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Rapid Isolation of cDNA Clones by Aliquot Testing via PCR Amplification

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In the last few years our laboratory has been involved in the identification and sequencing of genes in Xq24-qter.[1–4] During this work, a large number of data was obtained that could be analyzed with ad hoc software tools. From this analysis it was possible to identify specific fragments that had a high probability of containing coding sequences; however, direct proof of the existence of transcript still needed the labor-intensive approach of screening the cDNA libraries. In addition, the pattern of tissue expression of these putative transcripts was not known beforehand, and several tissues had to be tested, via Northern analysis, to detect the appropriate cell type, developmental stage, or tissue of expression.

Here, we report a rapid PCR-based method of identifying cDNAs from genomic sequences with a high probability of being transcribed. Prescreening cDNA libraries by PCR amplification with primers designed in putative exons is an efficient way of determining which library has to be screened for the isolation of the desired mRNAs. Additionally, performing PCR assays on aliquots of a discrete number of cDNA clones from nonamplified libraries, instead of performing traditional hybridization screening directly on millions of phages, is both fast and economical and allows the systematic screening of cDNA libraries too, as it has been reported for YAC and λ genomic DNA libraries.[5,6] These aliquots can be stored easily, used for an indefinite number of screenings, and maintained for an indeterminate period of time. The setting up of this procedure and its application using three cDNA libraries will be discussed.

MATERIALS AND METHODS

Genomic and cDNA Libraries

A cosmid library from the X3000.11 cell line, a human/hamster hybrid carrying the Xq24-qter chromosomal region as the only human DNA content,[7] constructed in pWE15 as described previously,[2] was the source of the genomic D8C6 and 430 clones. Human cDNA libraries from total brain, fibroblast, and embryo were either made[8] or purchased from Stratagene or Clontech. One million phages from each nonamplified library were split into 20 pools, and each single aliquot containing 50,000 PFU was plated. Then 10–12 ml of SM buffer from each plate were individually collected into separate tubes containing $CHCl_3$ (1:30 of volume) and maintained at 4°C or stored as described.[9]

PCR Assay

PCR was performed as described,[10] with the following minor modifications. Briefly, PCR testing of the libraries was carried out using 1 μl of phage lysate, boiled previously for 5 min and corresponding to 80–100 ng of human DNA template, in 50 μl final volume. The reaction cocktail was prepared with 1× buffer (Promega), 1.5 mM $MgCl_2$, 200 mM each of dATP, dGTP, dTTP, dCTP (Pharmacia), 250 mM of the appropriate primers, and 0.2 units of *Taq* DNA polymerase (Pharmacia). The samples were overlaid with 50 μl of mineral oil to prevent evaporation, and the PCR reaction was carried out in a Cetus thermal cycler under the appropriate conditions for each of the different primer pairs as reported below.

The PCR assay fidelity was monitored by performing each experiment with the published primer pairs from the housekeeping, low-abundant human HPRT transcribed sequence (as an internal control) under the conditions reported in Gibbs et al.[11] The search for genes was performed by testing directly the abovementioned cDNA collections; two primer pairs were used: The first was designed from genomic sequences of cosmid D8C6, and the second was from cosmid 430.

The D8C6 primer pair sequences used were ZF2 forward, 5′-aaaagaatcaaacactg-gaa-3′; D8C6 reverse, 5′-cccttcttctgtgga-agtttc-3′. The 430 primer pair sequences used were 430 reverse, 5′-gctgggtggggg-cggcaggtc-3′; 430 forward, 5′-agaagcagc-tgcccaggccg-3′. The amplification conditions were as follows: An initial denaturing step of 2 min and 30 sec at 95°C; denaturation for 1 min at 94°C, annealing for 2 min at 52°C (for D8C6) or at 60°C (for 430 primers), and extension for 2 min at 72°C for 30 cycles. The second PCR screening was then performed only on the 50,000 PFU aliquots from the positive libraries under the same conditions reported above. Prehybridization and hybridization, DNA sequence, and sequence analysis were performed as described.[12,13]

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RESULTS AND DISCUSSION

Pilot Scheme using HPRT Primer Pairs

A graphic representation of the technique is shown in Figure 1. The procedure consists of two PCR-based steps, followed by standard hybridization screening performed on a limited number of preselected clones. An initial test of this procedure was carried out using the HPRT primer pair as the amplification target in the human cDNA libraries. The effectiveness of the method is shown by the results obtained with the HPRT pair of primers 243–244.[11] The PCR product from the HPRT primers was of the expected size, as the correct 387-bp fragment was generated at the first screening step from the human fibroblast, total brain, and embryo cDNA libraries as in Figure 2A, (left). We then chose the brain cDNA library (lane 3) for further analysis.

We performed the second PCR screening using the HPRT primers on 10 discrete aliquots of the human total brain cDNA library, obtaining the expected amplification product from two aliquots as shown in Figure 2A (lanes 3 and 9, right).

To substantiate our method further we performed a complementary step of hybridization. We amplified 100 ng of human cDNA from each of the 10 aliquots of total brain cDNA library in separate tubes, using the appropriate vector primer pair with the intent of amplifying all of the cDNAs from each aliquot. The PCR-amplified products were run on an agarose gel, transferred to membranes, and hybridized to the HPRT cDNA fragment used as a probe. The same results were obtained using both PCR and hybridization assays (data not shown). Finally, the PCR product from aliquot 3 was subcloned using the TA cloning kit (Promega) and identified as the PCR product of the human HPRT gene by sequencing.

We used the HPRT PCR product as the positive internal control in both the first and the second PCR steps of screening for all of the libraries tested with different primer pairs.

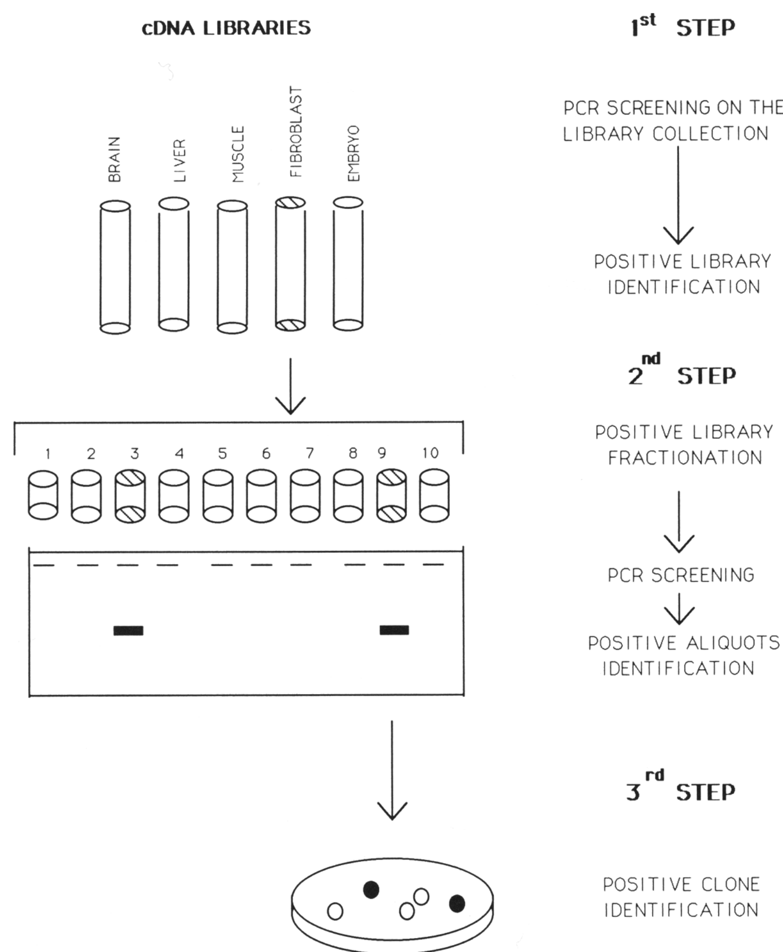

FIGURE 1 Schematic representation of the PCR-based aliquot screening method. Each single tube represents cDNAs from libraries of brain, liver, fibroblast, muscle, and embryo tissue, respectively. At the first step of screening the library containing the mRNA sequences was identified. In this particular case, the fibroblast library (patterned box) was positive. At the second step among the 10 screened, we selected the 2 positive aliquots (3 and 9) from the fibroblast library. At the third step the positive clone was identified by standard filter hybridization.

Identification of Genes Contained in Cosmids D8C6 and 430

We then applied our protocol to cosmids D8C6[2] and 430. Primer pairs were designed ad hoc from genomic DNA sequence with a high probability of representing coding segments.

The first level of PCR screening using the D8C6 primer pair was performed on the human cDNA libraries (fibroblast, total brain, and embryo). A PCR product of the expected size was found only in the human fibroblast cDNA library (Fig. 2B, left, lane 2). Therefore, we carried out the second PCR screening only on this library. To reach a high probability of identifying a middle abundant cDNA, we needed 600,000 recombinants split into 12 aliquots of 50,000 PFU each. As shown in Figure 2B (right) only 1 aliquot (lane 2) out of 12 plated contained the PCR product of the expected size (675 bp) amplified by the D8C6 primer pair. At the third screening level, 60,000 clones were plated from the same aliquot and then hybridized with the PCR product amplified from cosmid D8C6. Two cDNA clones were found, indicating that the mRNA in question was expressed at a low level. The cDNA clone D8C6-c1 was sequenced and fully analyzed, and was shown to be the cDNA ZNF75 belonging to the Krab subfamily of the *Krüppel* type ZF genes.[13]

This method was applied to the 430 primer pair in the same way. PCR was performed on the same set of human libraries; the total brain library gave a strong signal (Fig. 2C, left, lane 3). Aliquots of 50,000 PFU were prepared and screened by PCR. As expected, a PCR fragment of 553 bp was found to be present in 11 of the 14 aliquots tested, indicating that this transcript is very

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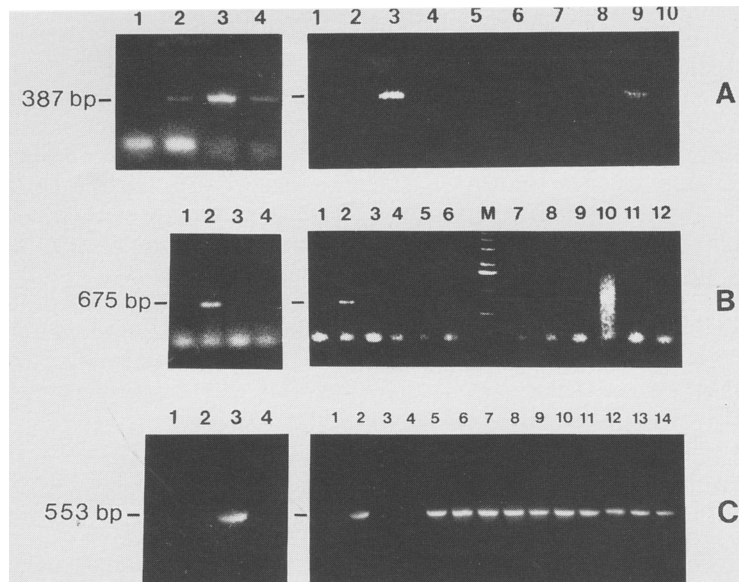

FIGURE 2 *(Left)* Library PCR screening (first step). (Lane *1*) Negative control. Libraries screened with HPRT (*A*), D8C6 (*B*), and 430 (*C*) primer pairs. (Lane *2*) Human fibroblast; (lane *3*) brain; (lane *4*) embryo. (*A*) HPRT cDNA, a ubiquitous mRNA, was amplified in all libraries tested. (*B*) The D8C6 cDNA was only amplified in the fibroblast library (lane *2*). (*C*) The 430 cDNA was amplified only in the brain library (lane *3*). *(Right)* PCR testing on aliquots (second step). (*A*) Two positive aliquots (lanes *3,9*) of the 10 screened were identified from the human brain library with the HPRT primer pair. (*B*) Screening of the 12 aliquots (lanes *1–12*) from the fibroblast library with the ZN75 primers pair. The only positive aliquot is shown in lane *2*. (*C*) Testing of the 14 aliquots (lanes *1–14*) from human brain library with the 430 primer pair. Of the 14 tested, 11 aliquots amplified the expected PCR product.

abundant (Fig. 2C, right). The high abundancy of the transcript was in concordance with the fact that a high number of clones was also obtained by conventional library screening.

The approach described here represents a general method for the rapid identification of tissue-specific and housekeeping mRNAs. The technique can be speeded up by increasing the number of levels of PCR screening and can be extended to other mammals such as mouse, rat, or primates. The fact that we need to have sequence information in advance to apply this method can be considered a limitation, but with the advances made today in sequencing procedures, we will soon be provided with a large amount of starting material for analysis. Application of this method will allow the rapid generation of expression maps of genomes, with the overall aim of identifying genes of biological and medical interest.

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