SSCP pattern was found in both subjects, and therefore they did not have the mutation present in their relatives (fig 2). However, there is still a possibility that II:6, and therefore III:10, are carriers of Fabry disease.

As patients with variant (cardiac) Fabry disease present mainly with cardiac symptoms resulting from cardiomyopathy, and because these patients have none or a mild form of the renal symptoms characteristic of the classic form of the disease, the correct diagnosis may be missed. The variant form is rare but may account for a few of the numerous cases of cardiac disease in men and should be considered when several cases of cardiac disease occur within a single family.

We thank Elsebeth Lund Christensen and Minna Becher for excellent technical assistance. This study was financially supported by the Danish Medical Research Council, Købmand i Odense Johann og Hanne Weimann f. Seedorffs legat, Signe and Peter Gregersens Mindefond and Novo's Fond.

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J Clin Pathol: Mol Pathol 1996;49:M312–M314

Novel primer specific false terminations during DNA sequencing reactions: danger of inaccuracy of mutation analysis in molecular diagnostics

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Abstract

The determination of nucleotide sequence is fundamental to the identification and molecular analysis of genes. Direct sequencing of PCR products is now becoming a commonplace procedure for haplotype analysis, and for defining mutations and polymorphism within genes, particularly for diagnostic purposes. A previously unrecognised phenomenon, primer related variability, observed in sequence data generated using Taq cycle sequencing and T7 Sequenase sequencing, is reported. This suggests that caution is necessary when interpreting DNA sequence data. This is particularly important in situations where treatment may be dependent on the accuracy of the molecular diagnosis.

(*J Clin Pathol: Mol Pathol* 1996;49:M312–M314)

Keywords: novel primer specific terminations, DNA sequencing, molecular diagnostics.

In the field of human genetics, every disorder studied at the molecular level requires nucleotide sequence determination. The information obtained from mutation analysis is often essential for understanding the structure and function of the proteins encoded by the genes under study. Identification of mutation(s) in a particular gene, which consistently segregates with the disease phenotype in pedigrees, provides proof that a given candidate gene is really the 'disease' gene in a specific inherited condition. The accuracy of mutation analysis is especially important for prenatal diagnosis where the continuation of a pregnancy may be dependent on the results of the molecular assay.

The dideoxy chain terminator DNA sequencing method is the most popular procedure because of its practical simplicity. It was originally described by Sanger *et al*¹ and many modifications to the original protocol have been introduced over the years. The two most common methods of DNA sequencing at

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Accepted for publication 18 July 1996

Figure 1 PCR and RT-PCR were performed as previously described.⁴ PCR products were purified using Wizard DNA clean-up columns (Promega) following the manufacturer's protocol. Two methods for generating sequence reaction products were used. (1) Cycle sequencing: 25–50 fmoles of purified PCR product was sequenced directly using the Promega fmol cycle sequencing kit and γ^{32} P end-labelled sequencing primer, as described by the manufacturers. (2) Sequenase sequencing: the T7 DNA polymerase Sequenase Version 2.0 kit (USB) was used with the following protocol. The sequencing primer was end-labelled as for cycle sequencing; 2 pmoles of labelled primer was annealed to ~250 ng of PCR product, in 1× Sequenase buffer in a 10 µl volume, by incubation at 100°C for five minutes followed by immediate quenching in dry ice/ethanol for five minutes. The tubes were microcentrifuged briefly to collect all condensate at the bottom. Sequenase enzyme mix (5.5 µl) (containing 7 µl TE, 10 µl water, 4 µl 0.1 M DTT, and 1 µl Sequenase) was added to the annealed primer and template. This mixture (3.5 µl) was then aliquoted to tubes, or microtitre tray wells, containing 2.5 µl of each of the ddNTP termination mixes. DNA polymerisation was performed at 37°C for 10 minutes. Reactions were terminated by addition of 4 µl formamide loading buffer. Sequencing reaction products from all reactions were analysed on buffer gradient gels⁵ followed by autoradiography. This figure illustrates the factor XIIIa exon 1 sequence observed with the 1R primer using the same PCR product template in Sequenase and Taq cycle sequencing. The T/A observed with Taq cycle sequencing is at position 176 from primer 1F and at position 208 from primer 1R.

present are the use of T7 DNA polymerase (Sequenase) and cycle sequencing using a thermostable DNA polymerase. The latter method has the particular advantage of requiring only fmoles of template DNA and is thus becoming the method of choice. Here, we present novel data on inherent primer related problems associated with DNA polymerases, which may lead to false sequencing results and discuss the level of caution necessary when undertaking molecular diagnosis.

Sequences analysed

Template DNA was generated by PCR and sequenced directly using Sequenase and Taq cycle sequencing. (1) Exon 1 of the factor XIIIa gene² was amplified using primers 1F (5'-dTACTCCCAGCAACTGGTTG-3') and 1R (5'-dCTGGCTCATAGGGTGCAC-3') to yield a PCR product of 383 base pairs (bp). (2) RT-PCR products corresponding to nucleotides 80 to 761 (codons 1 to 225) in the factor XIIIa cDNA³ were amplified from a normal (A1) and factor XIIIa deficient patient (A2). RT-PCR products A1 and A2 were generated, from cDNA, using oligonucleotides AF (forward primer: 5'-dCAAAAATGTCAGAAAC-TTCC-3') and AR (reverse primer: 5'-dCAG-CTTCTGGTCTTGATGT-3'). Each product was directly sequenced using primers AF, AR, AFn (5'-dAGGACCGCCTTTGGAGGC-3') which is nested immediately 3' to AF, and ARn (5'-dAATTACCCCGATGTCATT-3') which is nested by 19 bases 3' to AR.

RT-PCR product A1, from a normal subject, is 682 bp long and encompasses exons 2 to 5 of factor XIIIa cDNA³. RT-PCR product A2 is 493 bp long, and is generated from a factor

XIIIa deficient patient carrying a mutation at the exon 3/intron 3 splice junction.⁴ This mutation results in exon 3 being skipped during RNA processing.⁴ Hence, RT-PCR product A2 lacks exon 3 sequence and is consequently shorter by 189 bp than the A1 RT-PCR product. All 'incorrect' sequence data described herein could be consistently and reproducibly generated.

Results and Discussion

To ensure accuracy of data, DNA sequence analysis is always carried out both on the forward and reverse strands. Accuracy becomes an issue when forward and reverse strand sequencing generates contradictory results, particularly during mutation analysis in molecular diagnostics. This can sometimes be resolved by using a DNA polymerase of higher fidelity. We have observed a number of such instances and present one from analysis of exon 1 of the factor XIIIa gene.² During Taq cycle sequencing, the correct expected sequence was obtained with the forward primer 1F, but sequence analysis of the second strand using the reverse primer 1R gave two bands at position 176, giving the sequence 5'-1R³⁸³ ... C¹⁸⁰AGC T/A¹⁷⁶ GGA¹⁷³ ... 1F¹-3' (fig 1). When the same PCR products were sequenced using T7 DNA polymerase (Sequenase sequencing) and the reverse primer 1R, the misincorporation at position 176 was not observed (fig 1), stressing the higher accuracy of T7 DNA polymerase.

Taq DNA polymerase is known to have a higher misincorporation rate compared with other DNA polymerases,⁶ and the misincorporated bases are thought to be random. However, in cycle sequencing using Taq, the sequencing reaction products are not created by an amplification procedure. Thus, misincorporation in sequencing reactions does not have a significant effect on the final result, unless the dideoxynucleotide misincorporation is consistently at the same nucleotide in a major proportion of the sequencing template fragments in the reaction. It is only in PCR that errors during the early cycles can amplify and lead to errors in the end product. It is probable that the misincorporation seen with Taq cycle sequencing in fig 1 is not a random process, but may be specifically dependent on some as yet unknown local conformational factor. Also, the misincorporation may be influenced by the presence of the restriction enzyme PvuII palindromic sequence (fig 1).

Bands at the same position in two or three lanes, occurring at specific regions, are often due to band compression where a DNA strand is forming secondary structure during gel electrophoresis, leading to anomalous migration.⁷ This cannot be the explanation for the misincorporation band seen with Taq cycle sequencing, in lane A in fig 1, as this band is clearly not present in the Sequenase sequencing reaction products analysed on the same gel.

RT-PCR products A1 and A2, described above, generated the same results when sequenced directly using Sequenase or Taq cycle

Figure 2 Total RNA was isolated from peripheral blood mononuclear cells, reverse transcribed and RT-PCR products A1 and A2 generated and analysed as described in the text and previously.⁴ In this figure RT-PCR products A1 and A2 were sequenced using primers (A) AF and (B) AFn. The heterozygous A/G band in A1 shown in (A) is at +126 bases from the forward primer AF, and at -10 bases from the 3' end of exon 2. The data presented in this figure were obtained in three independent sequencing reactions, one of which is shown.

sequencing. Sequence analysis of product A1 with primer AF (forward PCR primer) showed it to be heterozygous at position 126 (the first nucleotide of codon 40, AAC, in the factor XIIIa cDNA), from the forward primer, giving the sequence 5'-AF¹...¹²³GTC¹²⁶A/G AC¹²⁹C...⁶⁸²AR-3' (fig 2A). The sequence result in this position from RT-PCR product A2 sequenced with primer AF was as expected: 5'-AF¹...¹²³GTC¹²⁶A AC¹²⁹C...⁴⁹³AR-3' (fig 2A). When both the RT-PCR products A1 and A2 were sequenced with primer AFn (forward nested primer), each was found to be homozygous for 'A' at position 126 (fig 2B). Hence, the expected sequence was obtained. Also, sequence analysis of products A1 and A2 with the reverse primers AR and ARn confirmed that both products are homozygous at base 126. Thus, there is not a consistent PCR derived misincorporation present in these sequencing substrates and Sequenase can consistently catalyse specific incorrect termination which is primer dependent (present with AF, absent with AFn).

GTCGAC is a recognition sequence for the restriction enzyme Sall. Digestion of products A1 and A2 resulted in fragments correlating with the expected sequence (data not shown), thus confirming the absence of a Sall site around position 126 in A1 (fig 2A). The 'false stop', G, seen at base 126 thus results from a combination of the specific nucleotide se-

quence around this position in the RT-PCR product A1, the primer AF sequence, and its separation from residue 126.

A second consistent primer/template dependent effect on Sequenase sequencing reaction products was seen during analysis of the factor XIIIa exon 1 sequence described earlier. At position 94 the nucleotide obtained differed with the forward and reverse primers. The sequence obtained from the forward primer was always 5'-1F¹...GGGG⁹⁴C AAGT...³⁸³1R3', but the sequence obtained from the reverse primer was invariably 5'-1R³⁸³...ACTT⁹⁴A CCCC...¹1F-3', using the same sequencing substrate (data not presented).

In summary, our data show that both Sequenase and Taq DNA polymerases can generate consistently and reproducibly incorrect sequence data. The inaccuracy observed may be influenced by a combination of primer sequence, the local conformation around the site of misincorporation and the distance between the primer and the termination point. The primer related sequence errors seem more likely to occur with Taq DNA polymerase than with Sequenase.

Although the consistent misincorporation products described herein all involve addition of dideoxy nucleotide residues, which are known to have very different enzyme affinities to their dNTP counterparts, the phenomenon observed is sufficiently worrying to warrant comment. We are not aware that this highly specific effect has been described previously. Thus, it may be advisable to perform two independent assays for molecular diagnosis—for example, sequence and restriction analysis. In situations where a restriction site does not exist around the mutation site, and sequence analysis using forward and reverse primers gives contradictory results, it may be necessary to determine nucleotide sequence with a different, third sequencing primer. This would be particularly important in prenatal diagnosis where the decision to proceed with the pregnancy will generally depend on the diagnostic result obtained.

Work in our laboratories is supported by the Medical Research Council, Wellcome Trust, Northern and Yorkshire Regional Health Authority, West Riding Medical Research Trust, and Yorkshire Cancer Research Campaign.

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