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Amplification of Human Genomic DNA Sequences With Polymerase Chain Reaction Using a Single Oligonucleotide Primer

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We present two examples of exponential nucleic acid amplification with the polymerase chain reaction (PCR) in the presence of only one amplification primer. Cloning and sequencing of the PCR products generated by amplification of human genomic DNA revealed that the amplified sequence contained only one primer and its complement, at the two ends of the PCR product. Although these experiments were performed with primers derived from the sequence of the prostate specific antigen (PSA) gene and the normal epithelial cell-specific 1 gene (NES1), the amplified sequences were novel and had no homology with either PSA or NES1 DNA. While both PSA and NES1 genes reside on chromosome 19q13.3-q13.4, the amplified sequences were found by mapping to reside on chromosome 5q12 and 5p15.1-p15.3, respectively. When we examined the mechanism of amplification by PCR using one primer in these two cases, we found that there was a high homology between the PSA primer or the NES1 primer and the two regions flanking the amplified sequence of chromosome 5q12 or 5p15. This indicated that the single PSA or NES1 primer could anneal on both strands of the DNA of that region, and mediate the exponential amplification. Since this phenomenon occurred to us twice with a limited number of different PCR reactions performed in our laboratory (< 20), we believe that it may represent a common artifact of PCR. Moreover, it appears that the palindromic primer binding sites can anneal to each other forming DNA cruciforms. J. Clin. Lab. Anal. 13:69–74, 1999.

Key words: polymerase chain reaction; PCR artifacts; single primer PCR; DNA cruciforms; RNA loops; hairpins

INTRODUCTION

The polymerase chain reaction (PCR) amplifies exponentially DNA segments that reside between two oligonucleotide amplification primers (1). The practical applications of this technique are widespread and the method is now used widely for both research applications and routine diagnostics. The exceptional sensitivity of the method is due to the exponential amplification which usually exceeds factors of 10^6-fold in 30–40 cycles. Its specificity depends on the nature of the primers, the experimental conditions (e.g., annealing temperature, $MgCl_2$ concentration, etc.) and the complexity of the target to be amplified. It is generally possible to amplify very rare sequences embedded within the whole human genome. This exceptional specificity is partially due to the fact that a single mispriming event will not usually lead to exponential amplification. In order for a target to be exponentially amplified, the primers must bind to each one of the two DNA strands with their 3´-hydroxyl ends facing each other and the distance between the two primers should not exceed 1–2Kb.

In this paper we describe two examples of exponential PCR amplification of human genomic DNA segments mediated by one oligonucleotide primer. Since the primers used were not specifically selected for this application, we suggest that this phenomenon may be a common artifact of PCR.

MATERIALS AND METHODS

We have used the following target DNAs: (a) human genomic DNA isolated from blood lymphocytes, (b) a plasmid that contains a 5-Kb human genomic DNA sequence, generated by subcloning a PAC (P_1 artificial chromosome) clone, and (c) a PAC clone (~ 100Kb) isolated from a human genomic DNA library (2). The clone identity is PAC 731K2. The plasmid clone (from now on referred to as the SacI plasmid) and the PAC clone both contain a 450 base pair (bp) sequence of genomic DNA which was initially identified as

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described elsewhere (3). The 450-bp sequence was deposited in GenBank, accession number U90205. The 5-Kb sequence of the SacI plasmid has also been deposited in GenBank, accession number AF038385.

PCR Primers

The primers used in this study are as follows:

PSA-A1: 5′-TGCGCAAGTTCACCCTCA-3′
PSA-A2: 5′-CCCTCTCCTTACTTCATCC-3′

The above primers were designed from the sequence of the prostate specific antigen (PSA) gene and have been used previously to amplify PSA cDNA (4).

Primer U: 5′-GGTTATCCAGCAAGAAGC-3′
Primer L: 5′-GGACTACAAGGGCACACC-3′

These primers bind internally to the 450-bp DNA sequence described above (also shown in Fig. 1) and they generate a 325-bp PCR product with a template that is either genomic DNA, the SacI plasmid or the PAC clone 731K2.

Primer NES1-5′-5: 5′-GGACCCTTGCCAGAGTGAC-3′
Primer NES1-3′-5: 5′-GGATCAGCAGGAGCATAAC-3′

cgccagtgtg atggatatct gcagaaatca gctt**CCCTCT CCTTACTTCA**
TCCTGCCAAT GGGTTATCCA GCAAGAAGCA GATCAGAATA
TAGGAAATAT CACTCTTTTG ACATGAATGT AGTTGAAATC
CATTTTTAAG TTAGTTAAGG ACTAACTTAA AAATACCTAC
AAAATCCAGG GAGACTGACT TCAGTATTTG TCATGGATCT
TCAGAATACT GAATCCCAAA TTTCAATACT CGCTTCTCAA
ACAGGGAGAA CTGTTCTAAG GTGACCCAGG AATGCCAGCC
ATACAAATAT GCTCAAGACA TGGAAAAAAA CATTAAAATG
GATTTGCTGC AACTGATATT CTGTGTTCTG CAATGCTAGG
TGTGCCCTTG TAGTCCAAGA ACTAACACTG CAGACTGCAA
CTAGAATTGT GGTCCCTGGT CATGTCACTT CCAAGAAGTA
TCAGGTTTAA GAACTAAGCA GTATGTGTAA GCTGAAAAGG
GATTAAGAGT GAG**GGATGAA GTAAGGAGAG GG**aagccaat
tccagcacac

Fig. 1. Sequence of a cloned PCR product generated with PSA primers and cDNA from lung cancer tissue (3). Lower case letters indicate vector sequences and sequences in bold denote the sequence of amplification primer PSA-A2. Note the presence of primer PSA-A2 and its complement at the two ends of the PCR product.

These primers were designed using the computer program Oligo 5.0 (National Biosciences Inc., Plymouth, MN) for the purpose of amplifying the cDNA of the recently cloned NES1 gene (5). NES1 cDNA is deposited in GenBank, accession number AF024605. A plasmid containing the NES1 cDNA was used as a positive control.

Polymerase Chain Reaction (PCR)

PCR was performed on genomic, plasmid, and PAC DNA with either one or two primers. PCR was carried out in a 20 μL reaction mixture containing 250 ng primers and 2.5 units of AmpliTaq DNA polymerase (from Roche Diagnostic Systems, Branchburg, NJ) using the Perkin-Elmer 9600 thermal cycler. Other conditions were as described previously (4). For blank control, no DNA was added. The cycling conditions for PSA-A1 and PSA-A2 primers were 94°C, 5 min for 1 cycle followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec with the last extension at 72°C for 10 min. For PCR using primers NES1 5′-5 and 3′-5, AmpliTaq Gold DNA polymerase was used (Roche) instead of AmpliTaq. The cycling conditions were 94°C for 12 min to activate the AmpliTaq Gold DNA polymerase followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, the last extension being 72°C for 10 min. The PCR products were electrophoresed on 2.5% agarose gels.

Nested Polymerase Chain Reaction (Nested PCR)

Nested PCR was performed with internal primers U and L, specific for the 450-bp sequence. One μL of the previous PCR product which was derived from human genomic DNA amplified with PSA primers, was used as target. The PCR mix was the same as described above using the AmpliTaq Gold DNA polymerase. PCR cycling was as follows: 94°C for 12 min to activate the DNA polymerase followed by 30 cycles of 94°C for 30 sec, 62°C for 1 min, last extension at 62°C for 10 min. The PCR products were electrophoresed on 2.5% agarose gels.

Cloning and Sequencing of PCR Products

PCR products were cloned into the pCRII–TOPO cloning vector (092008, Invitrogen, Carlstad, CA) following the manufacturer's instructions. The cloned inserts were sequenced with vector-specific primers using an automated DNA sequencer.

RESULTS

In our previous study (3), we identified and sequenced a 450-bp novel sequence from a lung carcinoma cDNA. This sequence was amplified in a PCR reaction employing the prostate specific antigen (PSA)-specific amplification primers PSA-A1 and PSA-A2. When this PCR product was cloned and sequenced, we obtained the results shown in Figure 1.

The PCR product usually contains the sequence of one amplification primer at one end, the amplified target sequence, and a sequence complementary to the second PCR primer (1). Figure 1 shows that the PCR product contains primer PSA-A2 at one end and a sequence complementary to PSA-A2 primer at the other. This suggests that PCR amplification occurred with the action of one primer only, which annealed to both target DNA strands but in opposite orientations.

In order to verify that genomic DNA amplification is feasible with one primer only, we performed the following experiment. We amplified human genomic DNA using PSA primers PSA-A1 and PSA-A2, or PSA-A1 only or PSA-A2 only. When we analyzed the PCR products by agarose gel electrophoresis, no bands of the expected product were seen. In order to check if the sequence of Figure 1 is represented in the reaction mixture but in low abundance, precluding its detection by agarose gel electrophoresis and ethidium bromide staining, we performed nested primer PCR, using the internal primers U and L, on the previous three PCR reactions. The results are shown in Figure 2. The 325-bp PCR product, expected from the nested primer PCR, is seen in lane 1 (template is PCR product generated with primers PSA-A1 and PSA-A2), lane 3 (template is PCR product generated with primer PSA-A2 only), and lane 4 (template is genomic DNA used as a control with primers U and L in a non-nested PCR reaction). In lane 2, no PCR product with the nested primer PCR is seen (template generated with primer PSA-A1 only). The data suggest that a single PSA primer (PSA-A2) is sufficient to amplify the 450-bp sequence of Figure 1. The 450-bp PCR product from genomic DNA could not be detected on agarose gels after the initial PCR but its presence can be easily revealed by nested primer PCR specific for the 450-bp sequence, using primers U and L.

Further verification that this single primer PCR mediates exponential amplification of the 450-bp sequence was obtained with templates which are less complicated than genomic DNA. In Figure 3, we amplified a plasmid in which we cloned a 5-Kb human genomic DNA fragment, containing the 450-bp sequence and flanking genomic sequences. In lane 1, we used primers PSA-A1 and PSA-A2. In lane 3, we amplified the plasmid with primer PSA-A1 only, and in lane 5, we amplified the plasmid with PSA-A2 primer only. It is clear that the 488-bp expected product (450-bp internal

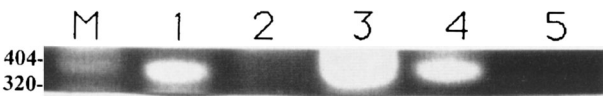

Fig. 2. Nested polymerase chain reaction (Nested PCR) with primers U and L. Targets were from PCR reactions described below. Lane M, DNA markers; lane 1, PCR product generated with primers PSA-A1 and PSA-A2; lane 2, PSA-A1 only; lane 3, PSA-A2 only; lane 4, genomic DNA control; lane 5, blank control. The expected 325-bp product is seen in lanes 1, 3, and 4 but not in lane 2.

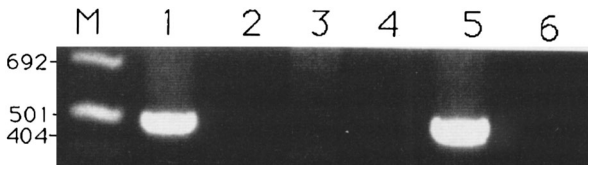

Fig. 3. Polymerase chain reaction (PCR) with the SacI plasmid as target. Lane M, DNA markers; lane 1, plasmid amplified with both PSA-A1 and PSA-A2; lane 2, blank control with PSA-A1 and PSA-A2; lane 3, plasmid amplified with PSA-A1 only; lane 4, blank control with PSA-A1 only; lane 5, plasmid amplified with PSA-A2 only; lane 6, blank control with PSA-A2 only. Notice the appearance of PCR products of the correct molecular weights in lanes 1 and 5 but not in lane 3.

sequence plus 2 × 19bp of primer sequences) is amplified with PSA-A2 alone (lane 5) but not PSA-A1 alone (lane 3). Similarly, we have used as a template a 100-Kb human genomic DNA fragment, containing the 450-bp sequence and flanking sequences, cloned in a PAC vector (Figure 4). In lanes 1 and 5, we obtained the expected 488bp PCR product using primers PSA-A1 and PSA-A2 and PSA-A2 alone, respectively. When PSA-A1 primer we used alone, no PCR product was obtained (lane 3).

We have also observed single primer PCR amplification with another set of primers, coded NES1-5´-5 and NES1-3´-5. These primers were derived from the cDNA sequence of the NES1 gene (5) which resides on chromosome 19q13.3-q13.4 (6). While other primers derived from the same cDNA sequence did amplify the expected region of genomic DNA, as verified by sequencing of the PCR products (data not shown), this primer pair amplified a 493-bp product which was cloned into a pCR II vector and then sequenced. The sequenced product is shown in Figure 5. We found that this product consists of the following: Primer NES1-5´-5 (19bp), an internal DNA sequence of 455bp and a 19-bp sequence complementary to primer NES1-5´-5. Thus, similarly to the data of Figure 1, a single primer was able to initiate expo-

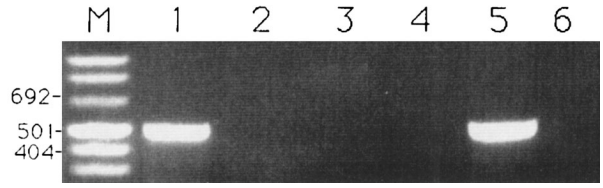

Fig. 4. Polymerase chain reaction using the PAC clone 731K2 as target. Lane M, DNA markers; lane 1, PAC amplified with both PSA-A1 and PSA-A2; lane 2, blank control with PSA-A1 and PSA-A2; lane 3, PAC amplified with PSA-A1 only; lane 4, blank control with PSA-A1 only; lane 5, PAC amplified with PSA-A2 only; lane 6, blank control with PSA-A2 only. Notice the appearance of PCR products of the correct molecular weights in lanes 1 and 5 but not in lane 3.

ccctt**GGAC CCTTGCCAGA GTGAC**TTTAT CATCAAGTGG AAGCCAAGGA
GCTTCATTTG TGCTTTTACA GTTATTTGTA ATTGCCAAAG TAAGATATTT
GTAAAATATA CTAAAACCTC ACCACATCTG CAAGCAGCAA ACATCCCTTC
CCCTGCCTGT CCCCTGAGGA GTCATTCCTG CACCTTTGGA CAGTGTCTTG
CCTCTGATGT TTGAACAAAA GAAACTGCCT GCCTGTGTGT TTTGGGTGAA
CTGGGGCCTC TGCAGCCCTC TGTGACATCC TGTACCCACC GAGGTCATCG
CTAACATTGT AAGCCAGATC CCTTGCTAAG GGTCTACATG GATTCTCTCA
CTCAAGCTGC ATTTTTGTCC ATAGAAACTA AGGCCTAGAA TGATGGAATA
ACTGGCCCCA TGTCACTCTG GCAGCTGTGA GCTTCAACAT TAGAAACTAA
GGCCTAGAAT GATGGAATAA CTGGCTCCAT **GTCACTCTGG CAAGGGTCC**a
agggcg

Fig. 5. Sequence of the cloned PCR product generated with NES1 primers and genomic DNA as target. Lower case letters indicate vector sequences and sequences in bold indicate the sequence of amplification primer NES-5′-5. Note the presence of primer NES-5′-5 and its complement at the two ends of the PCR product.

nential amplification of a genomic DNA sequence. The sequence of Figure 5 has no homology with any sequence deposited in GenBank and was recently mapped by us to human chromosome 5p15.2-p15.3 (data not shown); the NES1 gene resides on chromosome 19q13.3-q13.4.

In order to verify that primer NES1-5′-5 alone can amplify the sequence of interest, we performed the experiment shown in Figure 6. In lane 1, we amplified the expected 493-bp product with genomic DNA as target, with primers NES1-5′-5 and NES1-3′-5. In lane 2, we amplified NES1-cDNA with these primers and obtained the expected shorter PCR product of 214bp. In lane 4, we amplified genomic DNA with primer NES1-5′-5 only and generated the 493-bp PCR product. When we amplified genomic DNA with primer NES1-3′-5 only, no amplification was seen (lane 7).

The reason for the ability of one primer to initiate exponential PCR amplification was further investigated in both cases. Because we had available the SacI plasmid containing the 450-bp sequence of Figure 1 plus flanking sequences, we designed sequencing primers which allowed us to sequence completely this 4.8-Kb insert (this sequence was deposited in GenBank, accession number AF038385). We then aligned this sequence

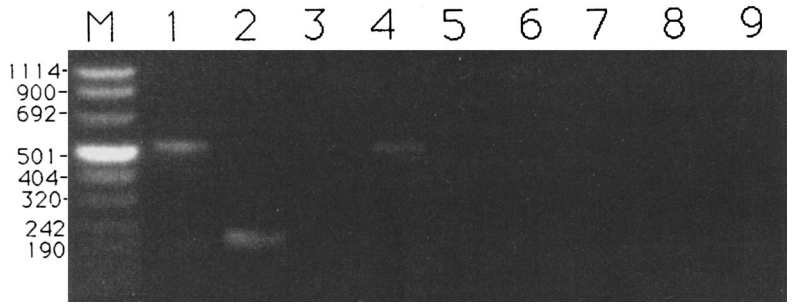

Fig. 6. Polymerase chain reaction (PCR) of DNA with NES1 gene-specific primers. Lane M, DNA markers; lane 1, genomic DNA amplified with both NES1-5′-5 and NES1-3′-5; lane 2, NES1 cDNA amplified with both NES1-5′-5 and NES1-3′-5; lane 3, blank control with both NES1 5′-5 and NES1 3′-5; lane 4, genomic DNA amplified with NES1-5′-5 only; lane **5,** NES1 cDNA amplified with NES1-5′--5 only; lane 6, blank control with NES1-5′-5; lane 7, genomic DNA amplified with NES1-3′-5 only; lane 8, NES1 cDNA amplified with NES1-3′-5 only; lane 9, blank control with NES1-3′-5. Notice the appearance of bands with lengths of 493bp in lanes 1 and 4 and a band with a length of 214bp in lane 2. For more discussion see text.

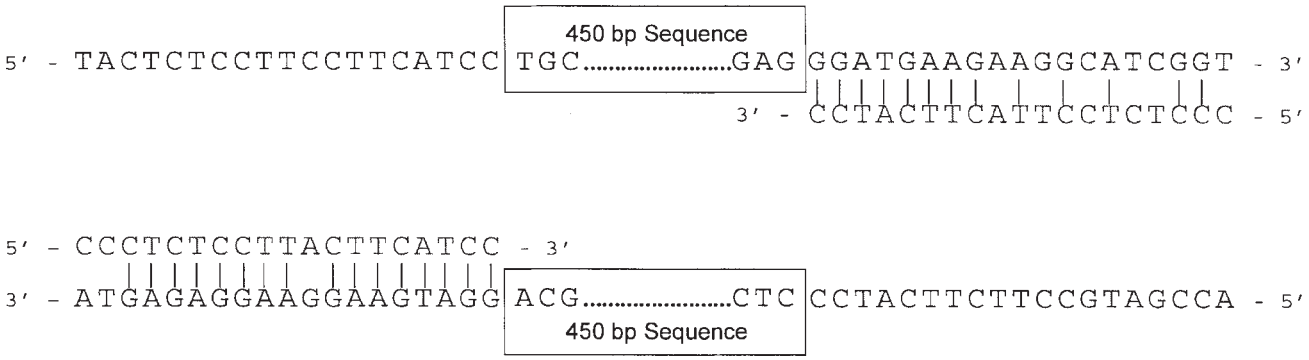

Fig. 7. Annealing of primer PSA-A2 on the two strands of DNA, at positions flanking the cloned 450-bp sequence. This annealing allows exponential amplification with a single primer.

along with primer PSA-A2 (Fig. 7). It can be seen that although primer PSA-A2 was designed from the sequence of the PSA gene, which resides on chromosome 19q13.3-q13.4, there is a highly homologous region to this primer (16/19 matching nucleotides) on chromosome 5q12 (where the 4.8-Kb sequence resides) flanking the 450-bp sequence. Interestingly, there is also a reverse complementary sequence spanning 8 contiguous nucleotides, flanking the other end of the 450-bp sequence, on the same DNA strand. The presence of these regions, flanking the 450-bp sequence, explain the ability of the PSA-A2 primer to exponentially amplify the 450-bp sequence, as shown in Figure 7.

We followed the same strategy to investigate the ability of primer NES1-5´-5 to exponentially amplify the sequence shown in Figure 5. We first identified a PAC clone containing this sequence by screening a human PAC genomic library (2) (PAC 42H21). We then subcloned this PAC into a plasmid vector and identified a plasmid clone (~ 10Kb) that contains the sequence of Figure 5. Using internal primers, we then sequenced regions flanking the sequence of Figure 5. When we aligned primer NES1-5´-5 with the newly generated sequence, we obtained the data shown in Figure 8. Again, although primer NES1-5´-5 was derived from the sequence of the NES1 gene on chromosome 19q13.3-q13.4, it is clear that there is a highly homologous region to this primer on chromosome 5p15.1-p15.3. Interestingly, there is also a reverse complementary sequence spanning 10 contiguous nucleotides, flanking the other end of the 455-bp internal sequence, on the same DNA strand. These regions allow exponential amplification of the internal sequence with one primer only.

DISCUSSION

The PCR method is used widely for research and clinical applications and, under appropriate conditions, it is a highly sensitive and specific technique. Nonspecific bands appear frequently in research applications. These bands usually originate from mispriming events which occur randomly, especially when a complex template, such as human genomic DNA is used as a target. Many mispriming events are not recognized since the PCR product may never reach enough concentration for detection on agarose gels. Recently, we identified a novel DNA sequence by PCR amplification with PSA gene-specific primers, followed by Southern blot hybridization (3). This 450-bp sequence had no homology with the PSA gene, from which the primers used for amplification were derived. After cloning and sequencing of this PCR product, it became evident that it originated by amplification with

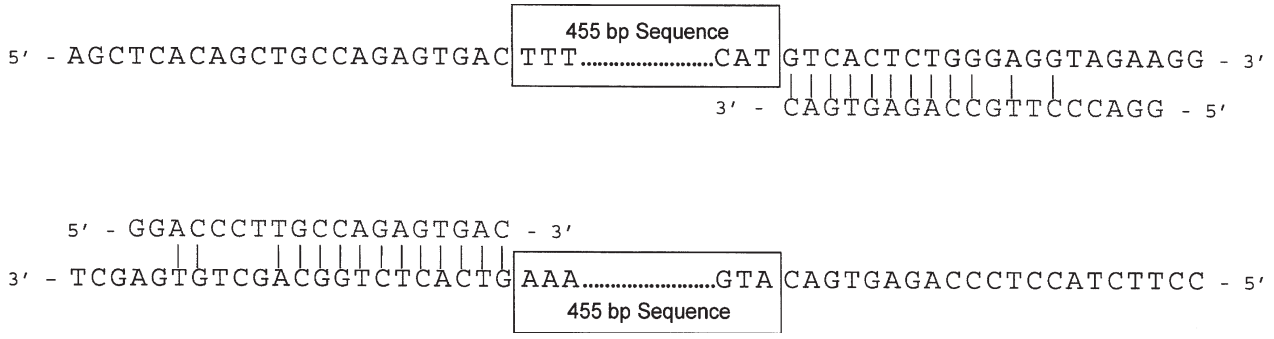

Fig. 8. Annealing of primer NES1-5´-5 on the two strands of DNA, at positions flanking the cloned 455-bp sequence. The annealing allows exponential amplification with a single primer.

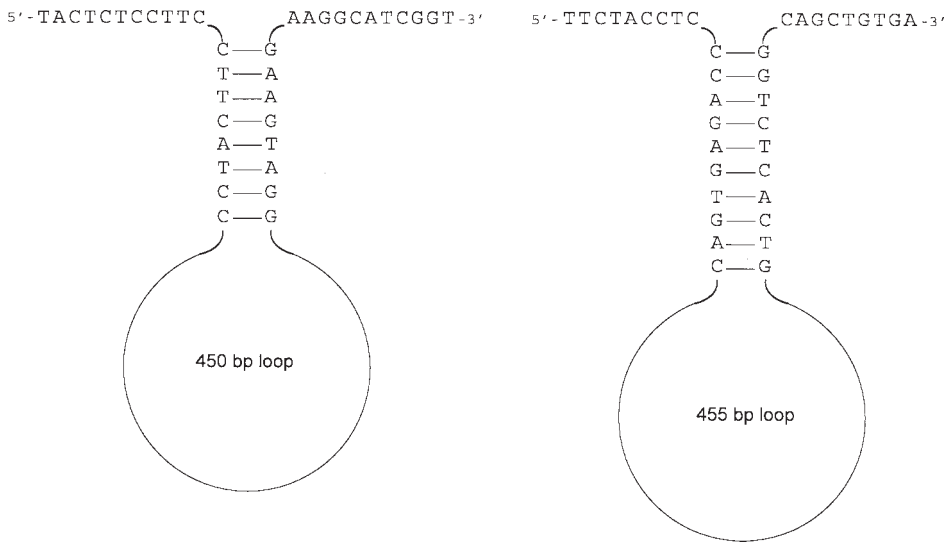

Fig. 9. Hypothetical loop structure of one strand of genomic DNA based on the reverse complementarity of the sequence flanking the 450-bp or the 455-bp sequence. If this sequence is expressed, similar looping may be seen on the mRNA as well.

only one PSA primer (PSA-A2). The amplified sequence was mapped to chromosome 5q12. A 100-Kb human genomic DNA PAC clone containing this 450-bp sequence was isolated by screening a library. This PAC DNA was then subcloned into a plasmid and a 5-Kb region, containing the 450-bp sequence, was completely sequenced.

By using nested-primer PCR, we were able to demonstrate that this 450-bp region could be amplified from genomic DNA by use of only one PCR primer (PSA-A2). We have further demonstrated the same phenomenon by using either PAC or plasmid clones as targets. An additional example was provided in which a genomic DNA region residing on chromosome 5p15.1-p15.3 was amplified with one primer designed from the sequence of the NES1 gene, which resides (as PSA does) on chromosome 19q13.3-q13.4. Similar to the first example, the 455-bp amplified region with the NES1-specific primer had no homology with the NES1 gene but represented a sequence not previously deposited in GenBank. These two examples indicate that amplification of genomic DNA regions with one primer, whose sequence was derived from a different region of the genome, is possible and it is likely a frequent event.

The mechanism of such amplification was examined by first isolating PAC clones from a human genomic DNA library which contained the single primer amplified regions. By obtaining additional sequence flanking the single primer PCR amplification product on these PAC clones, we were able to construct regional maps and examine the homologies of primers PSA-A2 and NES1-5′-5 with flanking sequences of the PCR products (Figs. 7 and 8). It is clear that the mechanism of PCR amplification with the single primer is the same on both occasions. Due to the presence of reverse complementary sequences on DNA (inverted repeats), separated by either 450 or 455 bases, the single primer could anneal to the two DNA strands as shown in the figures and mediate exponential amplification.

In theory, such reverse complementary sequences on DNA or RNA can cause nucleic acid looping **(**Fig. 9). In DNA, this looping of both strands leads to structures called “cruciforms” (7,8). In RNA, the looping will form a hairpin (7). The physiological role of such putative structures is currently obscure.

Since these two examples were identified from a limited number of different PCR reactions in our laboratory (< 20 different PCRs), we believe that this phenomenon is frequent and should be kept in mind when unexpected bands appear after PCR amplification.

REFERENCES

1. Mullis KB. The unusual origin of the polymerase chain reaction. Sci Am 1990;262:56–61.
2. Ioannou PA, Amemiya CT, Garnes J, et al. A new bacteriophage P1-derived vector for the propogation of large human DNA fragments. Nat Genet 1994;6:84–89.
3. Zarghami N, Levesque M, D’Costa M, Angelopoulou K, Diamandis EP. Frequency of expression of prostate specific antigen mRNA in lung tumors. Am J Clin Pathol 1997;108:184–190.
4. Zarghami N, Diamandis EP. Detection of prostate specific antigen mRNA and protein in breast tumors. Clin Chem 1996;42:361–366.
5. Liu XL, Wazer DE, Watanabe K, Band V. Identification of a novel serine protease-like gene, the expression of which is down-regulated during breast cancer progression. Cancer Res 1996;56:3371–3379.
6. Luo L, Herbrick J, Scherer SW, Beatty B, Squire J, Diamandis EP. Structural characterization and mapping of the normal epithelial cell-specific gene 1 (NES1). Biochem Biophys Res Commun 1998;247: 580–586.
7. Lewin B. Genes VI. New York: Oxford University Press; 1997. 105–106 p.
8. Courey AJ, Wang JC. Cruciform formations in a negatively supercoiled DNA may be kinetically forbidden under physiological conditions. Cell 1983;33:817–829.