available to isolate the full-length 5′-sequence of a new isoform of cytochrome c oxidase subunit IV (3) that was only transcribed in low amounts in the available tissues, as was later confirmed by quantitative PCR.

After 5′-RACE PCR was completed, the PCR products consisted almost exclusively of truncated fragments (Figure 2B, left gel), clearly smaller in size than expected. Therefore, bands for DNA extractions were excised in the expected size range (Figure 2B, left gel, arrow), even if no DNA was visible in that region after ethidium bromide staining, and cloned. Because of the poor separation properties of agarose gels, most of the isolated DNA represented the main (truncated) PCR products. To circumvent the onerous search for full-length clones by analyzing many individual plasmids, 300 insert-containing colonies were directly PCR-screened in 10 individual reactions. For each reaction, 30 colonies were picked (about half of the cellular mass), combined in 300 µL of double-distilled water, and mixed. Cells were cracked in the open tubes with two 20-s microwave pulses, and 1 µL of this solution was used without further purification as template in a PCR, utilizing the gene specific innermost primer and Qiinner under similar conditions as described above for the nested PCR. The observed band pattern in the subsequent agarose gel revealed mainly truncated fragments except for one band of the expected size (Figure 2B, right gel, arrow). This band was excised, the DNA isolated, and directly sequenced with the gene-specific primer and Qiinner showing the full-length 5′-region of the tuna COX IV-1 cDNA (AF204870), including 34 bp of 5′-untranslated region.

In summary, these protocols allow the rapid characterization of full-length cDNAs, despite the presence of mRNA secondary structures, partially degraded template RNA, or tissue with low expression levels of the target cDNA.

REFERENCES


Chimeric Gene Construction without Reference to Restriction Sites


Sequence databases now abound with open reading frames related to gene products of known function. Functional analysis of the numerous “homologs” undoubtedly will involve various chimeric constructs between the characterized gene product and the unknown open reading frame. Traditionally, the exchange has been performed by cutting out a segment from the donor DNA, using restriction sites common to the two genes, and ligating it into the recipient DNA. However, more often than not, such convenient restriction sites are not available, and chimeras are sometimes constructed with great ingenuity (1). One way to overcome this challenge is to engineer a restriction site by site-directed mutagenesis, but because convenient sequences must be found for the alterations, the precise manipulation of DNA is not always possible.

Here we present a universal method to exchange practically any segment of two homologous sequences without regard to restriction sites. Toward that end, we take full advantage of the unique properties of Class IIS restriction enzymes. By employing our new method, only two to four nucleotide homologies are necessary to exchange segments between genes, with no requirement for common restriction sites.

Class IIS restriction enzymes recognize non-palindromic sequences, and cleave at sites that are separate from their recognition sequences (2,3,7). The latter trait gives Class IIS enzymes two useful properties. First, when a Class IIS enzyme recognition site is engineered at the end of a primer, the site is cleaved off when digested. Second, overhangs created by Class IIS enzymes are template-derived and thus unique. This is in clear contrast to regular Class II restriction enzymes such as EcoRI, which creates an enzyme-defined overhang that ligates to any EcoRI-digested end. The unique overhangs produced by Class IIS enzymes can be ligated only to their original partner.
Methods to introduce DNA fragments without restriction digestion and ligation have been reported before. Overlapping sequences were utilized in “fill-in PCR” to construct a promoter-gfp reporter (4). In addition, a PCR-amplified mutant DNA fragment that contained an altered sequence with annealing stretches at the 5′ and 3′ ends (sticky feet) was hybridized to a single-stranded vector to exchange antibody domains (5). Contrary to these approaches, our method allows multiple segments to be exchanged simultaneously, making our approach suitable for medium-throughput screening for protein properties.

Figure 1 illustrates, in schematic form, an example of our experimental approach. CAX1 and CAX2 are cation/proton antiporters from the plant Arabidopsis thaliana. CAX1 appears to transport calcium, whereas CAX2 appears to transport heavy metals (6). We are interested in understanding which domains function in conferring ion specificity in these transporters. The transporters are similar in size (400 amino acids) and primary amino acid sequence (46.7% identical, 60.8% similar). However, with 56.1% identity at the nucleic acid level, common restriction sites are rare. We wanted to divide the two genes into four segments of approximately equal sizes (Figure 1A) and exchange each segment to create eight different chimeric constructs. Because there are no convenient restriction sites, a traditional approach is impossible. Figure 1 shows how the domains 2A and 1B are ligated, as an example. Note that the open reading frames can be divided into as many or as few segments as desired, provided there are 4-bp (the number depends on the restriction enzyme used) homologies at the site of digestion.

We incorporated a BsmBI (a Class IIS enzyme) site at the 5′ end of
primers to amplify 2A and 1B by PCR (Figure 1B). PCR was performed in a 100-µL volume containing 100 ng supercoiled plasmid template, 50 pmol each forward and reverse primers, 20 nmol dNTP, 1.5 mM MgCl₂, and 2.5 U Expand™ High Fidelity DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). The thermal cycler (MJ Research, Waltham, MA, USA) was programmed for 30 cycles with denaturation at 94°C for 30 s, annealing at 65°C for 1 min, and elongation at 72°C for 1 min. The PCR product was gel-purified using a QIAEX® II gel extraction kit (Qiagen, Valencia, CA, USA).

The purified PCR products were then digested with DpnI (New England Biolabs, Beverly, MA, USA). This enzyme digests only methylated template DNA and leaves the PCR products intact (8). Following column purification, the DNA was further digested with BsmBI (New England Biolabs). This digestion created a four-base cohesive end common to both genes, and the small DNA piece containing the BsmBI sequence was discarded in the gel purification process (Figure 1C). The overhang sequences were template-derived and unique to the position in the genes, properties that allowed ligation of the segments only in the correct order. A desired combination of segments (e.g., 2A, 1B, 2C, and 2D) was mixed along with the vector for ligation (Figure 1D). The ligation resulted in the correct creation of chimeric genes at a high frequency (12 out of 18).

We believe this new technique to be a novel application of Class IIS restriction enzymes. The technique not only overcomes the difficulties inherent in segment exchange but also enables the simultaneous creation of numerous chimeras, thus facilitating medium-throughput screening for protein domains of interest.

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