to obtain equal amplification factors for TRIIS1 and FC in a PCR reaction were determined and found to be identical, proving that both templates were comparably free of lesions. The differences in strand displacement amplification could therefore be explained only by different replication efficiencies.
Evolution consists of repeated cycles of amplification, mutation (often coupled with amplification) and selection of advantageous mutants. Evolution has classically been observed in vitro with RNA using the replicase of the Escherichia coli RNA phage Qβ (Mills et al., 1967; Spiegelman, 1971; Kramer et al., 1974; Biebricher et al., 1985). This RNA polymerase is able to copy both the plus and minus strand of its template in a cell-free environment with a high error frequency of about $3 \times 10^{-4}$ per base per replication cycle (Eigen, 1993). By applying the method of serial transfer, replication can be prolonged indefinitely (Mills et al., 1967). Error accumulation results in the formation of a broad quasispecies of nearly neutral mutants that is able to adapt to a variety of different selection pressures (Spiegelman, 1971; Eigen, 1993; Rohde et al., 1995). This experience of evolutionary mechanisms at the molecular level has highlighted our understanding of RNA virus evolution; e.g. quasispecies distributions have been shown to make possible the fast adaption of RNA virus populations to the immune response of their host (Domingo et al., 1985; Eigen, 1993).

Here, an in vitro evolution experiment on double-stranded DNA with selection for triplex formation ability has been performed. Amplification and mutagenesis were done by isothermal strand displacement amplification (SDA; Walker et al., 1992) starting from a defined sequence.

**Discussion**

Evolution consists of repeated cycles of amplification, mutation (often coupled with amplification) and selection of advantageous mutants. Evolution has classically been observed in vitro with RNA using the replicase of the Escherichia coli RNA phage Qβ (Mills et al., 1967; Spiegelman, 1971; Kramer et al., 1974; Biebricher et al., 1985). This RNA polymerase is able to copy both the plus and minus strand of its template in a cell-free environment with a high error frequency of about $3 \times 10^{-4}$ per base per replication cycle (Eigen, 1993). By applying the method of serial transfer, replication can be prolonged indefinitely (Mills et al., 1967). Error accumulation results in the formation of a broad quasispecies of nearly neutral mutants that is able to adapt to a variety of different selection pressures (Spiegelman, 1971; Eigen, 1993; Rohde et al., 1995). This experience of evolutionary mechanisms at the molecular level has highlighted our understanding of RNA virus evolution; e.g. quasispecies distributions have been shown to make possible the fast adaption of RNA virus populations to the immune response of their host (Domingo et al., 1985; Eigen, 1993).

Here, an in vitro evolution experiment on double-stranded DNA with selection for triplex formation ability has been performed. Amplification and mutagenesis were done by isothermal strand displacement amplification (SDA; Walker et al., 1992) starting from a defined sequence.
Previously it has been shown that SDA resembles rolling-circle replication, its cycles comprising nicking of a double-stranded DNA, elongation of the derived 3’ end and displacement of the downstream copy strand, followed by conversion of the displaced single-stranded intermediate to a double strand again (Figure 1; Walker et al., 1992; Walter & Strunk, 1994). Rolling-circle replication and closely related mechanisms play an important role in the replication of DNA genomes (Gilbert & Dressler, 1968; Ilyina & Koonin, 1992). There is evidence that they account for the in vivo generation
Figure 6. Fluorescence curves of strand displacement amplified sequence variants. (a) A standard SDA reaction mixture was initiated with the indicated concentration of mutants TRIIS1, FC and MC, and the increase of TO fluorescence upon replication monitored in a fluorometer (see Materials and Methods). Since the three mutants varied in sequence length, the identity of replication products could be confirmed by high-resolution denaturing polyacrylamide gel electrophoresis. (b) From the fluorescence curves, rate constants in arbitrary units could be extracted by the method of Biebricher et al. (1985, 1991). Briefly, the kinetics in the linear phase were characterized by the slope $\Delta I/\Delta t$, while kinetics in the exponential phase, that mainly occurs below the fluorescence detection limit, were analyzed using the displacement $t_{\text{dif}}$ between two curves initiated with different mutant concentrations. When the concentrations differ by a factor of 1000, then the exponential kinetics can be measured by $\kappa = \ln 1000/\tau_{\text{dif}}$.

Table 2. Selection values of initial and selected templates in the exponential ($\kappa$) and linear growth phase ($\rho$)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>$\kappa/10^{-3}$ (s$^{-1}$)</th>
<th>$\rho/10^{-2}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIIS1</td>
<td>2.4±0.3</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>FC</td>
<td>3.2±0.3</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>MC</td>
<td>3.1±0.3</td>
<td>2.0±0.2</td>
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$\kappa$ and $\rho$ were extracted from the fluorescence curves as described for Figure 6(b).

of plasmids in prokaryotes (Gruss & Ehrlich, 1989) and eukaryotes (Dasgupta et al., 1992), of single-stranded DNA viruses like filamentous bacteriophages (Baas & Jansz, 1988), plant geminiviruses (Laufs et al., 1995) or animal paroviruses (Berns, 1990), of double-stranded DNA viruses like bacteriophage $\lambda$ (Hendrix et al., 1983) and herpes simplex virus (Skaliter & Lehman, 1994) and of certain transposons (Mendiola et al., 1994).

In the case of Escherichia coli plasmid and bacteriophage replication, DNA polymerase I takes part in lagging strand synthesis by filling the gaps between the Okazaki fragments generated by DNA polymerase III, a process that leads to a higher susceptibility to mutations than in leading strand synthesis (Veauve & Fuchs, 1993). In the present study, the enzyme applied for replication and mutagenesis is the exonuclease-deficient large (Klenow) fragment of Escherichia coli DNA polymerase I (exo$^-$ Klenow polymerase). Its proofreading activity is suppressed by five orders of magnitude due to a double mutation in the 3'-5'-exonuclease domain of the large fragment (Derbyshire et al., 1988). Since proofreading by excision of misincorporated nucleotides plays a major role in replication fidelity (Goodman et al., 1993), the error rate of exo$^-$ Klenow polymerase under standard conditions is with $10^{-4}$ errors per base per replication about four to seven times higher than that of Klenow polymerase (dependent on the error of interest; Bebenek et al., 1990). Under the in vitro conditions applied in the present study, the determined error frequency is even an order of magnitude higher than this value. This may be due to the artificial conditions of SDA reaction under fluorescent detection, utilizing dATP$\alpha$S and thiazole orange. dATP$\alpha$S is known to suppress any residual exonuclease activity (Kunkel et al., 1981), while fluorescent dyes are mutagens (Kramer et al., 1974). Both effects could increase the error frequency. Though they may also limit the direct applicability of acquired results on to the in vivo situation, the obtained high error rates are the only chance to produce a broad quasispecies of mutants and to observe evolutionary processes with short nucleic acids replicated in vitro (Eigen et al., 1989).

After SDA in the exponential and linear growth phase without selection pressure other than for fast replication, large deletions dominate the obtained quasispecies, as was already found for continuous serial transfer under exponential growth conditions (Walter & Strunk, 1994). Deletions can be formed by primer misalignments (often after nucleotide misincorporation; Bebenek & Kunkel, 1990), via a “slipped mispairing” mechanism, as postulated for single-stranded phage and bacterial plasmids.
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(Horiuchi, 1983; Vilette et al., 1992) or in a more complex form by “strand switching” in quasi-palindromic sequence contexts (Ripley, 1990). Deletions are favored for low processive polymerases like the Klono fragment (Polesky et al., 1990; Wang & Ripley, 1994) and may be induced by fluorescent dyes like thiazole orange or acridine (Streisinger et al., 1966). They inevitably lead to loss of information content and are natural antagonists for complex functionality of genomes.

To counteract loss of sequence information, nine selection rounds with SDA replication coupled to selection for triplex formation were performed. The obtained quasispecies demonstrates that alternating replication and triplex selection yields mutants adapted to both selection pressures. The system essentially conserves the homopurine-homopyrimidine sequence tract capable of oligonucleotide binding that was already contained in the starting molecule TRIIS1 (Figure 2(b)), indicating that it did not find a superior alternative. This is not unexpected, because it is known that the utilized natural nucleobases exhibit only a limited number of possible triplets (Thuong & Hélène, 1993). Moreover, triplex selection via gel electrophoresis implies selection of strands that show a defined mobility shift and thus a roughly fixed sequence length. Heavily deleted sequences are selected against. Nevertheless, the selected sequences indicate (Figure 4) that single base exchanges or deletions at the end of the triplex-forming region seem to be tolerated under the experimental conditions (even a C-to-T change in position 25, four bases downstream from the duplex/triplex junction, is found in one clone). This is in accordance with earlier footprinting data (Stonehouse & Fox, 1994).

In order to essentially maintain their triplex-forming tract and at the same time enhance replication efficiency, mutants to be selected had to optimize their sequence for amplification by SDA. The obtained final consensus sequence FC (Table 1) carries one G-to-T base substitution and an adjacent point deletion in comparison with TRIIS1, resulting in a less stable secondary structure (Figure 5(c)). The G-to-T base substitution was also frequently found earlier under exclusively exponential growth conditions without triplex selection (Walter & Strunk, 1994). In the present study, the two mutations of FC were found to be accompanied by an increase of amplification efficiency in the exponential growth phase by 33% (Table 2). The easiest explanation for these findings would be facilitated binding of primers due to the less compact secondary structure as the dominant step in exponential growth. At the same time, amplification efficiency in the linear phase is reduced for FC against TRIIS1 by about 20%, which could be explained by a less efficient primer elongation and strand displacement of the downstream complement (Figure 1). As a consequence, TRIIS1 outgrowths FC under the SDA conditions used in the selection experiment. MC (Table 1), the most abundant and obviously most rapidly copied mutant, carries two more point deletions. This enhances its replication efficiency against the starting molecule in the exponential phase by 30% and in the linear phase by 33% (Table 2). MC seems to be the best adapted mutant, eventually selected after 270 molecular generations of amplification, mutation and selection.

Observing the described evolutionary phenomena in an in vitro amplification system for DNA like SDA has important implications. First, it proves that evolution in principle can take place in any isothermal reproductive system, as already shown for RNA replication by Qβ replicase (Spiegelman, 1971) or by self-sustained sequence replication (Breaker & Joyce, 1994). When the error rate of polymerization is high, a quasispecies of mutants is rapidly produced. When all steps of amplification occur simultaneously at a single temperature, different mutants start competing for resources and optimized amplicons will outgrow the mixture. This process can be coupled to a separate selection step by simple alternation with amplification. Selected mutants are then adapted to both selection pressures and it remains to be analyzed which selection pressure accounts for which mutation. Moreover, the most abundant mutant may be the best adapted sequence rather than the averaged consensus sequence. These findings have to be taken into account when interpreting evolution experiments on nucleic acids (Joyce, 1994).

Second, it gives a hint how selection in the analogous rolling-circle replication and related strategies of DNA virus and plasmid replication in vivo may contribute to evolution of rapidly reproducing genomes. As demonstrated in vitro, single base exchanges and point deletions can result in higher amplification efficiencies. As long as they are compatible with functionality of the genome in gene expression (as with triplex formation ability in the in vitro experiment), they will be incorporated into the sequence by evolutionary forces. Being able to model these properties of natural replicative systems in vitro could give the chance for a detailed understanding and finally the development of novel antiviral strategies as was already the case with the Qβ replicase system.

Materials and Methods

Materials

Exonuclease-deficient large fragment of Escherichia coli DNA polymerase I (exo- Klono polymerase; Derbyshire et al., 1988) was purchased from U.S. Biochemicals Corporation (Cleveland, OH) at a concentration of 5 to 10 units/μl, HincII (at 50 to 75 units/μl) from New England Biolabs (Beverly, MA), DNase I from Boehringer (Mannheim, Germany), and TdT DNA polymerase from Perkin-Elmer (Weiterstadt, Germany). 2'-Deoxyadenosine-5'-O-(1-thio-triphosphate; dATPpS; diastereomer mixture) and all other nucleotides were obtained from Pharmacia (Freiburg, Germany). Thiazole orange (TO) was from Molecular Probes (Eugene, OR).
and its concentration was determined by absorption measurement at 476 nm (ε\text{ACR}(TO) = 30,000 M\text{-}1 cm\text{-}1; Lee et al., 1986). All oligodeoxynucleotides were either synthesized on a Milligene Expedite Synthesizer and purified by gel electrophoresis or synthesized and HPLC-purified by NAPS (Göttingen, Germany). The 2-methoxy-6-chlor-9-aminacridine (ACR) moiety of oligodeoxynucleotide TRIPEL1 was attached in-line using the phosphoramidite, while tetramethylrhodamine (TMR) was attached post-synthetically via an amino-hexyllinker (NAPS, Göttingen, Germany). Aerosol Resistant Tips (ART) from Biozym (Hessisch Oldendorf, Germany) were routinely used to avoid contamination of SDA reaction mixtures with previously amplified products.

SDA reaction with fluorescent on line detection

The very first SDA reaction was initiated with the 59mer oligodeoxynucleotide TRIIS1 as template (Table 1). SDA buffer contained 50 mM potassium phosphate (pH 7.4), 3 mM MgCl₂, 4 mM dithiothreitol, 0.01% (v/v) Triton X-100, 3% (v/v) 1-methyl-2-pyrrolidinone (NMP), 0.25 mM each of dGTP, dCTP, dTTP and dATP. 10 mM MgCl₂, and 100 mM NaCl for potassium phosphate buffer (pH 6.5 or 7.0; see the legend (Table 1) in 10 μl of the 5' ACR-labeled oligodeoxynucleotide TRIPEL1 or of a dsDNA solution was incubated with 2 to 5 μM of carrier oligodeoxynucleotides (TMR) was attached post-synthetically via the phosphoramidite, while tetramethylrhodamine (TMR) was attached post-synthetically via an amino-hexyllinker (NAPS, Göttingen, Germany). Aerosol Resistant Tips (ART) from Biozym (Hessisch Oldendorf, Germany) were routinely used to avoid contamination of SDA reaction mixtures with previously amplified products.

Analysis of SDA products

SSCP analysis of SDA products was performed in an MDE-hydrolink gel (Serva, Heidelberg, Germany) as described by the manufacturer. For sequence analysis, either 1 μl of a terminated SDA reaction solution or the purified triplex DNA was further amplified in a final volume of 100 μl PCR reaction mixture (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatine, 0.04 units/μl Taq DNA polymerase) containing 0.5 μM each of primers SDAPCRP1 and SDAPCRP3 with HindIII and BamHI restriction sites, respectively (Table 1). Thermocycling was performed in a TRIO-Thermoblock cycler (Biomatex, Göttingen, Germany) for a total of 16 cycles with a temperature program of 94°C for 30 seconds, 30°C (in the following 14 cycles) for 30 seconds and 72°C for 30 seconds. The products were purified by non-denaturing polyacrylamide gel electrophoresis, restricted with HindIII and BamHI, cloned into pUC18 vector and transformed into Epicurian coli SURETM competent cells (Stratagene, Heidelberg, Germany). Finally, the plasmid DNA from single clones was prepared using the WIZARDTM miniprep protocol (Promega, Heidelberg, Germany) according to the manufacturer’s instructions, further purified by precipitation in ethanol, and sequenced as described (Walter & Strunk, 1994).

Non-radioactive DNase I footprinting

For non-radioactive detection, an isotopic footprinting assay (Stonehouse & Fox, 1994) was adapted to fluorescence detection of the digested fragments. TMR-labeled dsDNA was generated by PCR-amplification of templates TRIIS1 and FC as described above using 1 μM primers SDAPCRP1 and SDAPCRP3 (Table 1; the latter is a 5’ TMR-labeled form of primer SDAPCR3). Seven thermal cycles with 94°C for 30 seconds, 30°C (in the first two cycles) or 68°C (in the following five cycles) for 30 seconds and 72°C for 30 seconds were performed, the main product band identified by non-denaturing polyacrylamide gel electrophoresis, excised, diffusion eluted, and desalted. 25 ng (0.36 pmol) of this PCR-product were incubated with 0 to 5 μM TRIPEL1 and 20 to 15 μM of carrier oligodeoxynucleotide TSDAB1 (Table 1) in 10 μl of 50 mM sodium acetate (pH 5.5), 10 mM MgCl₂ and 100 mM NaCl for 15 minutes at 37°C, followed by 15 minutes at 16°C and five minutes at 22°C. A volume (2 μl) of a solution of 0.025 units/μl DNase I dissolved in 20 mM NaCl, 2 mM MgCl₂, 2 mM MnCl₂ was added, the mixture incubated for two minutes at 22°C and digestion stopped by addition of 138 μl of 20 mM EDTA (pH 8.0). Two-thirds of this mixture was twice extracted with phenol/chloroform, precipitated with ethanol, the precipitate washed, and loaded onto a 6% sequencing gel to be analyzed by electrophoresis on a model 373A DNA sequencer (Applied Biosystems, Weiterstadt, Germany). TMR-labeled fragments showed up in the “T”-signal. The ACR-labeled oligodeoxynucleotide TRIPEL1 (visible in the “G”-signal) does not interfere with the TMR-signal.

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References


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