cycling and using dye terminators for AmpliTaq DNA polymerase) and is about 25% of the signal strength of the AmpliTaq FS thermal cycling reaction with 0.3 pmol of template and conventional primer. The signal strength of the dye terminators for Sequenase cannot be compared with that of dye terminators for Taq DNA polymerase because of the differences in laser detection, optics and chemistry.

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Sequencing of 3′ cDNA Clones Using Anchored Oligo(dT) Primers

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Dideoxyribonucleotide chain termination is a predominant approach in current DNA sequencing practice (4). The successful sequencing reaction depends on a sequence-specific oligonucleotide as a primer to anneal to the site upstream of the region of interest. The extension of the nucleotide chain starts from the primer. To sequence cDNA clones, the most commonly used primers are specific to the flanking regions of cloning vectors. Because most cDNA syntheses are performed using an oligo(dT) primer, some cDNA clones contain an unusually long poly(A) stretch, which frequently causes problems in 3′-end cDNA sequencing, such as premature chain termination in the sequencing reaction. Sometimes, even if the chain extension proceeds beyond the poly(A) tail, the reaction may become very weak, and the sequencing ladders are unreadable. To overcome these problems, we have designed two oligo(dT) primers with degenerated anchored nucleotides as follows: Oligo 1 (17-mer): 5′-TTTTTTTTTTTTTVN-3′ and Oligo 2 (17-mer): 5′-TTTTTTTTTTTTTAC-3′, where V = A, C and G, and N = A, C, G and T. The extra nucleotides following the T stretch serve as an anchor to fix the chain start site. Oligo 1 contains two anchored nucleotides, while Oligo 2 contains three anchored nucleotides. Similar to the anchored polymerase chain reaction (PCR) primers for RNA fingerprinting (3), these anchored oligo(dT) primers would anneal to the poly(A) sequence, but only the annealing to the very 5′ end of the poly(A) sequence allows chain extension because the annealing to the middle of the poly(A) sequence leaves 3′ overhang
nucleotides that would not be extended. The use of degenerated nucleotides at the 3′ end of the oligo(dT) primers is to ensure the universality of the primer to perfectly match all possible 3′ poly(A) regions of cDNA clones.

To test the effectiveness of these two primers in 3′ DNA sequencing, a few cDNA clones that had problems in normal 3′ sequencing were selected. Double-stranded DNA sequencing reaction was performed using the T7 Sequencing Kit according to the instruction manual of the manufacturer. (Pharmacia Biotech, Piscataway, NJ, USA). As shown in Figure 1, both Oligo 1 and Oligo 2 generate clear sequencing patterns. The efficiencies of the two oligo(dT) primers are about the same, and thus two anchored nucleotides (Oligo 1) are sufficient to perform an accurate sequencing reaction. Generally, a 200-nucleotide sequence can be read easily from a single sequencing reaction, comparable to the result achieved using a regular 17-mer sequencing primer.

Currently, we are interested in identification of zebrafish genes by sequencing 5′ ends of randomly selected cDNA clones (2). This approach enables us to identify zebrafish genes based on the conserved 5′ coding sequences. For some interesting clones, the 3′-end sequences are also desired for genetic mapping by PCR single-strand conformation polymorphism (SSCP) (1). Design of a universal sequencing primer for 3′-end sequence greatly facilitates the study. There are several advantages to using the anchored oligo(dT) primer over the vector primers. First, it can be used to sequence difficult clones with a long 3′ A tail. Second, longer effective sequences [5′ of the poly(A) sequence] can be obtained by a single sequencing reaction since the vector primer is much further from the effective sequence. Third, the 3′ sequence can be obtained without the need to determine the orientation of the cDNA insert.

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Post-PCR Labeling Using Taq DNA Polymerase

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For most polymerase chain reaction (PCR) applications, product detection/quantitation is required. Typically, the products and reactants are first separated by gel electrophoresis. Subsequently, detection can be achieved in a variety of ways. The product can be detected by staining with ethidium bromide or silver, by autoradiography or by hybridization with a labeled oligodeoxyribonucleotide probe. If hybridization is performed with an enzyme-linked probe, colorimetric or chemiluminescence-based detection is possible. Of all the detection methods, ethidium bromide staining is most commonly used because of its convenience. However, in cases where high-detection sensitivity is required, one will often have to use an isotope-based detection method.

Of the isotopic detection methods, direct labeling of the product is preferred over hybridization-based methods. For direct labeling of the product, three approaches are possible: (i) the primer(s) can be labeled prior to PCR amplification; (ii) labeled NTPs can be incorporated into the product during amplification; or (iii) the product(s) can be labeled after the amplification. Labeling after amplification (post-PCR labeling) has the advantage that only a fraction of the PCR product need be labeled for analysis. The remaining unlabeled portion can be conveniently handled in subsequent manipulations. Post-PCR labeling is often performed by 5′ labeling using T4 polynucleotide kinase and [γ-32P]ATP. This generally requires some form of post-PCR purification because polynucleotide kinase is sensitive to pH and is inhibited by a variety of salts.

Taq DNA polymerase has been shown to possess an “extendase” activity; that is, the ability to add an extra nucleotide to the 3′ end of a blunted DNA fragment (2,3). This activity may be utilized for 3′ labeling of blunt-ended PCR products generated by a thermostable DNA polymerase that lacks the extendase activity. We describe a procedure using Taq DNA polymerase to label a PCR product in a post-PCR labeling reaction. The PCR product was labeled directly after amplification in the PCR mixture without prior purification.

To utilize the extendase activity for 3′ labeling, the PCR product should be blunt-ended and preferably amplified using a DNA polymerase without an extendase activity. Therefore, we first tested the extendase activity of two thermostable DNA polymerases that have neither 5′→3′ nor 3′→5′ exonuclease activity, the Sequencing Grade (SG) Taq DNA Polymerase (Promega, Madison, WI, USA) and the Deep Vent® (exo-) DNA Polymerase (New England Biolabs, Beverly, MA, USA). Because these enzymes do not have 5′→3′ or 3′→5′ exonuclease activity, they would not remove the extended tag or shorten the PCR product during 3′ labeling. A 41-mer oligonucleotide (5′-GAG AGC GAC ACG GTG TTT CGC ACC TAT AGT GAG TCG TAT TA-3′) was labeled with [γ-32P]ATP at its 5′ end using T4 polynucleotide kinase (New England Biolabs) and annealed to