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Incorporation of dITP or 7-deaza dGTP during PCR improves sequencing of the product

Herman Dierick, Michel Stul, Wim De Kelver, Peter Marynen* and Jean-Jacques Cassiman
Center for Human Genetics, Campus Gasthuisberg, O&N6, Herestraat 49, B-3000 Leuven, Belgium

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Premature enzyme pausing due to regions of complex secondary structure is a common problem in sequencing reactions. This not only makes the sequence impossible to interpret at this particular region, but often decreases the signal intensity of the rest of the sequence as well. Different methods have been proposed to diminish secondary structure formation (1–4).

We tested some of these methods in the sequencing of PCR products of several 'problem-fragments' of the p53 gene without success, suggesting that the secondary structures in these particular isolated single strands were very stable.

It was previously reported that 7-deaza dGTP allows the amplification of stable hairpin loop structures (5). Moreover this nucleotide analog and dITP (a more economical alternative), are frequently used in sequencing reactions to prevent base compressions, caused by stacking of the sequenced fragments, which lead to abnormal migration patterns during gel electrophoresis (6). Therefore we used these nucleotides to generate PCR fragments containing one of these analogs instead of, or together with, dGTP. We anticipated that the isolated single strand template molecules would show less or no secondary structures, allowing the T7 polymerase to read through the whole sequence. However, it has been reported that dITP incorporation in PCR can decrease PCR efficiency and increase misincorporation by Taq polymerase. To prevent these unwanted effects, we applied the specific conditions as mentioned (7).

The different nucleotides were added to the PCR of a 560 bp fragment encompassing exon 4, and the effect on premature enzyme pausing during sequencing was examined. With all PCR conditions, electrophoresis and ethidium bromide staining showed that only full-length products were obtained. After strand separation using streptavidin coated magnetic beads, an FIT-C-labeled internal sequencing primer was used, generating sequences of approximately 400 bases. Figure 1 shows raw sequence data, obtained on an ALF sequencing device (Pharmacia), of the problem region of exon 4 using different conditions. All sequencing reactions were performed using the Autoread sequencing kit (Pharmacia) in which dGTP is replaced by 7-deaza dGTP as a standard feature. The problems occurring under normal conditions (A) (using 7-deaza DGTP during the sequencing reaction), are resolved when utilizing dITP (E) during PCR. When 7-deaza dGTP is included during the PCR, the problem region is shifted approximately 50 bases downstream (D). The use of 10% formamide (B) or DMSO (C) during sequencing, as reported previously (1, 2), was without effect; neither was sequencing at higher temperature (adding glycerol to the sequencing mix to protect T7 polymerase). Comparable results were obtained for the sequencing of the shorter fragment containing exon 7 of p53 and of the D-loop region of mitochondrial DNA.

In conclusion, incorporation of dITP or 7-deaza dGTP (to a lesser extent) in PCR reactions appears to be an easy and effective method to avoid premature enzyme pausing during sequencing of PCR templates even with 7-deaza dGTP in the sequencing mixes.

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* To whom correspondence should be addressed

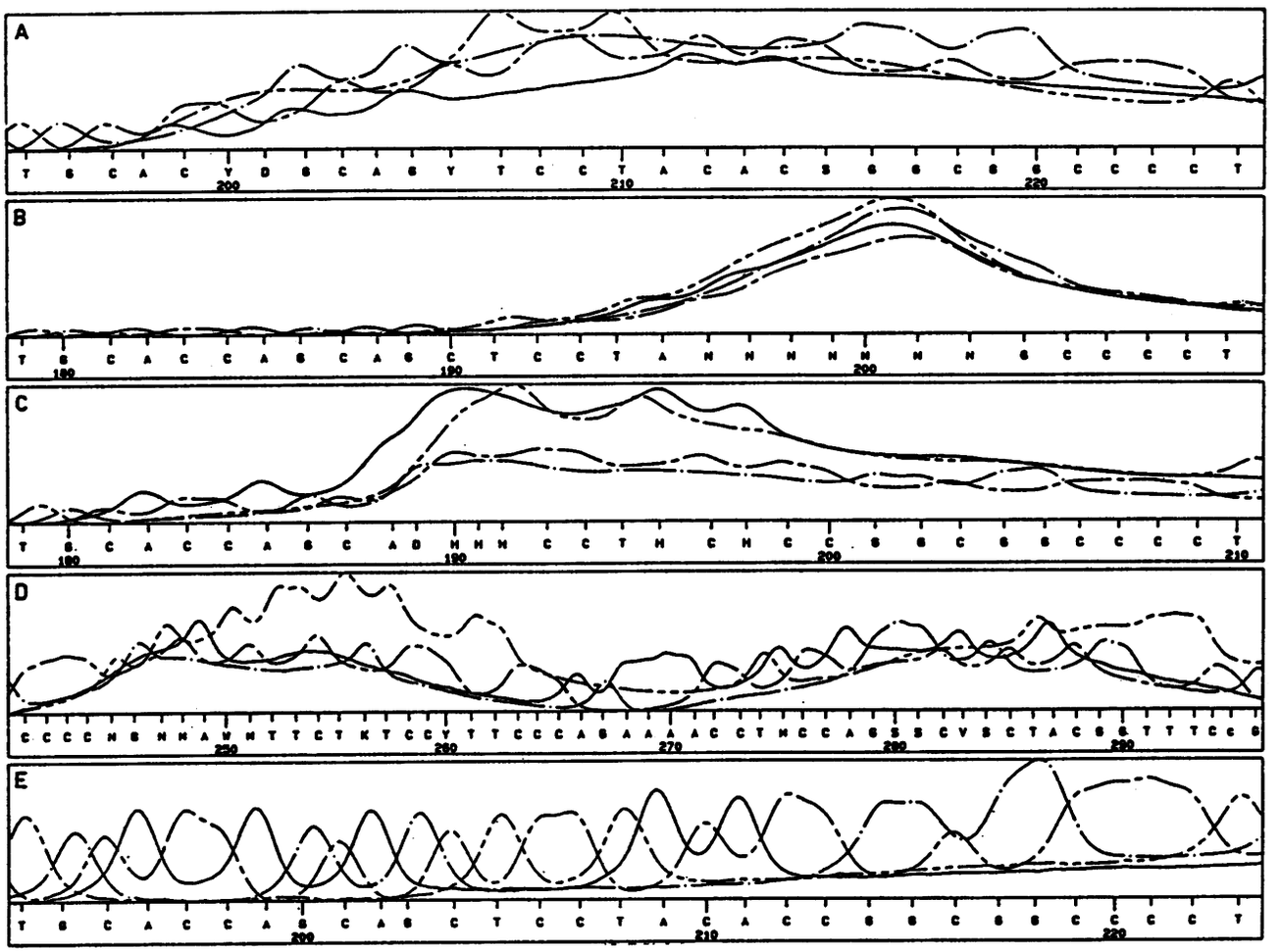

Figure 1. PCR of the 560 bp fragment of exon 4 of the p53 gene was performed under different conditions. The following reagents were used under normal conditions (A–C): 1 μg of genomic DNA, 20 pmol of each primer (3′ primer was biotinylated at the 5′ end), 200 μM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatin, 2.5 U Taq polymerase (BRL) in a final volume of 100 μl. In (D), dGTP was replaced completely by 7-deaza dGTP for the PCR, and in (E) dITP was added in a final concentration of 200 μM together with dGTP in a final concentration of 40 μM during the PCR. Thirty cycles of denaturation (40 seconds at 94°C), annealing (40 seconds at 60°C) and extension (50 seconds at 72°C) were performed on an automated heat-block (Pharmacia). Strand separation was performed on 80 μl of this PCR product using 50 μl of streptavidin coated magnetic beads (10 mg/ml) (Dynal). The isolated single strand was then sequenced with 2 μl of a 0.4 OD solution of a specific FITC labeled internal sequencing primer using the Pharmacia sequencing kit. In the DMSO-condition (C), DMSO was added after primer annealing prior to sequencing to a final concentration of 10% in the termination step. In (B) formamide was added to a final concentration of 10% during the annealing and termination step. The sequencing products were heated for 3 minutes at 85°C before loading and were run on a 6% Hydrolink gel at 34 W, 38 mA, 1500 V at a constant temperature of 42°C using an ALF DNA Sequencer (Pharmacia).