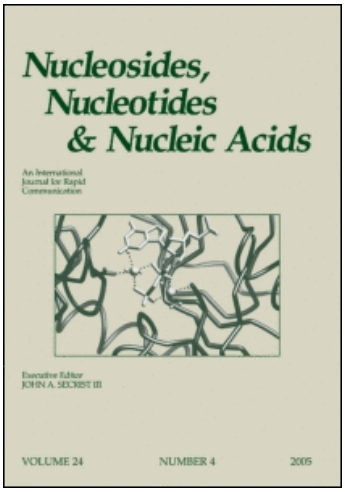

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Enzymatic Fluorescence and Biotin Labelling of Primers for Dna Sequencing and Pcr

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ENZYMATIC FLUORESCENCE AND BIOTIN LABELLING OF PRIMERS FOR DNA SEQUENCING AND PCR

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ABSTRACT. Using terminal deoxynucleotidyltransferase, it is possible to tag post-synthetically a sequencing primer with a fluorescent label. This procedure gives rise to a 3' terminally labelled oligodeoxynucleotide, elongated by a single labelled *ribonucleotide* that retains its substrate properties for a variety of DNA polymerases. A 3'-terminal biotin molecule may be incorporated into a PCR primers analogously.

INTRODUCTION

The rate of DNA sequence data acquisition for such large undertakings as the human genome project has been dependent on the development of rapid sequencing techniques, including automated data collection performed at present largely by the use of fluorescence-based detection systems.

The incorporation of the fluorescent label into the nascent DNA has been approached from several angles. At present, one may consider three sources of fluorescence, irrespective of the principle governing the design of the sequencing hardware (FIG. 1). a. Label attached at the 5' terminus of the primer[1]. b. Label incorporated as an appropriately chemically modified dideoxy-terminating nucleotide[2]. c. Internal labelling of the growing chain using fluorescent nucleoside triphosphates during the elongation reaction[3]. In practice, the source of the label is governed to a large extent by the nature of the available automated sequencer, or, more specifically, by the principle involved in the fluorescence detection. Labelled primers tend to give reliable data[4] irrespective of the detection system and their synthesis can be straightforward. In this case the availability of the corresponding phosphoramidite provides a convenient, if expensive, route to a 5'-terminal label during primer synthesis.

Using terminal deoxynucleotidyltransferase, it is possible to tag post-synthetically a sequencing primer with a fluorescent label. This procedure gives rise to a 3' terminally labelled oligodeoxynucleotide, elongated by a single labelled *ribonucleotide* (which must be complementary to the target sequence) (FIG. 1d), that retains its substrate properties for a variety of DNA polymerases[5]. Analogously, the enzymatic introduction of a 3'-terminal biotin molecule into a PCR primer permits the subsequent isolation of the biotinylated DNA single strand for, e.g. direct solid phase sequencing of PCR products. This approach offers a unique possibility of enzymatically introducing a defined (in terms of length and position) non-isotopic label into pre-existing oligodeoxyribonucleotides, while not blocking the 3' terminus for further elongation.

RESULTS AND DISCUSSION

Terminal deoxynucleotidyl transferase carries out the template independent and therefore random 5' to 3' elongation of DNA strands in the presence of doexynucleoside triphosphates. Under certain non-physiological conditions, the enzyme also accepts ribonucleoside triphosphates[6] but the addition of these substrate analogues is limited to a maximum of two residues. For the purpose of using primers modified in this way for DNA sequence analysis, one requires, ideally, that the reaction is limited to mono-addition.

Fluorescein labelling[7]. The use of fluorescein dUTP reduces the tailing length to no more than a few nucleotides. Furthermore, in the case of fluorescein riboUTP, the addition generates almost exclusively the mono-addition product[5]. Interestingly, the exact distribution of the tail length is determined by the nature of the base. In the case of fluorescein riboATP and, to a lesser extent fluorescein riboCTP multiple additions are observed[8]. However, by an appropriate change in the reaction conditions the preferential formation of the desired mono-addition product may be induced. The efficiency of the labelling reaction lies in the range of 50-70% for all three commercially available fluorescein riboNTPs.

Primers modified in this way are accepted by all DNA polymerases tested to date, including T7, Taq, Sequitherm and ThermoSequenase. Sequences obtained are qualitatively and quantitatively indistinguishable from those seen using conventional 5'-terminally labelled primers[5,8]. Nucleotide derivatives of other dyes are very much poorer substrates[8] and are not suitable for the described application. The lack of commercially available fluorescein-riboGTP, while limiting the use of pre-existing primers to some extent, is not a major handicap in the *de novo* design of primers.

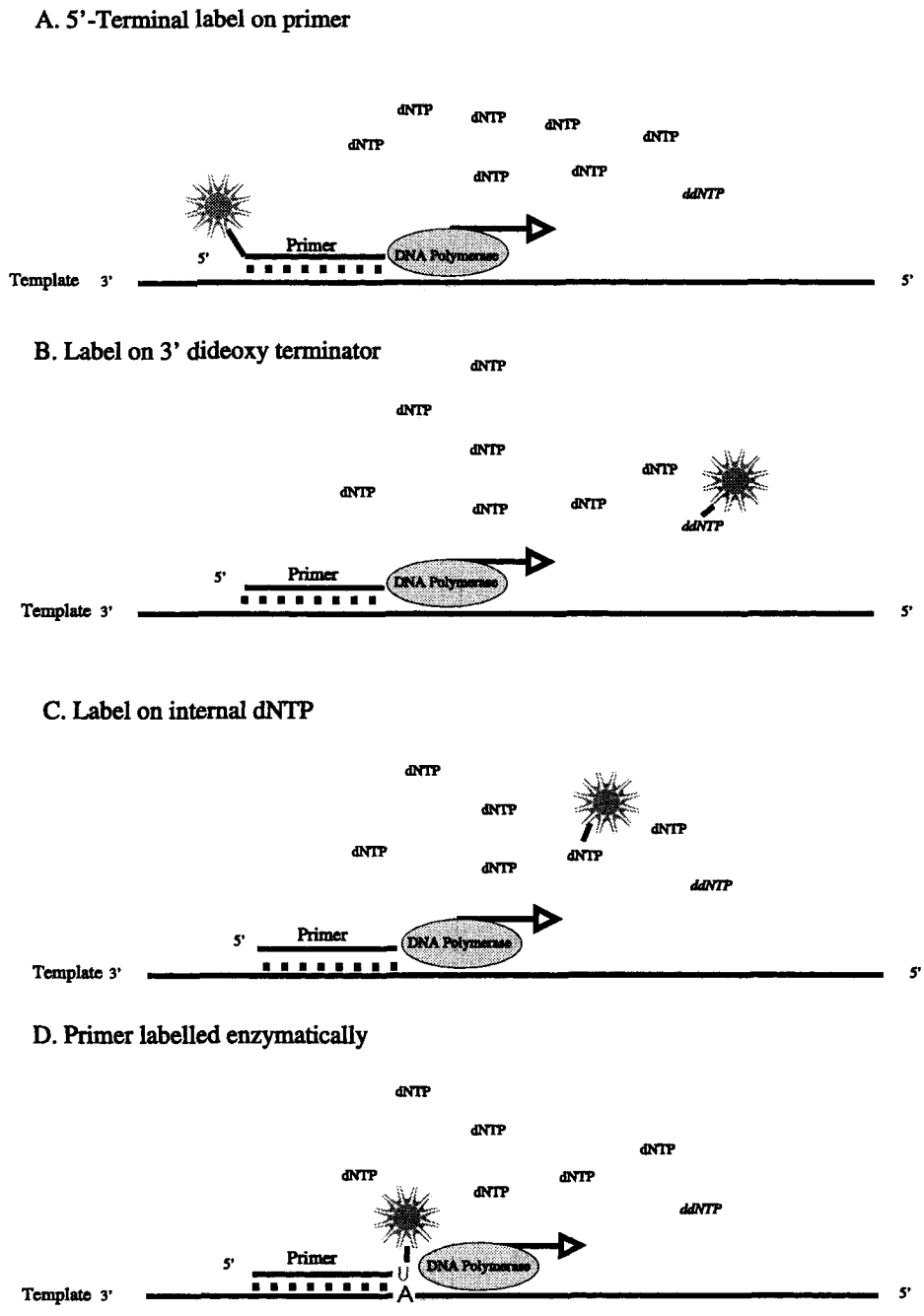

FIGURE 1. Alternative strategies for the incorporation of a fluorescent label during DNA sequencing.

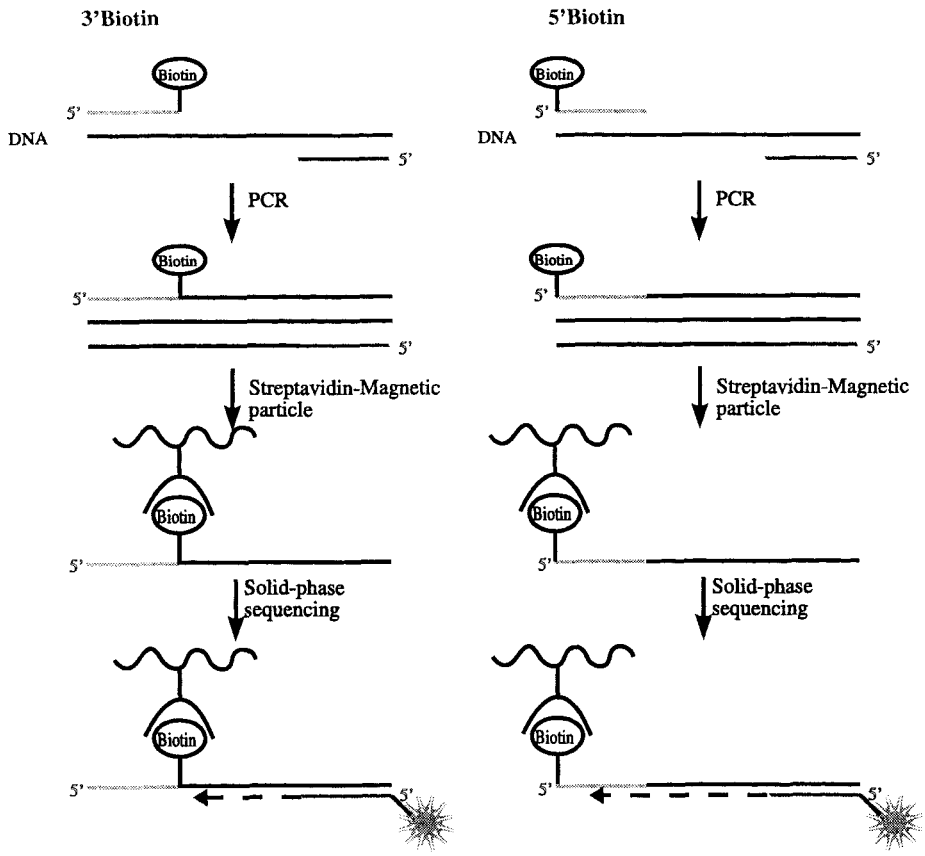

FIG. 2. Solid-phase DNA sequencing using PCR primers biotinylated at either the 3' or the 5' terminus, as indicated. After capture of the labelled strand, the DNA sequence is determined using a fluorescent primer (label denoted by a star). Note the alternative positions of the biotin in the PCR product.

Biotin labelling. For solid phase sequencing, one primer of a PCR primer pair must bear a biotin tag in order to permit the capture of the corresponding single strand by streptavidin-bound magnetic beads. Biotin-riboUTP fulfils the requirements for terminal transferase mediated 3'-terminal labelling of PCR primers, as set out above; a single nucleotide that does not interfere with the PCR reaction, is efficiently (>90%) added. The product of amplification (FIG. 2) carries the biotin at an internal position compared with the conventional 5'-terminal label. This, how-

ever, does not prevent its interaction with streptavidin-conjugated magnetic beads and biotinylated products are amenable to solid phase sequencing. In this case, as would be expected, the termination of the sequence reaction occurs just prior to the biotinylation site[5].

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