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Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides

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Summary. DNA amplification fingerprinting (DAF) is the enzymatic amplification of arbitrary stretches of DNA which is directed by very short oligonucleotide primers of arbitrary sequence to generate complex but characteristic DNA fingerprints. To determine the contribution of primer sequence and length to the fingerprint pattern and the effect of primer-template mismatches, DNA was amplified from several sources using sequence-related primers. Primers of varying length, constructed by removing nucleotides from the 5′ terminus, produced unique patterns only when primers were 8 nucleotides or fewer in length. Larger primers produced either identical or related fingerprints, depending on the sequence. Single base changes within this first 8-nucleotide region of the primer significantly altered the spectrum of amplification products, especially at the 3′ terminus. Increasing annealing temperatures from 15° to 70° C during amplification did not shift the boundary of the 8-nucleotide region, but reduced the amplification ability of shorter primers. Our observations define a 3′-terminal oligonucleotide domain that is at least 8 bases in length and largely conditions amplification, but that is modulated by sequences beyond it. Our results indicate that only a fraction of template annealing sites are efficiently amplified during DAF. A model is proposed in which a single primer preferentially amplifies certain products due to competition for annealing sites between primer and terminal hairpin loop structures of the template.

Key words: DNA amplification fingerprinting (DAF) – PCR – Primer-template interactions – Single oligonucleotide primer

Introduction

The most commonly used techniques for specific definition of DNA target segments are molecular hybridization (Southern 1975) and DNA amplification by the polymerase chain reaction (PCR; Mullis et al. 1986; Mullis and Faloona 1987). These techniques can be used to study variant or polymorphic sites for genotyping, or to construct genetic maps in the course of genome analysis. Detection of DNA polymorphisms by established fingerprinting procedures requires prior knowledge of DNA sequence, cloned and characterized probes, or considerable experimental manipulation. Amplification fragment length polymorphism (AFLP) analyses directed by arbitrary primers has been recently proposed as an alternative targeting tool for genetic typing and mapping (Williams et al. 1990; Welsh and McClelland 1990; Caetano-Anollés et al. 1991a). This strategy uses randomly generated primers to initiate amplification of discrete but arbitrary portions of a genome. The AFLP analysis procedure developed by Caetano-Anollés et al. (1991a), termed DNA amplification fingerprinting (DAF), offers the most advantages. It uses the most permissive amplification reaction conditions, the shortest primers (≥ 5 nucleotides), and provides the highest resolution of fingerprint products. Depending on the amplification parameters, DAF can generate a complex but characteristic spectrum of products, many of which are polymorphic (Caetano-Anollés et al. 1991b). While polymorphisms from simple banding patterns can be easily used as genetic markers and placed on genetic linkage maps, more complex (i.e. informative) patterns are better suited for DNA fingerprinting. The use of polyacrylamide gel electrophoresis and DNA silver staining can adequately resolve the spectrum of DAF amplified products into detailed and reproducible patterns that reveal more than just the few predominant products (Bassam et al. 1992a).

While many investigators have evaluated the effects of various primer-template mismatches in the PCR process, especially if they occur at the 3′ terminus of the primer (Sommer and Tautz 1989; Wu et al. 1989; Nichols et al. 1989; Ehlen and Dubeau 1989; Newton et al. 1989; Okayama et al. 1989; Kwok et al. 1990), little is known about overall primer length and sequence requirements.

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During primer-directed enzymatic amplification, several factors allow for a particular DNA target to be efficiently amplified. These include the number and location of primer-template mismatches, their stability at various annealing and extension temperatures, and the efficiency with which the polymerase can recognize and extend the mismatched primer-template duplexes. For example, thermodynamic and enzyme kinetic analysis showed that elongation of mismatched primer termini by *Drosophila* DNA polymerase α was inefficient (Petruska et al. 1988). Other factors like primer length, primer and template conformation, and reaction environment could affect the kinetics of primer-template duplex formation and thus influence the efficiency with which a particular genomic site is amplified. DAF represents a special in vitro reaction in which oligonucleotides are so short that they approach the functional limits for priming DNA amplification. In this study, we have investigated characteristics that are inherent to these very short primers and inferred primer-template interactions that are important for genome identification and the generation of molecular markers.

Materials and methods

Source of DNA. Escherichia coli strain Smith 92 and *Klebsiella pneumoniae* strain J7 were isolated from individual udder quadrants of mastitis-infected cows and were obtained from S.P. Oliver and B. Jayarao, Animal Science, The University of Tennessee, Knoxville. *Discula* sp. SC-101 was provided by R.N. Trigiano, Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville. Total DNA was isolated from bacterial and fungal DNA using a phenol-chloroform extraction method (Bassam et al. 1992b).

DNA amplification. Amplification was done using conditions optimal for genomes of low complexity (Bassam et al. 1992b). Under these conditions, no day-to-day experimental variability was observed; however, all experiments were repeated several times. Briefly, the reaction mixture (25 µl) comprised 3 µM primer, 0.3 units/µl of Stoffel fragment DNA polymerase (Perkin-Elmer/Cetus, Norwalk, Conn.), 200 µM of each dNTP (Pharmacia LKB Biotechnology, Piscataway, N.J.), 6 mM $MgCl_2$, 10 mM TRIS-HCl (pH 8.3), 10 mM KCl, and about 1 ng/µl of template DNA. To allow for adequate amplification, primers of 5 and 6 nucleotides in length were used at a concentration of 30 µM and 15 µM, respectively. Where indicated, amplification was with 0.1 units/µl of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus) instead of the Stoffel fragment. The amplification mixture was overlaid with two drops of mineral oil and amplified in a dual-block thermocycler (Ericomp, San Diego, Calif.) connected to a refrigerated water bath for 35 two-step cycles usually of 1 s at 96° C and 1 s at 30° C. The heating and cooling rates of the thermocycler were 23°/min and 14°/min respectively. One complete cycle took about 8 min if the annealing temperature was 30° C. Sample temperature was continuously monitored with a thermal probe. Oligonucleotide primers, synthesized with >99% efficiency, were used unpurified and behaved consistently from batch to batch.

DNA electrophoresis and silver staining. DNA amplification fragments were separated by polyacrylamide gel electrophoresis using a Mini-Protean II cell (BioRad, Richmond, Calif.). Separation was in 0.45-mm-thick slab gels of 5% acrylamide, 0.25% piperazine diacrylamide crosslinker, and 7 M urea (each from BioRad) supported on GelBond PAG polyester film (FMC, Rockland, Me.). Inclusion of urea in the gels gave superior band resolution. The running buffer was TBE (100 mM TRIS-HCl, 83 mM boric acid, 1 mM disodium salt of EDTA; pH 8.3). Usually, wells were loaded with 3 µl of a 1/10 dilution of amplification reaction (30–40 ng DNA) mixed with 3 µl of loading buffer (5 M urea and 0.02% xylene cyanole FF). Electrophoresis was at 100 V usually until the dye front was about 1.5 cm from the end of the gel. Analysis of small primer-dimer DNA fragments was done in gels of higher concentrations (10% polyacrylamide).

DNA was visualized using a silver staining procedure, which is fast and sensitive and is able to detect about 1 pg DNA/mm^2 band cross-section (Bassam et al. 1991). Briefly, gels were fixed with 10% acetic acid for 20 min, rinsed three times (2 min each) with double distilled water, impregnated with silver solution (0.1% $AgNO_3$ and 0.056% HCOH) for 30 min, rinsed quickly with water, and then developed at 8–10° C in an alkaline solution (3% Na_2CO_3, 0.056% HCOH and 0.0002% $Na_2S_2O_3 \cdot 5H_2O$). Image development was stopped in 10% acetic acid. Polyester-backed gels were preserved for permanent record by soaking in 50% ethanol for 10 min and drying at room temperature.

Isolation and reamplification of separated products. DNA fragments embedded in polyacrylamide were isolated directly from gels stained with silver. A small piece of gel containing the desired fragment was carefully excised from fresh or preserved gels with a scalpel, and placed in 25 µl of a standard reaction mixture. Optimum amplification was achieved with 30 cycles of 1 s at 96° C and 1 s at 50° C. The isolated fragment could be further purified by an additional round of isolation and amplification.

Results

We used related oligonucleotides differing in length or sequence, and templates that were engineered to have complementary or mismatched terminal sequences of varying length, as a system to test homology requirements important for the DAF reaction.

Related oligonucleotide primers of varying length

Sets of related oligonucleotides of decreasing length were designed by removing nucleotides from the 5′ end of

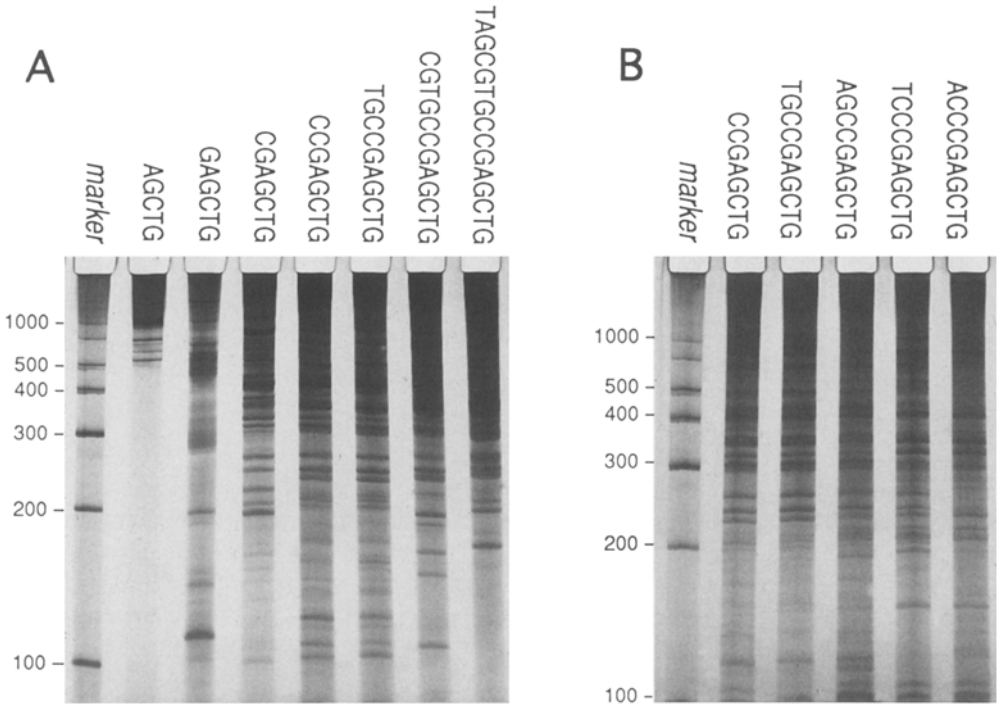

Fig. 1 A, B. Effect of oligonucleotide length among related primers. Total DNA from *Escherichia coli* was amplified with sets of: **A** primers varying in length between 5 and 15 nucleotides and sharing common 3′ sequences; **B** primers varying at the last 2 nucleotides from the 5′ terminus. Fragment sizes of the marker bands (Biomarker Low; Bioventures, Murfreesboro, Tenn.) are shown in bp

an arbitrarily chosen sequence. These oligonucleotides were used to direct amplification by both a DNA polymerase from *Thermus aquaticus* (AmpliTaq) and its truncated derivative, the so-called Stoffel fragment. The Stoffel fragment is a highly thermostable, recombinant DNA polymerase lacking the 289 N-terminal amino acids. It has a broad magnesium optimum, increased thermal stability, and no associated 3′–5′ or 5′–3′ exonuclease activity. These properties result in improved performance in DAF reactions. We find the Stoffel fragment more tolerant of reaction conditions, more efficient in the amplification of short products, and able to produce more informative fingerprints than the native Amplitaq (Bassam et al. 1992b).

Figure 1A shows fingerprints generated from *E. coli* DNA by the Stoffel enzyme and a set of primers originating from sequence TAGCGTGCCGAGCTG and varying in length down to 5 nucleotides. Patterns generated by related primers of 5, 6, 7, and 8 nucleotides in length were different in complexity and band distribution. Surprisingly, increasing primer length increased the number of amplification products. This trend was also observed using other oligonucleotide sets (not shown). In contrast, primers of 8 and 10 nucleotides in length produced virtually identical patterns. Longer primers produced patterns that were again divergent but showed some common bands. Increasing primer length produced a shift of these common bands toward higher molecular weight, due to additional nucleotides at each end of the amplification product contributed by the longer primers. These findings provide strong evidence that the products were amplified from the same template sequences and did not result from co-migration events.

Although patterns obtained with AmpliTaq differed from those obtained with the Stoffel fragment (Bassam et al. 1992b), the same overall trends in pattern distribution to those stated above were observed (not shown). These trends (Fig. 1A) also occurred when the above experiments were repeated with another set of related primers originating from the arbitrary sequence GAGCTGGGTAACGCC, and when using DNA from other strains of *K. pneumoniae* and *Discula* (not shown).

5′-Terminal primer sequences

Since patterns obtained with the octamer CCGAGCTG were almost identical to those obtained with the decamer TGCCGAGCTG, we examined whether sequence variation at the 5′-terminal portion of the decamer could produce variant patterns. Figure 1B shows that base substitutions in the last two nucleotides at the 5′ end produced several variant amplification fragments, but the overall patterns remained very similar. This indicates that sequences beyond the 8 nucleotides of the 3′-end region affect amplification only moderately and confirm the observations of Fig. 1A.

Annealing temperature

We determined the effect of annealing temperature on amplification directed by related primers. Primers of 7 to 15 nucleotides in length were used at annealing temperatures varying between 15° C and 80° C (Fig. 2). At 65° C, the heptamer failed to produce DNA amplification. At 70° C, only primers of 12 and 15 nucleotides in length produced detectable amplification products, and at 75° C only the 15-nucleotide primer amplified DNA. Temperatures over 80° C were unable to produce

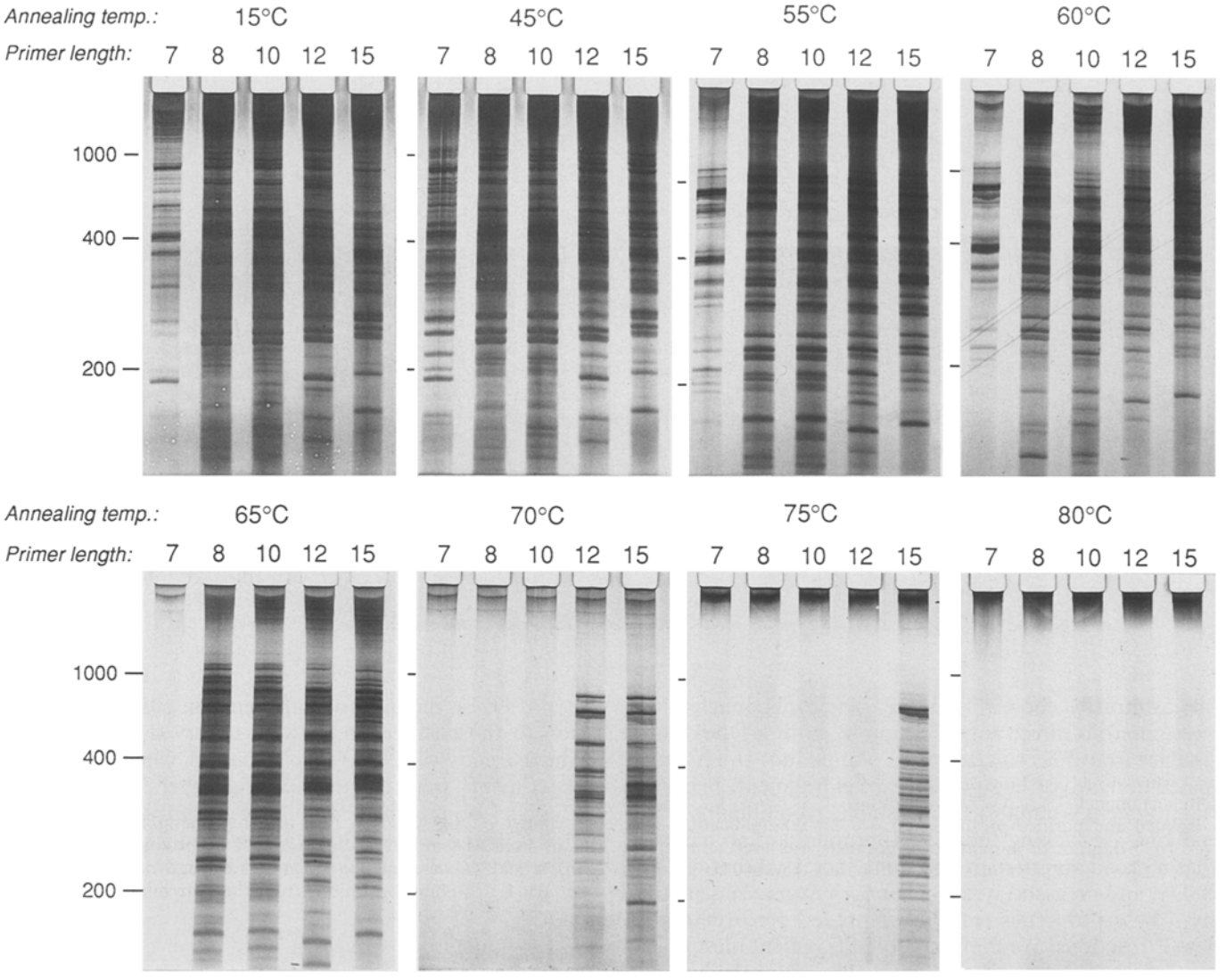

Fig. 2. Effect of annealing temperature on amplification directed by related primers of varying lengths. The lanes show patterns produced by primers that vary in length between 7 and 15 nucleotides (as indicated). Each primer shares common 3′-sequences as follows: 7, CGAGCTG; 8, CCGAGCTG; 10, TGCCGAGCTG; 12, CGTGCCGAGCTG; 15, TAGCGTGCCGAGCTG. The different annealing temperatures used were between 15° and 80° C as shown. All other cycling parameters were kept constant. Total DNA from *E. coli* was amplified in each case. Fragment sizes shown are in bp

profiles with any of the primers, probably because higher temperatures do not allow adequate primer annealing and extension. Surprisingly, we found that the trends in the pattern distribution shown in Fig. 1A were observed across the range of annealing temperatures examined. However, as annealing temperture increased, profiles tended to simplify due to a decrease in the number of low intensity bands.

Some amplification products produced at elevated annealing temperatures might have been derived from early annealing events occurring at room temperature, at the time when reaction components were mixed. To examine this possibility, both a heptamer and octamer primer were used to amplify DNA at an annealing temperature of 65° C. One essential reagent was deliberately excluded from the amplification mixture until it had exceeded the programmed annealing temperature. A similar "hot-start" approach is used in the PCR for amplifying very dilute DNA samples (Mullis 1991; Erlich et al. 1991). This approach precludes the generation of "spurious" products by preventing the nonspecific annealing of primers prior to amplification. When oligonucleotides were added to the reaction mixture at room temperature or at about melting temperature, the octamer produced amplification products with and without the "hot start" (data not shown). Patterns and product yield were similar when the reaction was initiated at both high and low temperatures. As expected, the control reaction using a heptamer at this high annealing temperature did not produce amplification products.

5′-Terminus overhangs

We then determined the effect of primer length on the amplification of a template with ends engineered to have

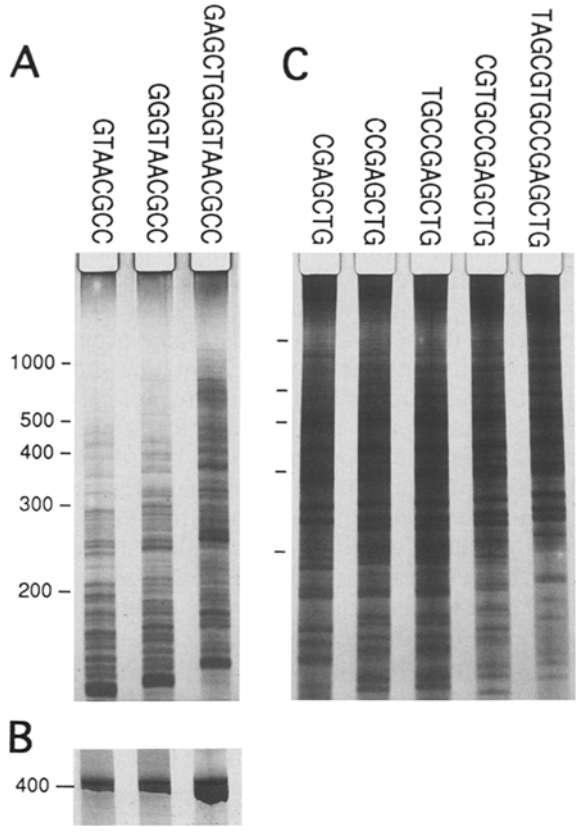

Fig. 3A–C. Effect of 5′-terminus overhang sequences on amplification of products with defined 8 bp termini. Primers of varying lengths derived from a common sequence (as in Fig. 1) were used to reamplify products originally produced from *E. coli* DNA using the octamers GTAACGCC (**A, B**) and CCGAGCTG (**C**). A single isolated and purified product was used for reamplification in **B**. A 10^{-4} dilution of amplification products was used as template in all cases. Of 25 products examined of less than 500 bp in length which were amplified with the octamer CCGAGCTG (**C**), 3, 1 and 7 products failed to amplify with primers of 7, 12 and 15 nucleotides in length, respectively. Fragment sizes shown are in bp

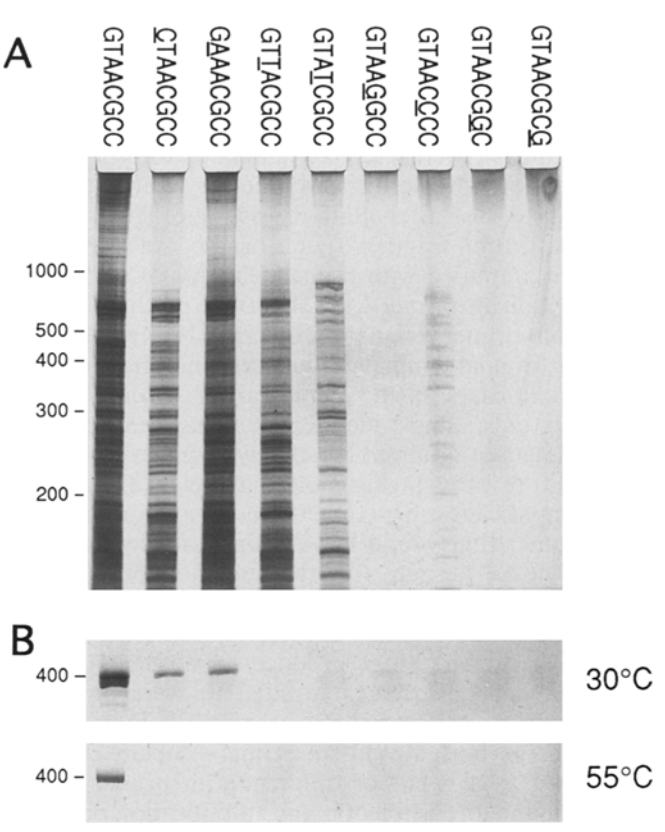

Fig. 5A, B. Effect of single base substitutions in the primer on amplification of products with defined 8 bp termini. Primers having single-base substitutions (*underlined*) and constant GC content were used to reamplify **A** all products, or **B** a single isolated and purified product, originally produced from *E. coli* DNA using the octamer GTAACGCC. A 10^{-4} dilution of amplification products was used as template in all cases. Of 36 products examined, 7, 6, 7, and 14 were missing when primers had base substitutions at the 1, 2, 3, and 4 positions from the 5′ terminus, respectively. Fragment sizes shown are in bp

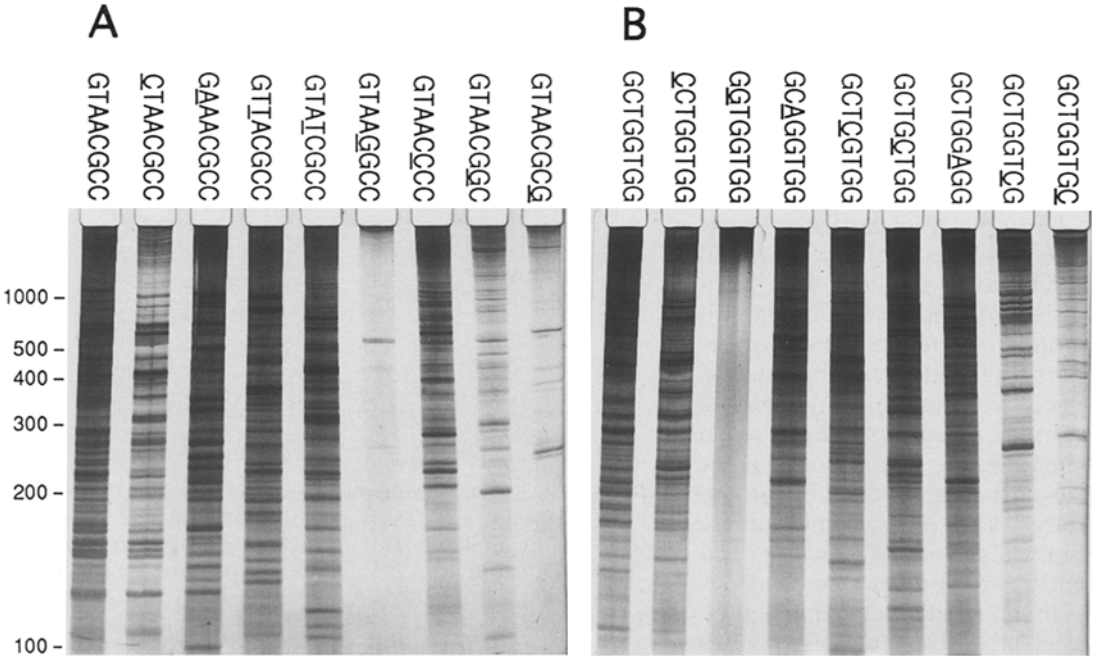

Fig. 4A, B. Effect of single base substitutions in the primer on amplification. *E. coli* DNA was amplified using related primer sets having constant GC content but single-base substitutions (*underlined*) in **A** GTAACGCC and **B** GCTGGTGG. Fragment sizes shown are in bp

perfect homology to the 3′ terminus of the primer. For this purpose, DNA fragments that had previously been amplified with octamers (in order to define the terminal 8 nucleotides), were used as templates for subsequent amplification with longer and shorter related primers. Initially, we used a template consisting of either a complex mixture of fragments or a single isolated 400 bp fragment amplified with primer GTAACGCC (Fig. 3A and B). Longer primers, which would produce 5′ overhangs on primer-template duplexes during annealing with the original template sequences, amplified template DNA in all cases. In the amplification of complex template mixtures, almost identical patterns were produced with primers of different lengths, with the expected mobility shift towards higher molecular weight (Fig. 3A).

Reamplification experiments using a shorter primer (a heptamer that would leave a single nucleotide overhang at the 3′ terminus of the template) produced an amplification pattern in which some of the expected dominant products failed to amplify (Fig. 3C). Similarly, longer primers (that produce primer-template duplexes having 5′ overhangs) resulted in several amplification products disappearing. These results indicate that overhangs in both strands of primer-template duplexes (produced as a result of differences in primer length) condition the formation of many amplification products, despite perfect complementarity.

Single base substitutions in the primer

Sets of primers with constant GC content were designed by substituting single bases at all positions in the sequence of an octamer. These primers were used to amplify DNA from *E. coli* and *K. pneumoniae*, in order to determine the contribution of each nucleotide in a primer to the specificity of the amplification reaction. All nucleotide substitutions caused a dramatic change in the spectrum of amplification products (Fig. 4). In general, less dramatic differences in patterns were observed when substitutions were towards the 5′ terminus (Fig. 4 and unpublished). The substitution of the 5′ terminal nucleotide produced patterns that shared several common bands.

Surprisingly, some primers produced markedly fewer amplification products than others. In particular, some produced very few or no products with the bacterial templates. These primers behaved similarly with plant and mammal DNA (not shown). The absence of amplification products was not due to the formation of primer-dimer artifacts, since short fragments were not detected in 10% polyacrylamide gels (not shown). Resynthesized primers were tested to discount faulty synthesis as an explanation for primer inefficiency. The reasons for the poor amplification are unclear, but may result from either extreme rarity of target sites or from interference by target site and/or product secondary structure. The existence of both extremely rare and very frequent oligonucleotide sequences in mammalian (Volinia et al. 1989) and other (Burge et al. 1992) genic regions has been demonstrated.

A set of primers with single base substitutions was used to reamplify DNA that had originally been amplified with the octamer GTAACGCC (Fig. 5A). Primers that would produce mismatches at the 5′-terminal region of the primer-template duplexes were able to amplify the majority of products. However, mismatches at the 5, 7, and 8 positions from the 5′ end of the primer failed to initiate amplification, while a mismatch at the 7 position produced few and weak amplification products. Once extension from a mismatched primer occurs, the resultant products (and their complements) have termini defined by the mismatched primer and are perfectly complementary in subsequent cycles of amplification. These molecules accumulate exponentially, while those with mismatches, accumulate from the original template only linearly. Based on these considerations, the observed reduction in product formation must reflect a dramatic decrease in the efficiency of mismatch extension. These results highlight the importance of the 3′-terminal region of the primer in the DAF reaction.

A template consisting of a single isolated 400 bp fragment with terminal sequences defined by the octamer GTAACGCC was also used in re-amplification experiments (Fig. 5B). Only mismatches in the first two nucleotides from the 5′ terminus were tolerated, but amplification was less efficient. However, when the annealing temperature was increased to 55° C, only the perfectly matched primer was successful in amplifying the synthetic template. Mismatches were thus less stable and therefore less tolerated during amplification at the higher annealing temperature.

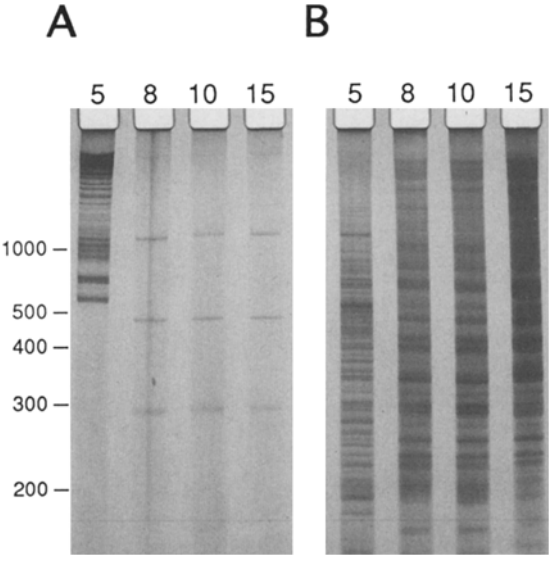

Fig. 6A, B. Effect of primer length on amplification of products having defined termini. The pentamer AGCTG in **A** or the octamer CCGAGCTG in **B** were used to reamplify products originally produced from *E. coli* DNA using primers of increasing length: 5, AGCTG; 8, CCGAGCTG; 10, TGCCGAGCTG; 15, TAGCGTGCCGAGCTG. A 10^{-4} dilution of amplification products was used as template in all cases. Fragment sizes shown are in bp

Effect of product terminal symmetry during amplification with primers of varying lengths

A set of related oligonucleotides of decreasing lengths originating from the oligonucleotide sequence TAGCGTGCCGAGCTG was used to reamplify DNA originally amplified with these same primers. These templates were thereby engineered to have terminal symmetry of between 5 and 15 nucleotides. When amplification was directed by a pentamer, only the template DNA with 5-base terminal symmetry was amplified (Fig. 6A). However, when an octamer was used to direct amplification, all templates were amplified (Fig. 6B). As expected, patterns generated from templates with 8- and 10-base terminal symmetry were indistinguishable and the pattern from the template with 15-base symmetry was slightly different. In contrast, the pattern produced from the template with 5-base terminal symmetry was much more complex than expected. This may result from products in very low abundance that were originally poorly amplified with the pentamer, but that are now stabilized as longer products during subsequent rounds of amplification with the octamer (see below). Similar results were obtained from the engineered templates with primers of 10 and 15 nucleotides in length (data not shown). In particular, the patterns obtained from each engineered template were virtually identical, regardless of the length of primer used for amplification.

Discussion

Very short arbitrary oligonucleotide primers can initiate amplification of discrete portions of a genome and produce characteristic fingerprints (Caetano-Anollés et al. 1991a). Each fingerprint reflects the successful primer-directed targeting of a set of sites in the genome. These amplified sites, also termed amplicons in the PCR (Mullis 1991), result from the annealing of the primer to complementary sites of each DNA strand, and depict a particular primer-template combination. The specificity of the DAF reaction was studied by varying primer length and sequence. We showed that for the primers studied: (i) the first 8 nucleotides from the 3′ terminus encompassed one basic domain; (ii) single base changes in the sequence within this domain significantly altered the spectrum of amplified products, especially towards the 3′ terminus; (iii) regions beyond the basic domain altered the amplified spectrum only moderately; and (iv) the boundary of the domain was not altered by increasing the annealing temperature.

The oligonucleotide domain was demarcated by a region (in our case of about 2 nucleotides) over which changes in primer length do not significantly alter the distribution of amplified products, and within which, single base substitutions produce highly variant amplification patterns. The existence of a 3′-terminal domain has several implications. It indicates that the crucial determinant of amplification by a DNA polymerase is the 3′ terminus of the primer, as has been observed for the PCR (Kwok et al. 1990). It also shows that a sequence of about 8 nucleotides from the 3′ terminus is sufficient to target genomic sites. Since addition of nucleotides to this domain does not increase the information content of the patterns significantly, longer sequences such as those used in other studies (Williams et al. 1990; Welsh and McClelland 1990; Parker et al. 1991) are not necessary.

In theory, primers of increasing length derived from a common sequence should target subsets of sites recognized by shorter primers. Thus, a common pattern should dominate fingerprints generated with these oligonucleotides. However, the short related primer sequences unexpectedly produced patterns that were relatively simple and highly divergent, rather than complex and cognate. The fact that observed patterns were divergent indicates that not all targeted sites are equally well amplified, and that some amplicons are amplified preferentially. This implies that while primer-template sequence homology defines a set of possible amplification sites, other factors that act during primer annealing, enzyme anchoring, or later in the amplification process, dramatically restrict their amplification. Moreover, shorter primers should theoretically produce a larger number of amplification products. However, we have observed an unpredicted decrease in the number of amplification products when primer length decreases.

To accommodate our observations, a general hypothesis can be postulated in which DNA amplification is modulated at two levels. In the first, the selection of target sites for amplification is determined by primer sequence and conditioned by the reaction environment. During the first few cycles, the primer "screens" the possible DNA target sites and anneals to many of them. In this early stage, annealing events may even include those with primer-template mismatches. Primer annealing is followed by enzyme anchoring and primer extension. Thus, the first few rounds of amplification select a subset of the many possible target sites to produce a defined population of amplification products. These products should then be efficiently amplified since their terminal sequences are defined by, and are therefore perfectly complementary to, the sequence of the primer. However, the internal sequences of target regions and their products may hinder the availability of their annealing sites, lowering the efficiency with which they are amplified.

The differential extent to which individual amplification products are further amplified constitutes the second level of modulation. Amplification products initiated by a single primer share the particular characteristic of having a region of terminal hairpin symmetry at least as long as the primer itself. In certain fragments, this symmetry may extend to internal regions thereby stabilizing the hairpin structures. For efficient amplification of these products to occur, the primer must displace these hairpin loop complexes long enough for the DNA polymerase to anchor and stabilize the duplex by strand extension. The extent of hairpin loop interference will be variable for each fragment, allowing some fragments to be preferentially amplified.

To test the hypothesis, a set of DNA fragments having variable regions of terminal hairpin symmetry (pre-

viously amplified with sequence-related primers) were challenged with primers of increasing length (Fig. 6). As predicted, a pentamer amplified only templates with 5-base terminal symmetry, but not templates having 8-, 10-, or 15-base symmetry, despite perfect complementarity. In contrast, an octamer was able to efficiently amplify fragments with terminal symmetry of up to about 15 bases. Furthermore, some products having 8-base terminal symmetry were not amplified with a related heptamer (Fig. 3) or with an octamer having a single base substitution at the 5′ terminus (Fig. 5). Our results suggest that when the primer is longer than the 8-nucleotide domain, it is better able to complete for annealing with hairpin loops.

The shorter the primer the more abundant will be its predicted annealing sites during primer screening. However as indicated by our results, shorter primers will have greater difficulty in displacing hairpin loop structures, especially when they are stabilized by additional base pair matching in internal regions of the loops. This should result in fewer amplified products than predicted from the total number of annealing sites. This is exactly the trend observed with primers of different lengths in Fig. 1. Since larger loops result in less stable hairpin structures (Xodo et al. 1991), it is conceivable that the fewer products amplified using short primers will be relatively long. Indeed pentamer primers produced very few if any products of low molecular weight (Fig. 1 and unpublished).

Temperature affects the formation, stability, and equilibrium between primer-template duplexes, hairpin loops, and other possible DNA molecular species formed during amplification (Fig. 7). Shorter primers require lower annealing temperatures to produce any amplification product (Fig. 2), although a pentamer can produce products at about 55° C (not shown). Based on considerations of thermal stability short primer-template duplexes should be unstable at these temperatures (Rychlik et al. 1990). For example, the pentamer AGCTG has a high duplex formation free energy ($\Delta G = -8.2$ kcal/mol) and a very low melting temperature ($T_m = -41.8°$ C, according to the nearest-neighbor method as modified in Rychlik et al. 1990). The fact that there is amplification at much higher temperatures indicates that while primer annealing events may be rare, the few that occur are quickly stabilized by enzyme anchoring and primer extension. Surprisingly, variations in temperature did not alter the boundary of the oligonucleotide domain, suggesting that the minimal primer sequence required to target a defined set of genomic sites is conditioned by factors other than primer annealing, such as hairpin loop stability.

Several apparent anomalies occurring in the DAF reaction have been explored. These anomalies include successful amplification at temperatures much higher than predicted, the unexpected production of fewer products with the shortest primers, and the fact that patterns generated with related primers of different lengths or sequence can show little resemblance to one another. In this paper we provide a model that can accommodate these and other observations. The model can serve as a basis for better design of primers and amplification parameters in future applications of the DAF technology. For example, in the construction of high-resolution genetic maps, the production of simple fingerprint patterns is often desirable. Here we show that this could be accomplished by decreasing primer length and increasing annealing temperature.

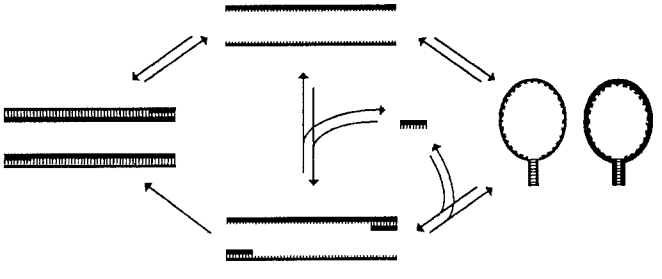

Fig. 7. A model of interactions between molecular species formed during DNA amplification with a single, arbitrary oligonucleotide primer. Following the template "screening" phase, a set of DNA fragments is synthesized. These first-round amplification products are initially single-stranded and have palindromic termini which allows the formation of hairpin loops. In subsequent rounds of amplification, the products can be in the form of template-template and primer-template duplexes, as well as single-strand and hairpin loops. The different species produced tend to establish an equilibrium, while enzyme anchoring and primer extension transform the relatively rare primer-template duplexes into accumulating amplification products

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