PCR, Southern Blotting and Oocyte Expression to Assess the Efficacy of Subtractive Hybridization

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Subtractive hybridization is a technique designed to isolate genes and transcripts abundant in or unique to a particular condition. The method has been utilized for constructing a subtracted cDNA library, differential cloning and identifying differentially expressed genes in specific cell lines (3–6,8,9). The analysis of the transcripts isolated by using this technology can be most difficult, in that even a subtracted library invariably contains an abundance of common transcripts. We therefore used the polymerase chain reaction (PCR), Southern blot analysis and the *Xenopus laevis* oocyte expression system to ascertain the efficacy of the subtraction technique.

Our goal was to utilize and expand the method as an alternative to traditional cloning techniques to facilitate the isolation of a growth-regulated renal sodium-dependent phosphate (NaPi) co-transporter, which we speculated was more abundant in the renal cortex of growing (3-week-old) than in adult (>12-week-old) animals. Due to the differences that exist in the renal handling of Pi between dietary Pi depletion and growth, we postulate that a transporter encoded by a growth-regulated mRNA different from NaPi-2, the transporter modulated during dietary Pi deprivation, accounts for the high rates of renal Pi transport observed during growth (7). Northern blot analysis performed in our laboratory had already confirmed that the expression of NaPi-2 was greater in the renal cortex of adult than in growing animals (1). Our aim was therefore to eliminate the common NaPi-2 transcripts and ascertain the presence of a novel and up-regulated NaPi transcript in the renal cortex of growing rats.

The protocol for subtractive hybridization used in our experiments was adapted from that of López-Fernández

and Del Mozo (4). Hybridization was sought between cDNA derived from renal cortex of >12-week-old rats (the “driver” condition) and poly(A) RNA obtained from the renal cortex of 3-week-old rats (the “tester” condition). Briefly, 10 µg poly(A) RNA from the renal cortex of >12-week-old rats were isolated on magnetic particles and used for first-strand cDNA synthesis. The cDNA generated binds to streptavidin-coated magnetic particles (Promega, Madison, WI, USA). Poly(A) RNA (10 µg) isolated from the renal cortex of 3-week-old rats was added to allow annealing of the mRNA to the cDNA for 20 min at 55°C. The subtracted poly(A) RNA after each passage was collected, and some aliquots were used for reverse transcription (RT)-PCR and Southern blotting, while aliquots after

the third passage were injected into oocytes.

RT-PCR (2) products for NaPi-2 (sense, position 1544–1565, CATGGC-CAAGGCACTGGGCAAA) and (anti-sense, position 1944–1966, TAGAGC-CGGGTGGCATTGTGGTG) (6) (423 bp) were abundant in the renal cortex of unsorted 3- and 12-week-old rats (internal control) (Figure 1A). RT-PCR for β-actin (control) of the same samples resulted in abundant product in the unsorted samples and steady removal after two subtracted cycles (Figure 1B). Because residual NaPi-2-specific RT-PCR product might have escaped detection because of lack of sensitivity of ethidium bromide staining, we blotted the gels (Figure 1A) onto nylon membranes and carried out Southern hybridization using a [³²P]dCTP-labeled full-length cDNA NaPi-2 probe. The results (Figure 2) confirm that poly(A) RNA, subjected to three cycles of subtractive hybridization, is completely depleted of NaPi-2 transcripts.

With knowledge that NaPi-2 could be effectively removed by subtractive hybridization, we investigated whether a conserved region of another NaPi co-transporter was present within the renal cortex of 3-week-old animals. RT-PCR amplification products of unsorted 12- and 3-week-old renal cortical poly(A) RNA and 3-week-old poly(A) RNA exposed to subtractive hybridization with 12-week-old renal cortical cDNA are shown in Figure 2. The primers used in these experiments were those specific for NaPi-2, and in addition, primers specific for a conserved region of all modulated (type II) NaPi

Figure 1. RT-PCR amplification products before and after subtractive hybridization. (A) RT-PCR of pre-subtracted renal cortical poly(A) RNA (3- and 12-week-old) and that of poly(A) RNA obtained from the renal cortex of 3-week-old rats exposed to hybridization (1, 2 and 3 cycles) with cDNA synthesized from >12-week-old rats, using primers specific for NaPi-2. (B) The same templates as in (A), assessing the presence of the control gene, β-actin. Note the elimination of NaPi-2 and the control, β-actin, after three cycles of subtraction.

Figure 2. Southern blot. Blot from the ethidium bromide-stained gel from Figure 1A. Data are consistent with RT-PCR (Figure 1A).

Benchmarks

Figure 3. RT-PCR amplification products for NaPi-2 and the conserved region. Pre-subtracted >12-week-old and 3-week-old poly(A) RNA and 3-week-old poly(A) RNA exposed to subtractive hybridization with >12-week-old renal cortical cDNA were used as templates. Primers for NaPi-2 (N) and for a conserved region of Type II NaPi transporters (C). Note the absence of NaPi-2 and the presence of the conserved region in the subtracted samples.

transporters (sense, positions 1278–1300, AGCATGACGTTTGTGTGCC-AGAG) and (antisense, positions 1645–1657, CACAAACCGTAAAGATACCGTCC). The results revealed apparent removal of all NaPi-2 mRNA transcripts after subtraction while the expression of the conserved region was abundant in all samples (Figure 3). One obvious question remains: if NaPi-2 and the putative transcript both contain this conserved region, why are both not removed by subtraction? First, note that the conserved region represents only a small segment of both NaPi-2 and the putative transcript. Since subtractive hybridization depends upon specific annealing of large segments of transcripts, this important but, nevertheless, relatively small region of homology will not result in removal of all transcripts with this region in common. In addition, we considered it likely that the growth-regulated co-transporter is significantly more abundant in 3-week-old rat renal cortex. Hence, as demonstrated by Mészáros et al. (5), it is unlikely that it can be completely subtracted out of 3-week-old renal cortical poly(A) RNA.

By sequence analysis, the RT-PCR amplification product that escaped subtraction was found to be 98% homologous with the corresponding segment of NaPi-2 cDNA.

To ascertain the effect of subtractive

hybridization on the capacity of the poly(A) RNA from the young animal to encode for Na⁺-Pi co-transport, we injected 50-μg samples into oocytes and measured the rates of Na⁺-Pi uptake. Fifty nanoliters of diethylpyrocarbonate-treated water were injected as a control. No difference in Na⁺-dependent Pi uptake was observed ($P > 0.3$) between oocytes injected with poly(A) RNA harvested after the third subtractive passage (253 ± 35 pmol/oocyte-h) and those injected with poly(A) obtained before subtraction (230 ± 40 pmol/oocyte-h). Both uptake rates were greater than that induced by poly(A) RNA of adult animals (163 ± 34 pmol/oocyte-h, $P < 0.05$) and that observed in water-injected oocytes (81 ± 14 pmol/oocyte-h, $P < 0.01$). Therefore, poly(A) RNA of the young, depleted of NaPi-2 mRNA by subtractive hybridization, preserved its ability to encode for Na⁺-Pi co-transport. The transcript detected in the subtracted sample by using primers designed to amplify a highly conserved region of the NaPi-2 mRNA, very likely represents part of the message for a growth-specific Na⁺-Pi co-transporter.

In conclusion, the PCR method in conjunction with Southern blot analysis and oocyte expression enhances one's ability to test the validity of subtractive hybridization. In addition, the method has permitted the isolation of a sub-

tracted cDNA library, which can be screened by routine means.

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