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A DNA cycle sequencing reaction that minimizes compressions on automated fluorescent sequencers

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ABSTRACT

We recently demonstrated that most band compressions (>70%) on DNA sequencing gels result from the presence of a single sequence motif, 5′-YGN$_{1–2}$AR-3′, where Y and R indicate base-pairing pyrimidine and purine residues, respectively. This finding raised the possibility that the use of 7-deaza-dATP instead of dATP in chain termination sequencing reactions would resolve most of the band compressions. Thus, we examined the effects of 7-deaza-dATP on DNA sequencing using thermostable DNA polymerases. The results indicate that the replacement of dATP with 7-deaza-dATP in conventional cycle sequencing reactions can successfully eliminate most band compressions without sacrificing sequencing performance.

The anomalous electrophoretic behavior of DNA fragments on DNA sequencing gels, known as band compressions, has been known to pose an obstruction to the high-speed and accurate determination of DNA sequences. Although band compressions have been vaguely ascribed to the formation of secondary structure at the growing end of sequencing reaction products (1), we recently discovered that there are only two types of sequence motifs that cause band compressions in a conventional sequencing system (2). Most of the compression sites (>70%) were found to include a unique sequence, 5′-YGN$_{1–2}$AR-3′, (where Y and R respectively indicate pyrimidine and purine nucleotides capable of base pairing) which we termed the M-type motif. The other sites carried various palindromic sequences with a three to four base–loop accompanied by a relatively long GC-rich stem, which we termed the L-type motif (2). Interestingly, Hirao and co-workers previously reported that two particular M-type sequences, 5′-GCGAAAGC-3′ and 5′-GCGAAGC-3′, were capable of forming extraordinarily stable mini-hairpin structures where the N7 atom of the A residue in a non-Watson–Crick type G–A base pair plays a key role in its folding (3–6). Thus, we deduced that the replacement of adenosine with 7-deaza-adenosine would destabilize and unfold the hairpin structures caused by the M-type motifs. However, it remained to be shown experimentally that this modification of conventional DNA thermal cycle sequencing reactions would effectively resolve band compressions without sacrificing the performance of the sequencing method.

We examined the effects of substitution of dATP with 7-deaza-dATP (c^{7}dATP) in a standard cycle sequencing reaction [containing 7-deaza-dGTP (c^{7}dGTP)] using Thermo Sequenase (Amersham Life Science, Inc., USA) on a 1 dye–4 lane DNA sequencer

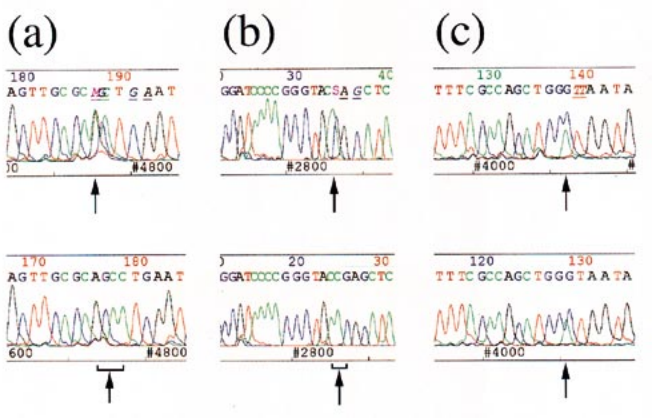

Figure 1. Effects of replacement of dATP with c^{7}dATP on band compressions. Cycle sequencing reactions were carried out using a Thermo Sequenase cycle sequencing kit from Amersham Life Science, Inc. The reaction conditions were essentially the same as described in the kit for the labeled primer-assisted method except that the nucleotide concentration of each dNTP in the final reaction mixture was raised to 150 µM while the ddNTP concentration was maintained at 0.75 µM for long reads. The template DNA was pUC19 and the primer was a fluorescein-labeled M13 reverse primer from Takara Shuzo Co., Ltd, Japan. The sequencing reaction products were analyzed on 4.2% Long Ranger^{TM} gels containing 7 M urea with a Shimadzu DSQ1000L DNA sequencer and the gel temperature was maintained at 40°C during runs. The upper charts were obtained by cycle sequencing reactions with a conventional set of nucleotides (dATP, dCTP, c^{7}dGTP and dTTP) and the lower charts were obtained under the same conditions except that dATP was replaced by c^{7}dATP. (**a**) A typical compression site containing the M-type motif; (**b**) an example of the L-type compression site resolved by the use of c^{7}dATP; (**c**) a typical compression site carrying the L-type motif.

(DSQ1OOOL, Shimadzu Corp., Japan). The detailed reaction conditions are given in the legend to Figure 1. First, it became evident that the use of c^{7}dATP actually eliminated the M-type band compressions completely (Fig. 1a). Band compressions at some of the L-type motif sites were also resolved probably because G–A base pairing in the loop contributed to their folding (Fig. 1b). In contrast, the compressions caused by most L-type motifs could not be removed by this modification, as expected (Fig. 1c). The replacement of dATP with c^{7}dATP did not result in any deterioration of the quality of sequence patterns with respect to readable length and band uniformity (Fig. 2). Another popular thermostable DNA polymerase, AmpliTaq® DNA polymerase FS (Perkin Elmer Corp., USA), also gave the same results on an ABI 373S DNA sequencer (4 dyes–1 lane system, data not shown) as those obtained using Thermo Sequenase. Thus, cycle sequencing reactions with c^{7}dATP, c^{7}dGTP, dCTP and dTTP as a set

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Figure 2. Comparison of sequence charts obtained by conventional and c^7dATP cycle sequencing reactions. The reaction conditions were as described in the legend to Figure 1. The entire sequence charts are shown in order to demonstrate that the replacement of dATP with c^7dATP did not affect the readability of the sequence over a region of 1000 bases.

of a deoxynucleotides worked well for dye–primer DNA sequencing using automated fluorescent sequencers. Also, the resolution of band compressions due to the presence of c^7dATP in the sequencing reaction mixture was consistent with our expectations.

c^7dATP has been used previously for reducing band compressions on sequencing gels when a non-thermostable DNA polymerase was used in the sequencing reaction. However, no theoretical framework as to how it worked was provided (7). The results in this study indicate that c^7dATP resolved band compressions by unfolding hairpin structures caused by the M-type motif. In addition, we show that c^7dATP can be used in cycle sequencing reactions using newly emerging thermostable DNA polymerases engineered for high quality DNA sequencing. Although dITP is also known to destabilize M-type hairpin structures, c^7dATP is better than dITP for dye–primer cycle sequencing since polymerase stoppage would be a problem if dITP were used (4). Although band compressions by classic L-type motifs were not resolved using sequencing reactions containing c^7dATP, L-type compressions occurred less frequently than M-type ones (<30% of total compression sites) and could be solved by computer, as they can be detected more easily due to the presence of a relatively long GC-rich stem region in their palindromic motif.

In conclusion, we have successfully demonstrated that the presence of c^7dATP besides c^7dGTP in cycle sequencing reactions can minimize band compressions on DNA sequencing gels, and that the technique represents a simple and robust alternative to conventional methods such as alterations of gel electrophoresis conditions (4,8–9).

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