A subtractive hybridisation method for the enrichment of moderately induced sequences

Uwe Konietzko and Dietmar Kuhl*

Zentrum für Molekulare Neurobiologie (ZMNH), University of Hamburg, Martinistraße 52, D-20246 Hamburg, Germany

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ABSTRACT

Moderately induced genes often escape detection in conventional subtraction hybridisation cloning. Here a modification of a phagemid subtraction protocol is described that overcomes this problem. The protocol uses low ratio hybridisation of driver to target sequences to allow enrichment of the sequences of interest, and back-hybridisation of the subtracted sequences with induced sequences to reduce the accumulation of false positive clones. The procedure takes advantage of the quantitative representation of cellular RNA populations in cDNA libraries, therefore, they may serve not only as renewable sources of driver and target sequences, but also as sources of population cRNAs used in northern blots and differential Southern blots.

A hallmark of synaptic plasticity in the brain is the requirement for RNA and protein synthesis (1). As a consequence a great deal of attention has been focused on identifying the specific genes that are induced in hippocampal neurons by plasticity inducing synaptic activity. Subtractive hybridisation is a procedure that increases the effective concentration of induced sequences expressed in an experimental RNA population (target) but not in a control RNA population (driver) (2). To implement subtractive hybridisation and cloning protocols for the isolation of activity-dependent genes, three main points have to be addressed which are of general concern for a variety of other applications. First, others and ourselves have previously shown that even genes that are induced several fold can have functional consequences on the properties of nerve cells (3–6). The commonly used 10–100-fold excess of driver sequences derived from the control cell population is, therefore, prohibitively large to enrich for potential genes of interest. Second, the complexity, i.e. the number of different RNA sequences transcribed in brain tissue, is considerably higher than in uniform cell cultures. As a result, the rate constant of the hybridisation reaction will be significantly smaller. To drive the hybridisation reaction to kinetic termination for low abundance genes, $R_{QK}$ values of >1000 have to be achieved (2). This requires a driver concentration of at least 3 µg/µl to limit the time of high temperature exposure during hybridisation. Since both driver and target sequences have to be isolated from the hippocampus, a relatively small area of the brain, the material required in standard subtractive protocols is difficult to obtain. Third, following subtractive hybridisation an enrichment of induced sequences is frequently accompanied by an accumulation of sequences unable to form heteroduplexes for purely technical reasons (7). Consequently the efficacy of the subtractive hybridisation is severely limited.

Here we implemented a phagemid subtraction protocol based on previous reports (7–10), and introduce modifications that address the above points. In addition, we report that cRNAs transcribed in vitro from cDNA libraries can be used as a representation of cellular RNA in virtual northern blots and in the preparation of virtual population cDNA probes.

As a first step in the subtraction protocol, cDNAs from poly(A)+ RNA of stimulated and control hippocampus were synthesised. These cDNAs were cloned into phagemid vectors to generate representative control and induced libraries. Restriction analysis of 100 individual clones of both libraries confirmed that 97% of the clones contained inserts of an average size of 1000–2000 bp. Representation of cDNAs in the control cDNA libraries in virtual northern blots showed that low ratio hybridisation was sufficient to effectively detect the library of GAPDH transcripts while moderately induced transcripts, as exemplified by t-PA, nur77 and c-fos, become highly enriched (Fig. 1A, lane S1 and B, lane 2:1). The enrichment

*To whom correspondence should be addressed. Tel: +49 40 4717 6275; Fax: +49 40 4717 6595; Email: kuhl@plexus.uke.uni-hamburg.de
of cRNA transcripts in cDNA libraries. Autoradiographs of virtual northern blot analysis 4 h post-PTZ. Control animals were injected with similar volumes of isotonic PTZ (50 mg/kg) were administered by i.p. injection. Animals that received on the left. Adult male Sprague-Dawley rats were used. CHX (120 mg/kg) and the RNA ladder (Gibco BRL) bands (1.35, 2.37, 4.40 and 7.46 kb) are indicated target ratio of 2:1. RNA amounts and abbreviations are as in (A). Positions of from a library that was generated after one round of subtraction with a driver to target ratio of 5:1. Lane 2:1, cRNA B for the enrichment of induced sequences. Virtual northern blots were hybridised to a probe specific for c-fos. Lane 5:1, cRNA from a library that was generated for nur77 (bottom). Lane C, cRNA from the control cDNA library prepared specific for t-PA (middle). An identical blot was hybridised to a probe specific for GAPDH (top) and, after stripping of the probe, rehybridised with a probe for the back-hybridised, subtracted library. Lane S2, cRNA from a library that was generated after one round of subtraction. Lane BS, cRNA from the back-hybridised, subtracted library. Lane S2, cRNA from a library that was generated after subtraction, back-hybridisation and a second round of subtraction. Positions of the RNA ladder (Gibco BRL) bands (0.24, 1.35, 2.37, 4.40 and 7.46 kb) are indicated on the left. (B) Driver to target ratio is critical for the enrichment of induced sequences. Virtual northern blots were hybridised to a probe specific for c-fos. Lane 5:1, cRNA from a library that was generated after one round of subtraction with a driver to target ratio of 5:1. Lane 2:1, cRNA from a library that was generated after one round of subtraction with a driver to target ratio of 2:1. RNA amounts and abbreviations are as in (A). Positions of the RNA ladder (Gibco BRL) bands (1.35, 2.37, 4.40 and 7.46 kb) are indicated on the left. Adult male Sprague-Dawley rats were used. CHX (120 mg/kg) and PTZ (50 mg/kg) were administered by i.p. injection. Animals that received CHX and PTZ were injected with CHX 0.5 h prior to PTZ and RNA was isolated 4 h post-PTZ. Control animals were injected with similar volumes of isotonic saline. Poly(A)^+ RNA from hippocampal of rats that had either undergone PTZ-induced seizures in the presence of CHX or were injected with saline, was selected by Dynabeads oligo (dT). The directional cloning protocol from Gibco BRL was used to generate the induced cDNA library in the pSPORT-1 vector and the control cDNA library in the pSPORT-2 vector. The orientation of the Norf-Sall segment of the poly linker in these vectors is reverts to avoid hybridisation of vector sequences during subtraction. Both libraries comprised 5 x 10^5 independent clones and were amplified in solid state (19) to minimise a bias for faster growing clones and maintain the original representation of clones in the amplified libraries. Driver RNA was transcribed in vitro from the Sall linearised pSPORT-2 control library following the Megascript T7 System protocol (Ambion) with the exception that the UTP concentration was reduced to 5 and 2.5 mM Biotin-21-UTP (Clontech) was added to the reaction. The biotinylated RNA was purified over Chroma Spin-100 columns (Clontech), precipitated and dissolved in TE buffer. Phagemid ssDNA was prepared as described (10). Further purification from contaminating double stranded (ds) DNA was achieved by using the single binding matrix (SSAM, Clontech), and a PuvD and BglII digest which cleave dsDNA but not ssDNA. To prevent non-specific hybridisation of target and driver sequences, the Poly(A)^+ region of the target was blocked in a primer extension reaction using the Not-oligonucleotide (GCGGCGGCCCCCAGG) as described (10). For the subtractive hybridisation reaction 80 µg of biotinylated driver RNA were denatured for 10 min at 65°C and placed on ice. Preblocked ss target library (50 µg) was spiked with 1.2 ng of tetracycline-resistance bearing plasmid (pBR322) with ampicillin resistance deleted and one fifth of this mixture (containing 10 µg ssDNA) was used in a mock hybridisation without the addition of RNA. Biotinylated RNA was mixed with the ssDNA in a ratio of 2:1 and precipitated with NH₄Acetate and ethanol. The pellet was dissolved in 7 µl of TE followed by the addition of 14 µl of hybridisation solution (75% formamide, 75 mM HEPES, pH 7.5, 3 mM EDTA, 0.3% SDS, 1.2 M NaCl) resulting in a driver concentration of 3.8 µg/µl. A 0.5 µl aliquot of this solution was photometrically measured to confirm complete solubilisation of the components. Hybridisation was performed at 42°C under vigorous shaking (300 r.p.m.) for 18 h to Rq values higher than 2000. To ensure the specificity of hybridisation the subtraction mix was adjusted to 33 mM NaCl in a 640 µl volume and incubated for 30 min at 60°C. This corresponds to a high stringency wash with 0.2x SSC. After the addition of 160 µl of 5 M NaCl, the subtraction reaction was incubated at room temperature four times in sucission with four times 3 mg Dynabeads-streptavidin (Dynal) for 20 min. Biotinylated RNA and RNA–ssDNA heteroduplex hybrids were removed using a magnetic tube holder (Dynal). After the fourth magnetic separation 200 µg of streptavidin (Clontech) were added and the solution incubated for an additional 30 min at room temperature, phenol/chloroform extracted and precipitated. The ssDNA pellet was dissolved in 10 mM Tris, pH 7.4 and dialysed. Conversion of the ss phagemid DNA to ds plasmid was as described (10). Following transformation of Electromax DH12S (Gibco BRL), bacteria were plated on ampicillin (100 µg/ml) and tetracyclin (50 µg/ml) containing plates. Comparison of mock subtraction and subtraction plates revealed that 77% of the clones were removed after the first round of subtraction. The subtracted cDNA library was used to produce single stranded phagemids used as target in a back-hybridisation reaction. Biotinylated driver RNA for the back-hybridisation reaction was transcribed in vitro from the Sall linearised pSPORT-1 induced library using the SP6 Megascript System protocol (Ambion) with the modifications described above. Samples (450 pmol) of an oligo complementary to the pSPORT-1 polylinker site (GGGCGGCCGCTCTAGAGGA TCCAAGCTTACGTACGCGTGCA TGCGAGTGTCACACGCGGCTAGCTCTTTC), were added to 60 µl of driver RNA, heated to 60°C for 15 min and placed on ice. Aliquots (30 µg) of subtracted ssDNA phagemid target were added and hybridisation was performed as described above. After 18 h hybridisation to a Rq-value >1000 the reaction mix was washed with 2x SSC at 60°C, and adjusted to 1 M NaCl before adding 6 mg Dynabeads-streptavidin (Dynal) and shaking for 15 min. Dynabeads bound ssDNA was captured via a magnetic holder (Dynal) and the supernatant incubated with an additional 4 µg of Dynabeads. The combined beads were washed four times with 1 M NaCl and re-suspended in 200 µl 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂. To release bound ssDNA the solution was first incubated with 10 µl RNase H (Gibco BRL) for 30 min at 37°C followed by the addition of 2 µl 10% SDS and 300 µg protease K (Serva). After incubation at 45°C for 30 min the solution was heated to 90°C for 5 min and the beads were removed with a magnetic holder. Bidestilled water was added twice to the beads with heating to 90°C. The combined supernatants were precipitated, dialysed, rendered double stranded and transformed into DH12S bacteria as described above. The resultant subtracted and back-hybridised library was converted into ss Phagemid target and taken through a second round of subtractive hybridisation. For virtual northern blots, cRNA was transcribed in vitro from Norf linearised libraries using the components and the protocol of the SP6/T7 Megascript System (Ambion). Gels and blots were as described for standard northern blot analysis (3).
from the subtracted library. After kinetic completion of the induced library and the target phagemids were prepared, the percentage of insertless clones in the original phagemids devoid of inserts or containing insert sequences of <50 bp. Whereas the percentage of insertless clones in the original library was 3%, this percentage increased to 52% in the first virtual, induced (+) cDNA population probe. cDNA inserts were released from 15 randomly picked clones of the back-hybridised, subtracted library. Lane F, cDNA insert released from c-fos plasmid (13), loaded as a control for an activity-induced gene. Arrows mark lanes of two clones from the back-hybridised library with strong differential hybridisation signals with the (+) probe. However, it is imperative that these clones be considered as candidates until the abundance of their corresponding RNA is examined by northern blots or another quantitative method. Standard northern blot analysis confirmed that expression of nine clones was induced by the stimulation protocol, two of which are reported to be induced by synaptic activity (3,12).

B) Figures 2 A, lane BS), were picked at random and analysed in a differential Southern blot. Radiolabelled plus and minus virtual cDNA population probes were generated by reverse transcription of cRNA that had been in vitro transcribed from the induced and control cDNA libraries, respectively. Virtual cDNA population probes eliminate the need to isolate poly(A)+ RNA from tissue and rely on the representation of the cellular RNA population in the cDNA libraries. Of 90 clones tested, 31 showed a differential signal. Figure 2 shows an example of a differential Southern blot using induced and control, virtual population probes. In this example two clones exhibited a dramatically increased hybridisation signal with the induced probe. An autoradiograph of a Southern blot hybridised to a control, virtual (+) cDNA population probe. (B) Autoradiograph of a duplicate blot hybridised to a virtual, induced (+) cDNA population probe. cDNA inserts were released from 15 randomly picked clones of the back-hybridised, subtracted library. Lane G, cDNA insert released from GAPDH plasmid (20) loaded as a control for a constitutively expressed gene. Lane F, cDNA insert released from c-fos plasmid (13), loaded as a control for an activity-induced gene. Arrows mark lanes of two clones from the back-hybridised library with strong differential hybridisation signals with the (+) probe. The stringent wash was performed with 0.1% SDS, 0.1% SSPE, 5× Denhardt’s, 0.1% SDS, 100 µg/ml herring sperm) for 16 h at 42°C. The stringent wash was performed with 0.2× SSC, 0.1% SDS at 65°C for 30 min. Exposure time of the autoradiographs was adjusted on the basis of the constitutively expressed GAPDH gene. Clones exhibiting differential hybridisation signals were examined in standard northern blots with cellular hippocampal RNA from control and experimental seizure animals. Sequence analysis of two positive clones shown in Figure 2 revealed that they are identical to t-P A and nur77.

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