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Heterogeneity of primer extension products in asymmetric PCR is due both to cleavage by a structure-specific exo/endonuclease activity of DNA polymerases and to premature stops

(eubacterial DNA polymerases/elongation/hairpins/PCR plateau)

GREGORY TOMBLINE[†‡], DINA BELLIZZI[§], AND VITTORIO SGARAMELLA[§¶]

[†]Laboratory of Molecular Genetics and Informatics, The Rockefeller University, New York, NY 10021-6399; and [§]Department of Cell Biology, University of Calabria, 87030 Arcavacata, Italy

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ABSTRACT **In PCR, DNA polymerases from thermophilic bacteria catalyze the extension of primers annealed to templates as well as the structure-specific cleavage of the products of primer extension. Here we show that cleavage by *Thermus aquaticus* and *Thermus thermophilus* DNA polymerases can be precise and substantial: it occurs at the base of the stem–loop structure assumed by the single strand products of primer extension using as template a common genetic element, the promoter-operator of the *Escherichia coli* lactose operon, and may involve up to 30% of the products. The cleavage is independent of primer, template, and triphosphates, is dependent on substrate length and temperature, requires free ends and Mg^{2+}, and is absent in DNA polymerases lacking the 5′→ 3′ exonuclease, such as the Stoffel fragment and the T7 DNA polymerase. Heterogeneity of the extension products results also from premature detachment of the enzyme approaching the 5′ end of the template.**

A better understanding of the factors contributing to processivity and fidelity of enzymatic DNA synthesis is desirable. The acknowledged inability of DNA polymerases to initiate *de novo* synthesis and the obligatory discontinuous replication of the lagging DNA strand make primer extension relevant in a variety of *in vivo* DNA transactions, such as replication, recombination, and repair. Also several important *in vitro* reactions, such as PCR, rely on primer extensions (1).

In principle, primers can be extended uninterruptedly on a circular template. On a linear template they may be extended all the way to its 5′ end, provided the polarities of polymerization and fork movement are the same. In practice, primer extension does not proceed beyond the incorporation of a few thousand nucleotides. Longer extensions, such as those required *in vivo* for the complete copying of leading strands, involve the cooperation of several enzymes and accessory proteins (1).

In vitro, primer extension generally stops after at most a few thousand nucleotides; in PCR, special combinations of thermoresistant DNA polymerases have to be used in order to push this limit to tens of kilobases (2). During primer extension several events can contribute to the appearance of products shorter than expected: increasing attention is being given to the cleavage of products and templates.

Problems affecting primer extension are of general interest also in relation to the variability of the resulting sequences compared to the template and thus to the appearance of mutations and/or rearrangements. For example, modifications of a template by UV or alkylating agents as well as the occurrence of mismatches at the growing end can affect primer extension and thus the fidelity of the ensuing repair synthesis (3). Recently hairpins have been postulated as substrates for a structure-specific nickase involved in V(D)J rearrangements (4). These effects could be relevant also in widely used analytical techniques, from dideoxy DNA sequencing (5) to the wealth of PCR based-protocols (6): they can reduce both the yield and the quality of the products.

Under typical PCR conditions and using the DNA polymerases from either *Thermus thermophilus* (7) or *Thermus aquaticus* (8), we have investigated the heterogeneity of the products of single primer extensions (also known as asymmetric or one-sided PCR; ref. 9) mostly directed toward the *lac* operon PO region (10) cloned as a part of the *lacZ* gene in pBS (11). This paper characterizes further the structure-specific cleaving activity (12, 13) of eubacterial DNA polymerases, as independently described by Dahlberg's group (14), and identifies its target in the stem–loop structure potentially assumed by the *lacPO* sense strand. Further, it provides evidence that in PCR a substantial contribution to the heterogeneity of the primer extension products can come also from premature termination (15). These findings may help lead to a better use of thermoresistant DNA polymerases in DNA amplifications (16), fingerprinting (17), and sequencing (18).

MATERIALS AND METHODS

DNA. The plasmid pBS(−) (henceforth pBS) was extracted from *Escherichia coli* XL1-Blu RecA$^-$ (11) using either Qiagen (Hilden, Germany), or Magic Minipreps (Promega). Molecular weight DNA markers *Msp* I-pBR322 and *Bst*EII-λ DNA were purchased from New England Biolabs (Beverly, MA). Primers were supplied by GenoSys (The Woodlands, TX) and had the following sequences (5′ → 3′): P1, CAACTGTTGGGAAGGGC; P2, TCCCGACTGGAAAGCGGG; P3, GCCACGTTCGCCGGCTTT; P4, TGCAGCTGGCACGACAGG.

Enzymes. Taq (8), Tth (7), and the Stoffel (19) DNA polymerases were from Roche Molecular Systems. Restriction endonucleases were from NEB. Sequencing was performed with Sequenase II (Upstate Biotechnology) (20) or by a cyclic procedure developed in this laboratory.

Primer extensions were run in a TwinBlock system (Ericomp, San Diego) through cycles of denaturation (94°C, 2 min), annealing (62°C, 2 min), and elongation (72°C, 3 min). One half (10 μl) of the mix, containing buffer, 5–10 pmol of primer (labeled, if terminal labeling was desired; otherwise unlabeled), triphosphates {unlabeled, at final 50 μM, or labeled, if internal labeling was sought, at 10 μM, with 1–10 μCi (1 Ci = 37 GBq) per tube of [α-^{32}P]dGTP or dATP[α-^{35}S]}, was overlaid with two drops of mineral oil and put to 94°C ("hot start"): the second half, containing buffer, template

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[‡]Present address: DNA Repair and Molecular Carcinogenesis Program, Jefferson Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107.
[¶]To whom reprint requests should be addressed.

(25 fmol) and enzyme (0.5–1 unit), prepared separately at 0°C, was added under oil to the first half while at 94°C, and the cycles were started immediately; buffers were as indicated by the manufacturer. Terminal labeling of the primers was obtained by standard phosphorylation of the 5′-OH ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB).

Gel Electrophoresis. The extension products were run on polyacrylamide gels next to sequencing reaction mixtures obtained with the same primer used for the extension and dideoxy chain terminators, either with Sequenase (20) or with Taq DNA polymerase (ref. 21; unpublished procedure).

RESULTS

Effect of Template Structure on Primer Extension. We first investigated the effect of the template structure on primer extension under PCR conditions. For a better characterization of the original extension products, we used only one primer per reaction: this reduces the chances of processing of the extension products, but leads to linear amplification. As templates we used the covalently closed circular plasmid pBS or linearized derivatives with the relevant 5′ terminus at variable distance from the priming site. The extensions were primed by one of the four primers, P1 through P4. Our experimental system is described in Fig. 1.

In Fig. 2 we present the results of extension of either P1 or P2 on a variety of templates; the other two, P3 and P4, gave similar results (not shown). Initially as templates we used the supercoiled plasmid: the overall extent of incorporation is similar to that obtained with linear templates. The autoradiographies of the gels (lanes "sc") show that each of the two primers P1 and P2 is extended into products at least 1–2 kb long; they migrate as a smear visible at the top of the gel. Low levels of faster discrete bands are visible, but we have not investigated them.

Primers P1 and P2 have also been extended on restricted templates. The distances between the annealed primers and the 5′ ends of these templates varied from about 100 bases to >1 kb. In the third and fourth lanes of Fig. 2 (lanes P1/P and S) we show the patterns obtained when P1 is extended on pBS

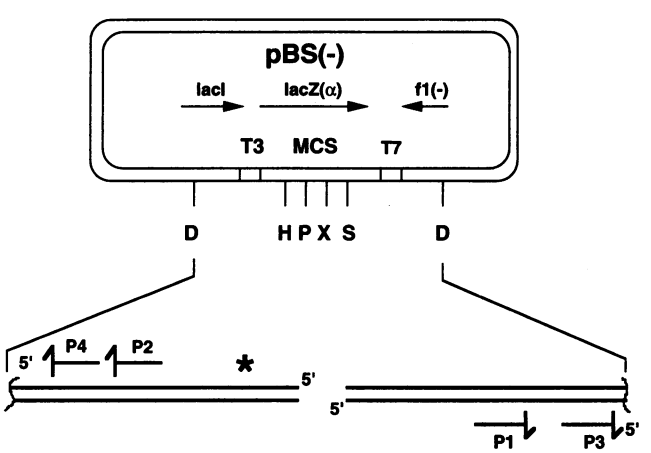

FIG. 1. Experimental system. At the top, pBS(−), an f1 phagemid derivative (11); its *lacPO* region is located at the beginning of the arrow below *lacZ*(α) (11). The linearized templates result from its restriction at the *Hin*dIII (H), *Pst* I (P), *Xba* I (X), and *Sma* I (S) unique targets in the multiple cloning site (MCS) or at *Dde* I (D: of the six *Dde* I sites only the two relevant to this research are indicated). The primers define a region including the *lacPO* region and the MCS: they are 18-mers with similar base composition (12 GC) except for P1, a 17-mer with 10 GC. The distance between the 5′ ends of the internal primers, P1 and P2, is 415 bases and between the external ones, P3 and P4, is 798 bases. P1 and P3 are 363 bases apart; P2 and P4 are 20 bases apart. Below are shown the ends of the linearized plasmid and the position of the four primers. The star indicates the palindrome (see text). The drawing is not to scale.

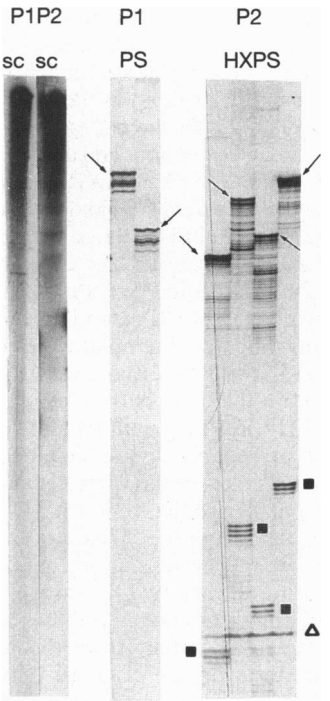

FIG. 2. Autoradiography after electrophoresis of primer extension products on a variety of templates. Lanes P1 and P2/sc, extension products of P1 and P2 on supercoiled pBS; lanes P1/P and S, extension products of P1 on pBS linearized with *Pst* I and *Sma* I; lanes P2/H, X, P, and S, extension products of P2 on pBS linearized with *Hin*dIII, *Xba* I, *Pst* I, and *Sma* I. Arrows indicate full extension; triangles, unique common band; solid squares, variable bands (see text).

linearized with *Pst* I (lane P) and *Sma* I (lane S). In the case of P1 the size of the extension products is determined by the distance between the 5′ ends of the linearized template and of the primer: with P1 it is 203 bases for *Pst* I and 182 for *Sma* I. On these templates the length of the slowest products (arrows) is as expected for full extensions. In addition there are variable amounts of shorter products (not further investigated). Similar patterns are obtained when P1 is extended on *Hin*dIII- and *Xba* I-cut pBS. Also with P3 we obtained mostly full extensions plus the series of progressively shorter ones (not shown).

When each of the two primers P2 and P4 is extended on linear templates, the patterns are more complex than with P1 and P3. As shown in Fig. 2 (lanes P2/H, X, P, and S indicate extension of P2 on templates cut, respectively, with *Hin*dIII, *Xba* I, *Pst* I, and *Sma* I), the slowest bands are again exactly as expected from full extensions (arrows). Similarly to the extension of P1 and P3, P2 and P4 also give a series of bands migrating just ahead of the full extensions: they contain progressively shorter sequences with a rich size distribution (not been further investigated). Differently from P1 (and P3), with P2 and P4 there are two additional sets of bands. One band (triangle) is unique, 102 bases long with P2 and common to all the four different templates. The other sets (solid squares) are composite: we refer to them as "variable" bands. For each template the sets of variable bands are different. Their overall patterns echo those of the full and quasi-full extensions. For example, in lane H (*Hin*dIII-linearized pBS) the extension of P2 gives, from the top, a band 201 bases long, which is the fully extended product (arrow), plus the series of the quasi-full extensions; below, we have the unique 102-base band (triangle); and then a group of continuously variable bands (solid square). The slowest of these has the mobility of 99 bases, the next of 98, and so forth. The patterns obtained with pBS linearized by *Xba* I, *Pst* I, and *Sma* I are comparable: they too contain the full (arrow) and quasi-full extension prod-

ucts, the common unique band (triangle), and the set of variable bands (solid square). In each of the four P2 lanes, the full extension band (arrow) shows a size equal to the sum of the common unique band (triangle) plus the slowest of the variable bands (solid square). With P4 the pattern (not shown) is similar to P2, except that the full extensions and unique band are 20 bases longer: this is due to the 20-base shift of P4 versus P2 (Fig. 1); with P2 and P4, the sets of the variable bands are identical.

All this is summarized in Fig. 3. Here we present only the gel regions corresponding to the unique common band and the variable bands. Panels P4 and P2 report the patterns obtained when *Taq* DNA polymerase is extending P4 and P2, respectively, on the four linearized templates produced by *Hin*dIII (lane H), *Pst* I (P), *Xba* I (X), and *Sma* I (S): the four lanes labeled gact contain the ladders from cyclic sequencing. The unique band is 122 bases long with P4 and 102 bases with P2: again this band is present also in the sequencing ladders (lanes gact). Conversely, the sets of the variable bands (marked with solid squares in Fig. 2) are the same with P4 and P2 but vary according to the position of the 5′ end of the templates. Thus, *Hin*dIII (its site is the closest to the primers) produces a template 8 bases shorter than *Pst* I (the next); between *Pst* I and *Xba* I (the third) the difference is 16 bases and between *Xba* I and *Sma* I (the most distant from the primers) 9 bases. Indeed, these are the pairwise distances between the slowest of these variable bands in the corresponding lanes. Panel T7 shows the sequencing pattern obtained with T7 DNA polymerase: with this enzyme, the extension of P2 (and P4) does not lead to the appearance of the unique common band visible with *Taq*: the arrow indicates its expected position. The four lanes at the right have been overloaded compared to the leftmost "g" lane, hoping in vain to evidence some signal.

In conclusion, the extension of the primer P1 (and P3) elicits the formation of only the full-size products (arrow), plus the series of quasi-full ones. Instead P2 (and P4) yields: (*i*) the full- (arrow) and quasi-full-size products: their lengths vary with both the primer and the template; (*ii*) a unique product (triangle) of either of two lengths, 122 bases with P4 and 102 with P2, independent of the template; (*iii*) sets of variable bands (solid square), dependent on the template, but not on the primer. The sum of *ii* and *iii* gives *i*.

Cleavage Versus Pausing in Extension. The heterogeneity of the extensions of P2 and P4 could be caused by cleavage, in agreement with recent findings on the 5′ → 3′ exo-activity in eubacterial DNA polymerases (14). It could be due also to pauses or premature terminations.

In order to distinguish between these possibilities we changed labeling protocol. With α-labeled triphosphates, all the products should be labeled, regardless of the mechanism of formation. If instead, either the 5′ or the 3′ terminus carries a label, only one of the cleaved products will show it.

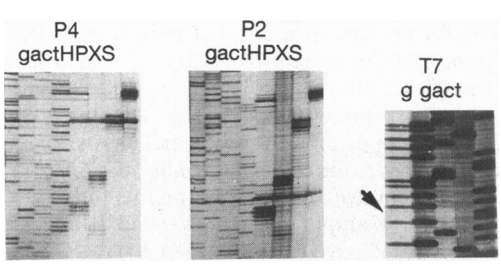

FIG. 3. Cleavage products in the extensions of P4 and P2 on linearized templates: for lanes HPXS, see Fig. 2. Lanes gact show sequencing ladders obtained in cyclic reactions with the same primers of the extensions and *Taq* DNA polymerase. T7, sequencing ladders obtained with P2 and T7 DNA polymerase (the rightmost gact lanes of T7 have been overloaded). The arrow points to the position expected for the common band.

Cleavage is demonstrated by an experiment in which the products are labeled at the 5′ end of the primers, and not internally, as before. Lanes 1 and 2 of Fig. 4 show the extension products of 5′ labeled P2 on either *Hin*dIII- or *Sma* I-linearized template: we have the full-size products (arrow), respectively, of 201 bases (lane 1) and 234 bases (lane 2) plus the quasi-full extensions; we have also the common unique band of 102 bases (triangle); but no variable bands are visible, even after overexposition of the gel (lanes 3 and 4). This supports the hypothesis that the variable bands derive from the unlabeled end (i.e., the 3′) of the extension products. A parallel experiment in which the label is internal confirms the origin of the variable bands (solid square) from the 3′ end (Fig. 4, lanes 5 and 6). Identical results are obtained with Tth DNA polymerase (not shown).

The presence of the unique common bands (triangle) as well as of the variable bands (solid square) following priming with P2 and P4 is thus due to a cleavage of the extension products (heterogeneous at their 3′ ends) into two portions: one, unique, carries the primer (and its 5′ label); the other, composite, lacks the 5′ label but carries the internal one, when present. It should correspond to the 3′ portion of the extension products. Indeed, the two sets of fragments together make up the length of the full and quasi-full extensions (see above). Conversely, the heterogeneity of the two groups of bands indicated by arrows and solid squares (Figs. 2 and 4) is likely due to the same events of premature termination, rather than to cleavage. The original extensions, with their fixed 5′ and variable 3′ ends, yield the longer products (arrow). Their cleavage originates the unique fragment from the 5′ moiety (triangle) and the set of variable bands (solid square) from the 3′ ends.

Further proof of the involvement of the 5′ → 3′ exo-activity of *Taq* polymerase was obtained with its derivative, the so-called Stoffel fragment (8, 19): this enzyme carries the polymerizing activity but no 5′ → 3′ exo. Extension of P2 and P4 on linearized templates leads to full-size fragments, without any

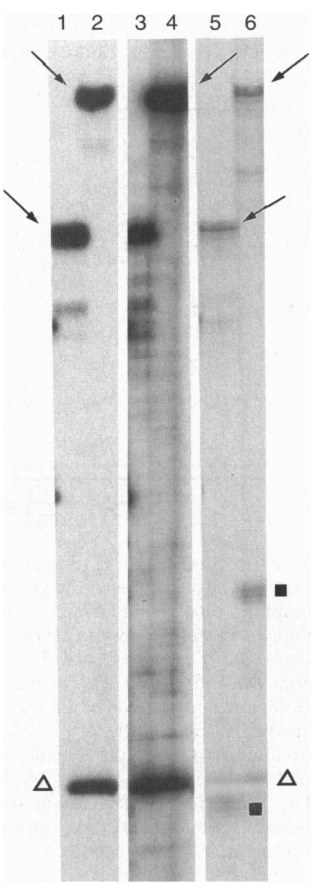

FIG. 4. Comparison of internally versus terminally labeled extension products. Terminally labeled P2 was extended on *Hin*dIII- (lane 1) and *Sma* I-linearized (lane 2) pBS; lanes 3 and 4 are overexposures of the same gel. Internally labeled products obtained on the same templates were loaded in lanes 5 and 6.

appearance of cleaved products (not shown). Internally labeled full extension products of P2 were synthesized with Stoffel on *Sma* I-linearized pBS, isolated from the gel and exposed to *Taq* as usual: the extended product was cleaved in the absence of added primer, dNTPs, and template. No cleavage was obtained if the reaction was carried on for 20 min at fixed temperatures (25, 40, 55, and 70°C) or if Mg^{2+} was omitted.

Requirements and Structural Specificities of the Cleavage with *Taq* and *Tth* DNA Polymerases. In the previous sections we have shown that the products of the extension of P2 and P4, but not P1 and P3, can be cleaved by the 5′ → 3′ exo of *Taq* and *Tth* DNA polymerases. The target lies within the *lacPO* region (Figs. 1 and 5) and is placed at the base of the hairpin where transcription is initiated, mostly at position T_{129} (Fig. 5) of the template strand (10).

If structural peculiarities of the target are involved, different substrate lengths could affect intra-strand folding and thus the cleavage of P2/P4 extension products. A simple way to alter the secondary structure and possibly interfere with cleavage could exploit different lengths of extension products. Such substrates can be obtained on templates restricted at different sites. For this we have used pBS cut with *Hin*dIII, *Pst* I, *Sma* I, *Dde* I, plus the supercoil (sc) as control: P4 was the primer (Fig. 6). The extension products are labeled internally with [α-^{32}P]dATP: again, the triangles indicate the unique common 5′ part of the cleaved extension products, the solid squares their heterogeneous 3′ complements. The lengths in bases of the templates under study are 221 (H), 232 (P), 257 (S), 1428 (D), and theoretically infinite in the case of intact, supercoiled pBS. Since the overall levels of synthesis are comparable (as shown also in Fig. 2), it seems that the longer the extension products, the less efficient the cleavage. Also Dahlberg's group

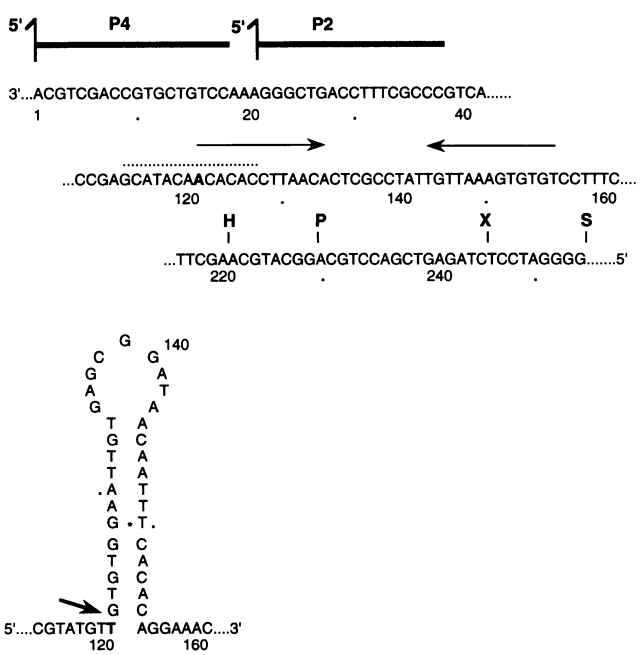

FIG. 5. Sequence of the template for the extension of P2 and P4 and possible structure of the product. Above the sequence of the template are indicated the positions of the two primers P2 and P4. The 5′ termini produced by *Hin*dIII, *Pst* I, *Xba* I, and *Sma* I on the template strand are indicated by the bold initial of the corresponding enzyme above the target sequence: cleaved bonds are marked by vertical lines. The alternating Pu/Py sequence is overlined with dots, and the inverted repeats originating the hairpin are indicated by two converging arrows. Below is shown the putative hairpin-like structure assumed by the primer extension products. The cleavage site is indicated by the arrow pointing at bold T_{122} at the beginning of the stem: note corresponding A in the template. Numbering starts with the first residue of the template complementary to the 5′ end of P4.

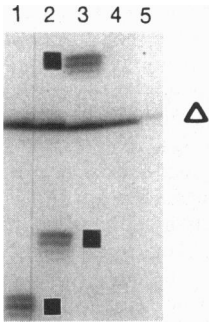

FIG. 6. Effect of extension products length on cleavage. Internally labeled products were synthesized using P4 and analyzed as before. Templates are pBS cut with *Hin*dIII (lane 1), *Pst* I (lane 2), *Sma* I (lane 3), *Dde* I (lane 4); as in Fig. 2, sc is supercoiled pBS (lane 5). Symbols as in Fig. 2.

(14) reported a similar effect, but with templates of lengths differing at their 5′ end.

DISCUSSION

The overall heterogeneity of the extension of the primers studied here is due to two distinct events: (*i*) generalized premature stops just ahead of the template 5′ terminus; and (*ii*) occasional cleavage of the products by an exonuclease associated with the polymerases.

The factors contributing to the occurrence and distribution of the premature stops vary with different terminal sequences but are as yet unknown.

We have focused our attention on the cleavage which occurs in the system shown in Fig. 5. Such activity may be considered an exo-endonuclease, since it requires, or at least is stimulated by, a free end but cuts well inside polynucleotide chains. We have evidence that the intact plasmid survives a similar exposure to the enzyme without cleavage (not shown). It is also possible that the intact plasmid is topologically constrained and cannot fold as required for cleavage.

Three features of the relevant region are noteworthy. One is the presence on the template of a row of 13 residues (115–127, 1 being the 5′ end of P4, top of Fig. 5). It is essentially made up by alternating Pu/Py, mostly A/C, except for the doublet $A_{121}A_{122}$; this region includes the −10 position (A_{119}) of the *lacP* (22). We have highlighted this sequence with a dotted line. It is interesting that a similar base composition is present in the system where Odelberg *et al.* (23) detect recombination during single cycle PCR: incidentally, they too report extensive cleavage of the template either in the presence or the absence of primer (see their figure 4). The second feature of the region is the presence of a palindrome: a stretch of 13 bases (122–134, thus partially overlapping with the alternating A/C run) can pair with a complementary T/G sequence, 144–156, with just one mismatch (C_{128}*A_{150}): the two sequences are overlined by solid converging arrows. Their pairing can originate a stem–loop structure: that assumable by the extension products is shown at the bottom left of Fig. 5. Note that here the mismatched pair is G*T. The third feature is that the pentanucleotide which primes the synthesis of new DNA chains in phage T4 is an RNA transcribed from a template sequence, 3′-GTT-5′ (24), which is central to our alternating Pu-Py stretch (positions 120–122 in top part of Fig. 5): relevant to this is that the 5′ → 3′ exonuclease of the eubacterial DNA polymerases can act on RNA (14). Thus this cleavage may be involved in the removal of the RNA primer at the 5′ end of the Okazaki fragments (1).

It seems most likely that our exo/endonucleolytic activity is the same as that described by Longley *et al.* (12), by Holland *et al.* (13), and more recently and thoroughly by Lyamichev *et al.* (14). Differently from them and from D. Gelfand (personal commu-

nication), we find that incubation at fixed temperatures elicits no cleavage: it is still possible that proper conditions for cutting of our substrate under constant temperatures can be found. We too find that cleavage depends on the substrate structure, requires Mg^{2+} but not dNTPs, templates, or primers, is absent in phage T7 DNA polymerase (acting at 37°C) and Stoffel fragment, produces 3′-OH ends: this is suggested by the finding that the cleavage products can be elongated if further cycles are allowed (not shown). Also our activity affects up to 30% of the original extension products; this may be an underestimation, since at each cycle the cleaved portions may compete successfully with the original primers, thermodynamically if not kinetically, be extended into longer products, and thus lower the net synthesis. This scenario may explain the so-called "plateau effect" which prevents most of PCRs from holding the exponential rate of synthesis for more than a few cycles (ref. 25; K. Mullis, personal communication): the 5′ cleavage products may function as primers and be extended in place of the original ones. Indeed, during symmetric PCR with the same primers the overall yields are lower than expected, and product heterogeneity increases with the number of cycles (unpublished).

That the phenomenon under study involves the cleavage of the original extension products is supported from the following observation: when internal label is used, in each lane the specific radioactivity of the unique band (triangle) approximates that integrated over the whole set of the variable bands (solid square), as from densitometric tracings (not shown).

In the course of our experiments, especially in cyclic sequencing, we have frequently noticed bands common to the four lanes: they could be instances of structure-specific cleavage. These lines must be pursued, with particular interest for those involving the two complementary strands of palindromic sequences. Obviously, the assumption of a secondary structure by a given DNA sequence does not mean that a complementary sequence will adopt a similar structure (26): it will be of interest to examine the susceptibility to cleavage of the products resulting from extension of clockwise primers flanking the *lacPO* region (Fig. 1). Appropriate mutations in the palindromic sequence may also provide clues as to the structural requirements of this cleaving activity.

As to the possible role *in vivo*, the finding that mammalian cells contain exonuclease(s) active on flap structures (27) may probably relate to the phenomenon reported here. Roles have been postulated for these nucleases in the context of DNA replication: it is conceivable that such an activity may be involved also in the processing of the 3′ ends of the Okazaki fragments, through strand displacement, branch migration, and template switching (1) as well as in recombination and possibly in repair of DNA. An example of template switching during primer extension under one cycle PCR has been recently described (23).

Even if the eukaryotic DNA polymerases lack a $5' \rightarrow 3'$ exonuclease (1, 14), similar activities are often found in proteins known to interact tightly *in vivo* with, e.g., calf DNA polymerase ε (28). Interestingly, stem–loop DNA structures are substrates of a "hairpin nickase" in mammalian cells (4). A structural and functional homology between mammalian DNase IV and the $5' \rightarrow 3'$ exonuclease domain of *E. coli* DNA polI has been reported (29). A somehow similar specificity has been proposed as responsible for the *in vitro* processing of heteroduplexes by T4 endonuclease VII (30). A structure-specific endonuclease has been described by Friedberg and coworkers for the yeast enzyme Rad1/Rad10 (31). In relation with the effects of these activities on genetic variation, it has been found that stem–loop structures are frequent mutational sites for mammalian DNA polymerase α (32).

Structure-specific exo/endonucleases could also be of use *in vitro* for fingerprinting DNA as such, in AP-PCR (33) and RAPD (34) and/or after a variety of treatments such as UV irradiation which could interfere with primer extension.

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