were in progress, data that was neither smoothed nor processed. The combination of Fam and Tamra reporters can have important applications for “lab-on-a-chip” devices that cannot use large, expensive and high-powered fluorescence detection systems.

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the plasmid and another one designed from the known DNA sequence (2,10).

P1 DNA clones containing the rat Tage4 gene were isolated by the screening of a rat PAC DNA library (RPCI-31, created by Pieter de Jong and Peng Yeong Woon, Roswell Park Cancer Institute, NY, USA). A 1150 bp fragment of the rat Tage4 CDNA was used to probe the filters. Eight clones were isolated and further identified by hybridization of EcoRI-digested DNA. One of these clones (clone RPCIP12-N22604Q2; http://resource.rzpd.de) was selected for further analysis. DNA (5 μg) amplified from this clone was digested with 50 U of Alul, Ddel or Hinfl for 2 h at 37°C or with 50 U of BstOII at 60°C. These enzymes have been chosen because they are 4- or 5-base cutters (AG1CT, C2TNAG, G1ANTC and CC1WGG, respectively). These sites are common in mammalian genomes, thereby eliminating the need to have any prior knowledge of restriction enzyme sites surrounding the gene of interest. Furthermore, short DNA fragments are generated facilitating their cloning and amplification.

Following purification by Wizard® PCR Preps DNA Purification System (Promega, Lyon, France), the restriction fragments were incubated at 72°C for 1 h with 5 U of Taq DNA polymerase in a total reaction volume of 50 μL containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 0.75 mM MgCl2 and 50 nM of each dNTP. Finally, the fragments from each digestion were purified and 1/10 of this product (ca. 500 ng DNA) was ligated with 50 ng of pGEM®-T (Promega) at 4°C for 2 h in a 10 μL reaction. Using this strategy, a unique cloning vector harboring 3'-T overhangs that allow the insertion of products with overhanging A residues can be used for all of the different restriction enzymes. In addition, preparation of linearized and phosphatased vector for each restriction enzyme is avoided. More importantly, this strategy allows the use of enzymes of high cutting rate, which would otherwise cut the cloning vector into several pieces.

Fragments of interest are then amplified by a “hot start” PCR, performed with 2 μL of each ligation product with one primer on the pGEM-T plasmid (M13Fw=5' CGCCAGGGTTTTCCCAGTCACGAC-3' and one specific primer sitting on the 5' end of the known sequence (Figure 1). The M13Fw primer is located 5' to the cloning site of the plasmid. Similar results were obtained using the universal M13 reverse primer, which is located on the opposite side of the cloning site. The specific primers were designed to have a melting temperature (Tm) above 65°C. This allowed us to use an annealing temperature of 63°C during the PCR and thus to reduce nonspecific priming events.

The PCR mixture contained 2 μL of ligation product, 10 pmol of M13Fw, 100 pmol of specific primer, 10 mM of Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 50 nM of each dNTP, 2 mM MgCl2 and 1.25 U of Taq DNA polymerase incorporated into wax beads (Promega) in a total reaction
volume of 50 µL. The optimal ratio of primers (M13Fw vs. the specific oligonucleotide) was found to be 1:10, which is in agreement with previously reported data (2). The inserts were amplified by denaturation at 92°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 3 min for 35 cycles in a Crocodile™ II Thermal Cycler (Appligene-ONCOR, Illkirch, France). The first cycle was preceded by an initial step at 94°C for 3 min, and the last cycle was followed by a final extension step at 72°C for 3 min. Aliquots (5 µL) of each amplification reaction were analyzed by agarose gel electrophoresis stained with ethidium bromide. Figure 2 shows an illustration of a typical walk, corresponding to the fourth walk of Figure 1. The PCR products were purified and sequenced with the primers used for the amplification.

Applying this protocol, we performed nine different walks. All of them were successful, indicating that the reliability of this procedure is high. When applied to the promoter region of the Tage4 gene, four successive walks allowed us to get sequence information on approximately 2 kb of DNA (Figure 1). The length of the walks we performed ranged from approximately 100–800 bp. This is in agreement with what can be expected using these frequent cutter enzymes. We have not observed any false positive amplification (i.e., PCR products that would not partially overlap the known sequence). Limited background amplifications were seen in very few reactions. Figure 2, lane 3 presents one such reaction. In such cases, the major band was gel purified before sequencing.

A single day is sufficient to prepare the ligation reactions. Then, the same digestion/ligation can be used repeatedly to walk in both directions from a known sequence. Indeed, the use of frequent cutter restriction enzymes results in the identification of short sequences. Therefore, in most cases, several walks will be necessary to overcome the limitation of this approach.

In summary, the method we report is simple and versatile for short genomic walks from any known sequence. We found it most convenient to quickly obtain information on the promoter and exon-intron boundaries of a given gene.

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Received 14 June 1999; accepted 17 August 1999.

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Improved Band Resolution, Loading Reliability and Reduced 32P Contamination in Mobility Shift Assays by Retention of Unbound Probe

BioTechniques 27:1122-1126 (December 1999)

Electrophoretic mobility shift assays (EMSAs) are widely used to study DNA-protein interactions (1–4). EMSA detects the binding of small amounts of transcription factors to gene promoters. Multiple proteins of similar molecular weight can bind the same DNA probe, so it is important to achieve good band resolution. One problem we encountered while separating higher molecular weight complexes and supershifted proteins is the loss of unbound probe into the tank buffer because of long running times. This run-off prevents comparison of unbound probe loads and produces