0273-2289/94/4401—0015$03.00

Interference by Complex Structures of Target DNA with Specific PCR Amplification

ZHENGLIAN ZHANG,[1] KAI HUANG,[2]
YUYING ZHANG,[2] NIANJUAN LIU,[2] AND KAIYU YANG*,[2]

Chinese Academy of Sciences, [1]Institute of Biophysics, and [2]Institute of Microbiology, Beijing, China

Received May 5, 1993; Accepted June 4, 1993

ABSTRACT

It has been considered that target DNA is a forgiving component for PCR amplification. Herein we present evidence to demonstrate that secondary structure located at the end of a template may interfere with the specificity of amplification. Experiments indicate that nonspecific amplification results from a long stretch of stem and loop structures at the 3′ end of prochymosin cDNA. Based on the sequence of mRNA coding for prochymosin, it is argued that the sequence responsible for the formation of the complex structure described here is most likely generated during synthesis of the second cDNA strand.

Index Entries: Specificity of PCR amplification; complex structure of prochymosin cDNA.

INTRODUCTION

The polymerase chain reaction (PCR) has become a widespread technique applied both in molecular biology and biotechnology. Many papers and monographs (*1,2*) have dealt with the optimization of the parameters of PCR to achieve success for diverse purposes. Among these parameters, most attention has been focused on the primer, buffer, cycling, and enzyme, but less on the target DNA, since the DNA template is considered

*Author to whom all correspondence and reprint requests should be addressed.

as a more forgiving reaction component. During work on site-specific mutagenesis of the prochymosin gene, we found the complex structure of target DNA strongly complicated the PCR amplification. The evidence is presented in this paper.

MATERIALS AND METHODS

Preparation of Template DNA and Oligonucleotide Primers

The *Eco*RI-*Eco*RI fragment containing codons for Cys250–Cys283 (pepsin numbering) was obtained from the expression plasmid harbouring bovine prochymosin cDNA PTaAC 5 (*3*) and used as the template DNA. The following oligonucleotide primers P1–P4

P1 5′ GACATCGACGACGACAACCT 3′ (1)
P2 5′ AAGCCACTGGTTCCGAAGCCCTGGT 3′
P3 5′ GAATTCGACGGGATCCTGGGGATG 3′
P4 5′ TGAATTCGAGCTCGGTACCCGGGGA 3′

where synthesized with the ABI 381A DNA synthesizer in trityl-off mode. After deprotection with concentrated ammonium hydroxide, the primers were desalted on a Sephadex G-50 minicolumn.

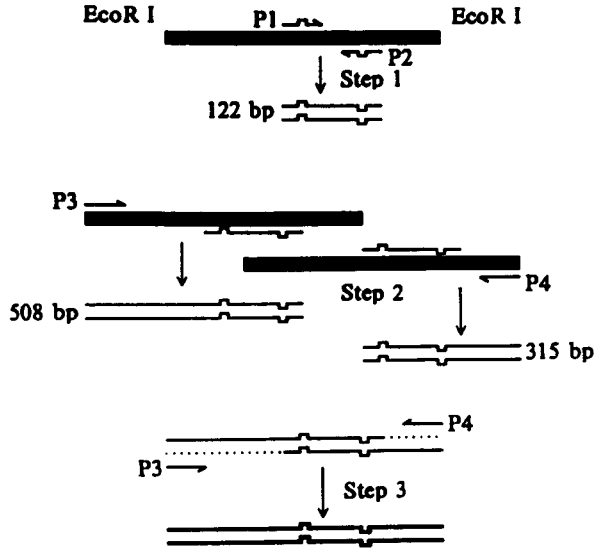
PCR Amplification

The strategy for PCR amplification to generate mutagenesis is shown in the following schematic diagram.

Reaction mixture (100 μL) contained 50 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 2.5 m*M* $MgCL_2$, 0.1% w/v gelatin, 0.20 m*M* each of dNTPs, 0.25 mg template DNA, 1 m*M* each of primers, 2.5μ Taq DNA Polymerase (Perkin-Elmer/Cetus Instruments, Norwalk, CT). Thirty PCR cycles (94°C × 1 min, 45°C × 1 min, 72°C × 2 min) were performed after initial denaturation (94°C × 5 min). A 5-μL aliquot was drawn out for 1.5% agarose gel electrophoresis to analyze the PCR products.

Sequencing

The Promega T7 DNA Polymerase Sequencing System was used to determine the sequence of the 3′ end of PTaAC 5 on double-stranded plasmid DNA. The annealing primer was PUC/M13 forward primer (24mer).



Scheme 1.

RESULTS AND DISCUSSION

Amplification of 122-bp and 508-bp Fragments

The distance between the codons for Cys250 and Cys283 within the chymosin structural gene is estimated to be 99 bp. When P1 and P2 were used as primers to initiate the PCR amplification, a 122-bp fragment should be produced. This is true, as shown in Fig. 1A. Extension from the primers P3 complementary to the 5′ end sequence and 122-bp fragment will result in the accumulation of a 508-bp fragment. As expected, a homogeneous product was obtained exclusively (data not shown).

Amplification of 315-bp Fragment

From the schematic diagram of strategy for site mutation, it is anticipated that the primer P4 complementary to the 3′ end sequence and 122-bp fragment should direct the synthesis of a 315-bp fragment. However, against expectation seven bands were revealed in Fig. 1B. When primer P1 was used instead of the 122-bp fragment to pair with primer P4 for extension, six products with different size were produced (data not shown), indicating nonspecific amplification occurred once again.

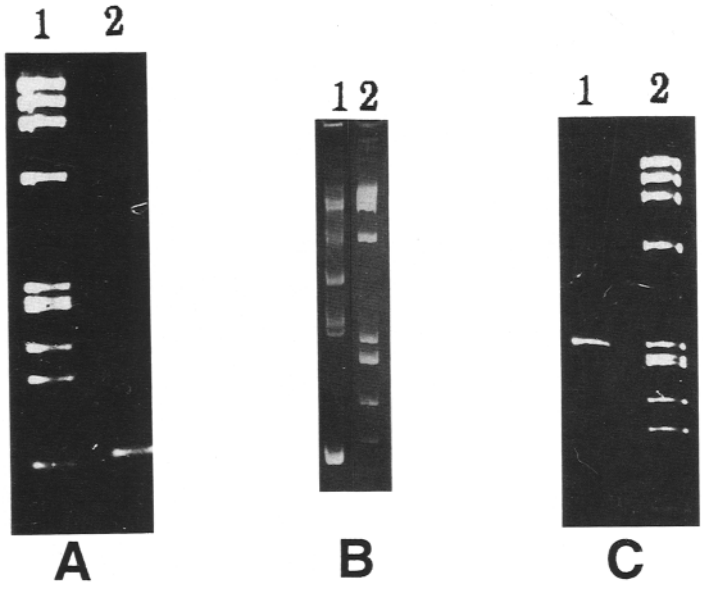


Fig. 1. Electrophoresis of PCR-amplified products. (**A**). PCR-amplified 122-bp fragment (Lane 2). P1, P2 used as primers. Lane 1: Φ174/*Hae*III used as marker. (**B**). PCR-amplified fragments (Lane 1). P4, 122-bp fragment used as primers. Lane 2: Φ174/*Hae*III used as marker. (**C**). PCR-amplified fragment (Lane 1). P1, P4 used as primers, *Eco*RI-*Eco*RI fragment with deletion of 102 bp used as template. Lane 2: Φ174/*Hae*III used as marker.

Interference by the Complex Structure of the Template with the Specific Amplification

Since computer analysis has indicated that all the base-pairings between each primer (P1–P4) and its corresponding complementary sequence on the template are specific, it is reasonable to assume that the failure of specific amplification of the 315-bp fragment could be related to the unknown structure at the 3′ end of the target DNA. Previous experiments showed that whenever the *Eco*RI-*Eco*RI fragment from plasmid PTaAC 5 was cloned into the M13 vector, a fragment of approx 100-bp within the 3′ untranslated region was deleted. Therefore, we took advantage of this fact and prepared *Eco*RI-*Eco*RI fragments with about 100 bp deletions from the M13 mp19 subclone for further PCR amplification. As shown in Fig. 1C a single product of about 310 bp was produced, suggesting that a 3′ untranslated sequence from PTaAC 5 interfered with the specific amplification.

To characterize its structure, the sequence of the 3′ untranslated region was directly determined on the double-stranded plasmid PTaAC 5 by using the dideoxy chain termination method. Based on the primary sequence it is rational to assume a special secondary structure with a 53 bp stem and

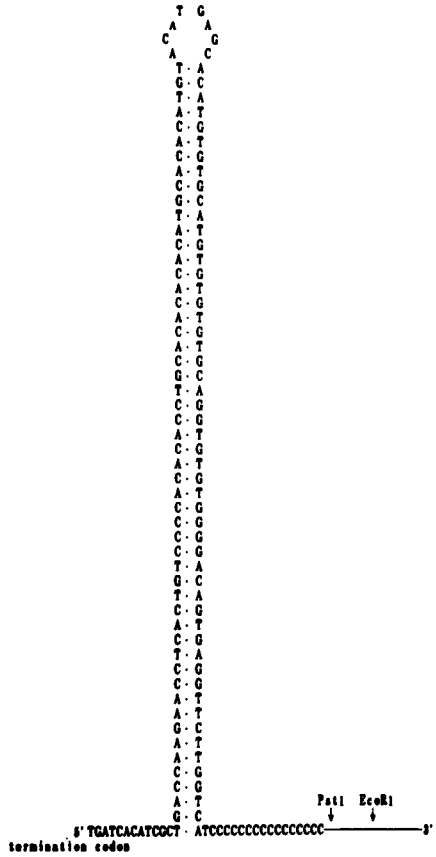


Fig. 2. Sequence and potential secondary structure of 3′ untranslated region from PTaAC 5.

an 8-nucleotide loop (Fig. 2). Most likely, the secondary structure with the long stretch of stem was restored partially during annealing or/and extension, and in turn impeded the specific amplification. An incomplete extension through this region could give rise to a number of products that could "reprime" upstream and produce several fragments larger than 315 bp.

Compared with the 3′ untranslated region of the bovine prochymosin cDNA reported by Harris et al. (4) the first 80 nucleotides from both sources are identical, whereas the others are quite different (Fig. 3). In our case the signal for polyadenylation, AATAAA, and the poly A tail were missing. Instead, the last stretch of sequence was complementary to the preceding sequence with the center at positions 66–67 from the termination codon TGA. Obviously, the 3′ untranslated sequence reported by Harris et al. represents the natural sequence, whereas its counterpart obtained in this laboratory most likely resulted from the snapback of the second-strand cDNA during its synthesis.

```
Harris's    TGATCACATCGCTGACCAAGAACCTCACTGTCCCCACACA
This lab's  TGATCACATCGCTGACCAAGAACCTCACTGTCCCCACACA

            CCTGCACACACACATGCACACATGTACATGAGCACATGTG
            CCTGCACACACACATGCACACATGTACATGAGCACATGTG

            CACACACACAGATGAGGTTTCAGACAGATGATTCTCAATA
            TGCATGTGTGTGTGCAGGTGTGTGGGGACAGTGAGGTTCT

            AACGTTGTCTTTCGTCAAAA
            TGGTCAT··· ··· ··· ··· ··· ··· ···
```

Fig. 3. Comparison of the 3′ untranslated sequences of prochymosin cDNA determined by Harris et al. (4) and this laboratory.

In fact, the natural sequence around positions 66 and 67 contains repeated doublets of CA and GT. This specific sequence may be able to form a secondary structure. Therefore the formation of snapback DNA is not simply an artificial event. In this situation we prefer to emphasize that it is wise to pay attention to this problem during construction of a cDNA library to facilitate screening of authentic and natural cDNA of the gene of interest.

REFERENCES

1. McPherson, M. J., Quirke, P., and Taylor, G. R. (1991), *PCR: A Practical Approach*. Oxford University Press, Oxford.
2. Saiki, R. K. (1989), In *PCR Technology*, Henry A. Erlish (ed.), Stockton Press, New York, pp. 7–16.
3. Zhang, Y. Y., Zhou, W., Liu, N. J., and Yang, K. Y. (1991), *Chin. J. Biotechnol.* **7**, 195.
4. Harris, T. J. R., Lowe, P. A., Lyons, A., Thomas, P. G., Eaton, M. A. W., Millican, T. A., Patel, T. P., Boss, C. C., Carey, N. H., and Doel, M. T. (1982), *Nucleic Acids Res.* **10**, 2177.