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G Houge

Genome Res. 1993 2: 204-209
Access the most recent version at doi:10.1101/gr.2.3.204

Simplified Construction of a Subtracted cDNA Library Using Asymmetric PCR

Gunnar Houge

INSERM U-301, SDI No 15954.I CNRS, Centre Hayem, Hôpital St. Louis, F-75010 Paris, France

A novel method for the direct construction of subtracted plasmid cDNA libraries in the plasmid pBluescript is presented. Two libraries in λ-ZAP were compared starting with general phagemid excision from both libraries. Thereafter, single-stranded (ss) plasmids from one library were subtracted with biotinylated cDNA molecules generated by asymmetric PCR on ss plasmid templates from the other library. The nonsubtracted plasmids were used to transform *Escherichia coli* directly, thus making a subtracted plasmid library. Preliminary data suggest that the specificity of the method is around 25%. The method is sensitive enough to detect low-abundance mRNAs. In contrast to other subtractive methods based on λ-ZAP, the bias introduced using PCR in this case only affects the method's specificity and not its sensitivity.

The detection of differentially expressed mRNA molecules is still a considerable challenge. The cloning and characterization of such molecules is often desired in experimental systems where a given treatment changes cellular properties and where this change seems to depend on protein synthesis. Traditionally, the radioactive labeled cDNA pool from treated cells (called $cDNA^+$) is hybridized to an excess of mRNA from nontreated cells (called RNA^-), and nonhybridized cDNA is selected by hydroxyapatite chromatography. This subtracted cDNA can be used as a probe to screen a cDNA library or to make a subtracted library.[1]

Here I present a subtractive method based on λ-ZAP cDNA libraries and asymmetric PCR. The method was used to detect novel mRNA species induced after cAMP treatment of a myeloid leukemia cell line. Briefly, phagemid excision was used to prepare circular single-stranded (ss) cDNA in pBluescript from both libraries (− and +). Circular ss DNA^+ was hybridized to biotinylated linear $cDNA^-$ made by asymmetric PCR using universal primers on circular $ssDNA^-$ templates, and the subtraction was done with streptavidin-coated magnetic beads. The circular $ssDNA^+$ that remained after subtraction was used to transform *Escherichia coli* directly. The specificity of the method is sufficiently high to allow direct screening on Northern blots of potentially subtracted cDNA sequences.

MATERIALS AND METHODS

Poly(A) RNA Preparation and cDNA Library Construction

Total RNA was prepared from 500×10^6 IPC-81 rat leukemia cells[2] before and two hours after stimulation by 0.2 mM 8-(4-chlorophenylthio)-adenosine 3′;5′-cyclic monophosphate (8-CPT-cAMP) using the acid-guanidinium-phenol extraction method.[3] Leukemia cells were pelleted and homogenized by resuspending the pellet in 1.8 ml of 25 mM sodium citrate buffer pH 7.0 containing 4 M guanidinium thiocyanate, 0.5% sodium lauroylsarcosine (wt/vol), and 1% 2-mercapto-ethanol (vol/vol) per 50×10^6 cells. Further purification and, in some cases, Northern blotting of RNA was done as described previously.[4] Poly(A) RNA purification was done using the PolyATract mRNA purification kit (Promega), following the manufacturer's protocol. Stratagene's Uni-ZAP XR/Gigapack II Gold Cloning Kit was used to make + and − cDNA libraries according to the manufacturer's protocol, with the exception that the cDNA pellet was air dried at the bench top and not vacuum dried after the different modification steps.

Preparation of ss Circular DNA from the λ-ZAP Libraries

The phagemid excision was done as described in the pBluescript protocol (Stratagene) with small modifications.[5] XL1-Blue bacteria infected with only helper virus (VCS-M13) and λ-phage from the λ-ZAP cDNA library served as controls for the efficiency of the triple selection with antibiotics (the *E. coli* XL1-Blue's F′ episome confers tetracycline-resistance, VCS-M13 helper phage infection confers kanamycin-resistance, and λ-ZAP infection confers ampicillin resistance). After 16 hr of incubation at 37°C in the shaker, growth was only seen in the doubly infected bacterial suspensions. The phages in the supernatant of the bacterial culture (λ-ZAP, wild-type

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VCS-M13, and recombinant VCS-M13) were precipitated as described.[1] Further DNA purification was done using a classical method.[1] CsCl gradient centrifugation was not used to separate phagemids from λ-virus.[5] The purified DNA was instead run on a 1% agarose gel, and the DNA between the VCS-M13 band and the nonrecombinant pBluescript band was excised and purified by GeneClean (see protocol below).

Preparation of Bacteria for ss Plasmid Transformation

The *E. coli* TG1 bacteria (RecA$^+$) used for generating the subtracted plasmid libraries were made competent by the hexaminecobalt(III)chloride method,[1,6] obtaining a transformational efficiency of approximately 10^5 colonies/μg ss circular DNA. Freshly prepared bacteria should be used for maximal efficiency of transformation. The transformations were done by carefully mixing 1–5 μl of ss plasmid suspension with 200 μl of ice-cold competent bacteria (in 15-ml Falcon tubes), followed by 15 min incubation on ice and 90 sec heat-shock in a 42°C water bath.[1] Other strains of bacteria, like the RecA$^-$ XL1-Blue and INVαF, were less efficiently transformed by ss plasmids.

Protocol for Subtraction

A schematic presentation of the method is given in Figure 1. For the convenience of the reader who wants to repeat the experiment, the method is described in the form of a protocol:

1. Prepare poly(A) RNA from the cells one wants to compare.

2. Construct λ-ZAPII unidirectional cDNA libraries.

3. Excise ss circular pBluescript plasmids caged in the λ-ZAPII.[5] This ss DNA represents the noncoding strand of the ds cDNA.

4. Precipitate phages and purify the DNA in the phage pellet using standard protocols for phage precipitation and DNA purification.[1] Dissolve the purified DNA pellet in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

5. Run an aliquot (e.g., 50 μg) of the DNA on a preparative 1% agarose gel. Excise the DNA smear between the VCS-M13 band (=8.7 kb ssDNA, corresponding to ~5 kb on a dsDNA ladder) and nonrecombinant ss pBluescript DNA band (=3.0 kb ssDNA, corresponding to ~1.7 kb on a dsDNA ladder). Purify the ssDNA in the excised fragment (representing ss pBluescript plasmids with cDNA inserts) using a standard procedure, e.g., GeneClean. Aliquot the purified circular ss plasmid DNA and freeze at −20°C.

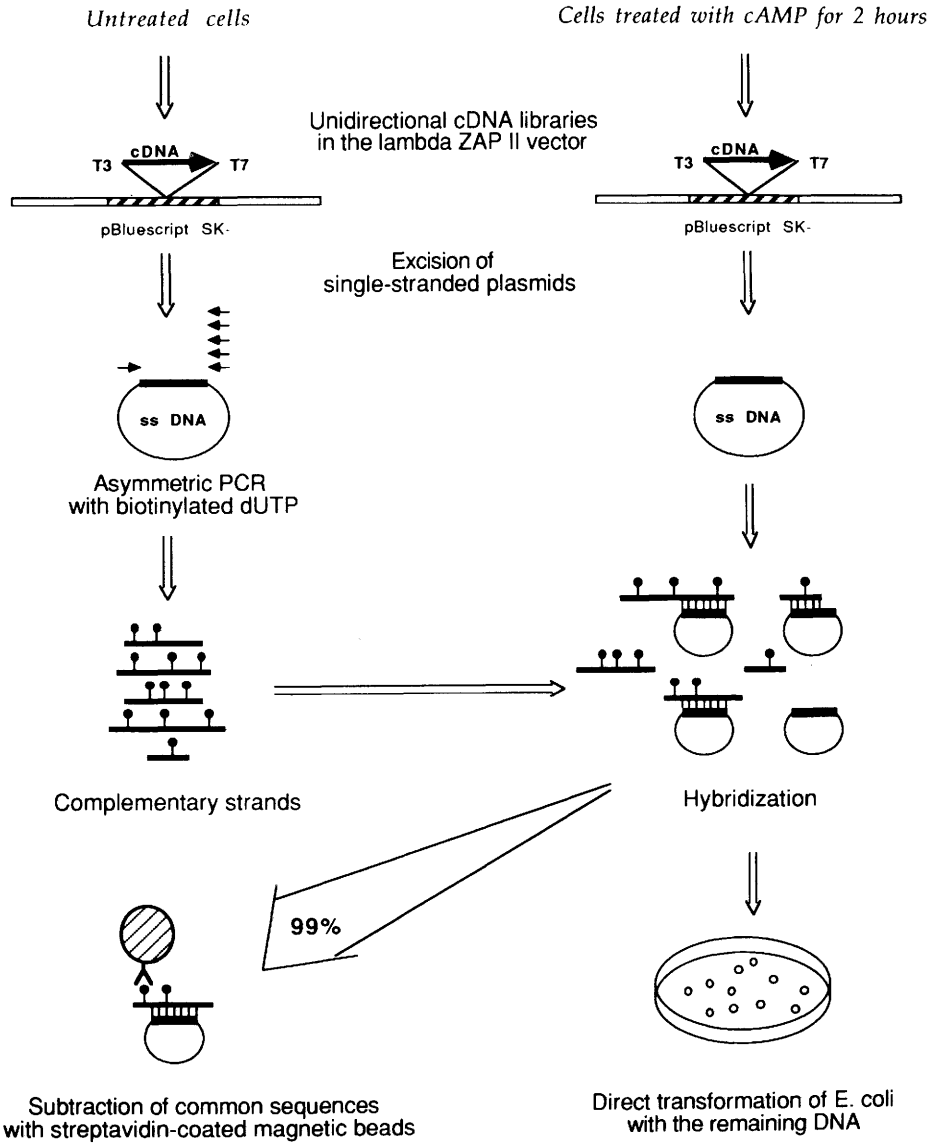

FIGURE 1 Outline of the protocol for making a subtracted plasmid library.

6. Make the subtractive tool by doing asymmetric PCR on 40 ng of ssDNA$^-$ with a preferential amplification of the coding cDNA strand. Set up a 100-μl PCR reaction with the following final concentrations: 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.15 mM dTTP, 0.05 mM 16-biotin-dUTP (Boehringer), 0.5 μM T3 primer (ATTAACCCTCACTAAAG), 0.01 μM T7 primer (AATACGACTCACTATAG), 1× *Taq* buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatine), and 2.5 units of *Taq* DNA polymerase. PCR conditions: one cycle at 96°C 60 sec/45°C 90 sec/72°C 10 min; following 29 cycles at 94°C 90 sec/45°C 90 sec/72°C 3 min, and finally 72°C 10 min. Chloroform-extract and ethanol-precipitate the product of the PCR reaction. The product of an asymmetric PCR is shown in Figure 2.

7. Hybridize the biotinylated DNA$^-$ made above with no more than 40 ng of circular ssDNA$^+$ plasmids in 20 μl of hybridization buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 0.75 M NaCl, 0.1% SDS). Hybridize under mineral oil for 24–48 hr at 65°C. Add 30 μl 10 mM HEPES, pH 7.5, 1

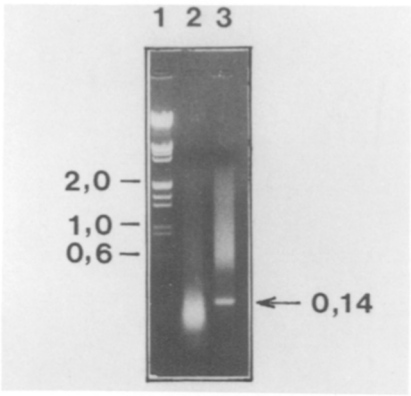

FIGURE 2 The cDNA inserts in the single-stranded plasmids made by phagemid excision were amplified by asymmetric (lane *2*) and symmetric (lane *3*) PCR as described in Materials and Methods. Note the 140-bp band in lane *3* amplified from ss pBluescript plasmids without cDNA inserts. The size marker (lane *1*) is *Eco*RI–*Hin*dIII-cut λ-DNA; size indications are in kilobases.

mM EDTA, extract the mineral oil with chloroform, and precipitate the DNA.

8. Resuspend the hybridization product in 50 μl of 0.5× SSC and add the solution to one aliquot of prewashed (with 0.5× SSC) Streptavidin MagneSphere paramagnetic particles (Promega). Incubate for 15–30 min at room temperature with occasional gentle mixing, and remove the streptavidin-coated particles on a magnetic separation stand. Remove the supernatant carefully (50 μl) and repeat the subtraction once with another aliquot of magnetic particles. For highest efficiency, the final supernatant (50 μl) should be used immediately for transformation of *E. coli* TG1. If not, aliquot and store at −20°C.

9. Use 1–5 μl of the supernatant to transform highly competent *E. coli* TG1. Plate transformed bacteria on an LB plate containing 100 μg/ml ampicillin. Pick resistant colonies and screen for cDNA inserts on an X-Gal/IPTG plate.

10. The final screening can be done using several different procedures.[1,5,7] We chose to make plasmid minipreparation from randomly selected white colonies. The cDNA inserts were amplified with PCR using T7/T3 primers (same conditions as above) and analyzed on a low-melting-point agarose gel (Fig. 3). Thereafter, the desired bands were excised and used as substrates for probe labeling (we obtained excellent results by using random nanonucleotide primed labeling with T7 DNA polymerase and adding 2 pmoles of T7 primer to the 50-μl labeling mixture, e.g., by using Pharmacia's ^{T7}Quick Prime Kit). The radioactive probe was used to screen for subtracted sequences on Northern blots.

RESULTS

After subtracting 40 ng of ssDNA$^+$ with the product of one asymmetric PCR reaction (Fig. 2), 25–200 ampicillin-resistant colonies were obtained after transformation of *E. coli* TG1 with 5 μl of the solution remaining after the subtractions (e.g., 1/10 of the total volume). The number of colonies obtained after transformation of *E. coli* TG1 with the same amount of nonsubtracted ssDNA$^+$ varied from 2500 to approximately 20,000. The best results were obtained using freshly subtracted ssDNA that had not been frozen. Repeated freezing/thawing of ss circular plasmids or storage at 4°C for several weeks lead to a dramatic reduction in transformational efficiency. Assuming that there is a linear relationship between the number of ss plasmids and the number of colonies obtained after transfection, the subtractional efficiency is around 99%. Using electroporation, Rubenstein et al. found an efficiency per microgram of plasmid that was higher for dilute ss plasmid concentrations.[8] If this is also the case for the described chemical method for bacterial transformation,[6] the efficiency of subtraction is even higher. However, possible degradation of ss circular DNA molecules during the 24–36 hr of hybridization at 65°C will also affect the calculated efficiency. In two different experiments, the loss of ss circular DNA due to nicking or shearing was estimated to be no more than 50% of the ss circular plasmid population after 36 hr of incubation. This is in agreement with the results of Rubenstein et al.[8]

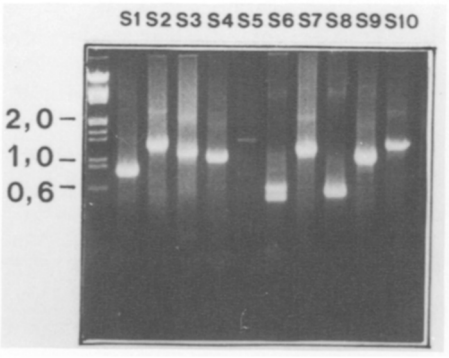

FIGURE 3 cDNA inserts in 10 plasmids prepared from randomly picked white colonies, named S1–S10, were amplified by PCR. The amplified bands were excised; the DNA was purified by GeneClean and was thereafter used for probe-labeling and PCR-based sequencing. The size marker on the left is *Eco*RI–*Hin*dIII-cut λ-DNA.

After screening 254 subtracted colonies on an X-Gal/IPTG plate (from a total of approximately 6000 subtracted colonies, obtained after three different subtraction experiments), 21 white colonies were randomly picked for analysis. Eight white clones obtained after transfection with nonsubtracted ssDNA$^+$ were randomly picked as controls. The cDNA inserts were amplified using PCR directly on a tiny amount of boiled bacteria or on plasmids obtained after plasmid minipreparations. After analysis on a 1% low-melting point agarose gel (Fig. 3), the cDNA bands were excised and used as templates for probe preparation (see step 10 above). The isolated PCR fragments can also be sequenced directly using one of the many PCR-based sequencing kits available.

None of the eight nonsubtracted cDNAs hybridized to mRNAs showing differential expression on Northern blots prepared from IPC-81 cells treated with 0.2 mM 8-CPT-cAMP for 1, 2, 3, or 4 hr (data not shown). Eleven of the 21 subtracted cDNAs gave similar negative results, and most of these false-positive cDNA clones gave strong hybridization signals. These clones might represent abundant cDNA molecules that are amplified by asymmetric PCR with low efficiency. Ten clones hybridized to mRNAs showing differential expression upon treatment of IPC-81 cells with 0.2 mM 8-CPT-cAMP (Table 1). Some of these clones lacked a polyadenylation site and a continuous poly(A) tail in the 3′ end (S12, S13, and S16) and could represent cloning artifacts. Probes from two of these clones (S12 and S16) gave several bands on Northern blots, both of low and very high (>20 kb) molecular weight. Some of these bands were differentially expressed and therefore the clones are included in Table 1. Clone S3 has a poly(A) sequence (on complementary strands) in both the 3′ end (42 A's) and the 5′ end (24 A's) but no typical polyadenylation sites (AAUAAA) in front of them (expressed pseudogenes with

TABLE 1 Differentially Expressed Clones

Clone number	Approx. cDNA size (kb)	Number of bands recognized on Northern	Presence of poly(A) tail	Homology to partial sequence	Particular features
F1-2	0.96	2 (sense) or 4 (antisense)	yes	not found	sense and antisense expression
S3	1.0	4	yes	not found	poly(A) sequence in each end of clone but no polyadenylation signals
S7	1.1	1	yes	not found	
S8	0.37	1	yes	not found	
S10	1.2	1	yes	RATRPPO	nearly 100% identical with the rat acidic ribosomal protein P0
S11	1.7	2 (<0.5 kb apart)	yes	not found	
S12	0.8	5	no	in several rat genes	contains 80-bp rat specific sequence found in the middle of many introns
S13	0.14	2	no	not found	recognizes same 4.7-kb band as S3 (see Fig. 4)
S15	1.2	2 (<0.5 kb apart)	yes	not found	
S16	0.6	3	no	not found	contains hairpin with perfect 55-bp stem and a 7-base loop

kb = kilo base, bp = base pairs, b = base.

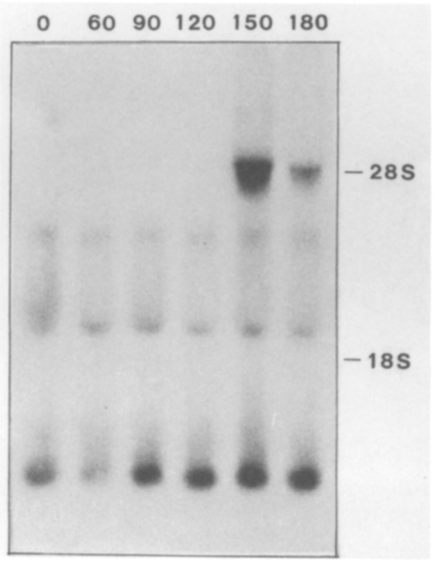

FIGURE 4 A 4.7-kb RNA species (probe made from S3 in Fig. 3) abruptly induced in cells after incubation for 150 min with 5 μM 8-CPT-cAMP. Numbers along the *top* indicate minutes of incubation with 8-CPT-cAMP. The positions of 28S and 18S rRNA are indicated on the *right*. The same probe hybridizes with lower-molecular-weight mRNA species that are almost unaffected by the cAMP-treatment. Total RNA (25 μg) was applied to each lane of a denaturing 1.5% agarose gel. Membrane exposure time was 24 hr.

complementary sequences?). It is thus possible that the 4.7-kb band abruptly appearing after 2.5 hr of cAMP treatment does not represent an ordinary mRNA band (Fig. 4). The other clones in Table 1 have cDNAs from apparently ordinary mRNAs, giving one or two bands upon hybridization to Northern blots (Figs. 5 and 6). These clones represent mRNAs in the low to moderate abundance group. It is noteworthy that none of the nonsubtracted $cDNA^+$ clones revealed complex expressional patterns. These negative controls all recognized only one major band on Northern blots (data not shown).

DISCUSSION

When this work was in progress, two other methods based on directional cDNA cloning in λ-ZAP were published.[5,7] Owens et al. subtracted the circular $ssDNA^+$ with photobiotinylated RNA^-. This RNA^- was made in vitro by T7 RNA polymerase on linearized λ-ZAP DNA templates. PCR with universal primers was used to amplify the cDNA sequences remaining after the subtraction, and the PCR product was subcloned into pBluescript to make a subtracted plasmid library. Klar et al. employed a variation of this method, using in vitro RNA synthesis from both libraries as the basis of subtraction.[7] The cDNA molecules remaining after two rounds of biotinylated-RNA/cDNA-based subtraction (about 2% of the starting material) were PCR amplified and subcloned into λ-ZAP arms to make a subtracted λ-ZAP library. In both cases, the final subtracted libraries were screened with + and − cDNA probes to find clones that were differentially expressed.

The method presented here is faster (subtracted clones can be available after 2 weeks of work starting with poly(A) RNA) and sufficiently specific to justify direct screening of the clones in the subtracted library on + and − Northern blots (+ and − refer to RNA from the stimulated cells and the nonstimulated cells, respectively). It also avoids the use of PCR for amplification of the subtracted product. The subtractional efficiency is at least as good as that obtained with RNA/DNA-based methods.[5,7,9] After having purified circular ss plasmid DNA from the two λ-ZAP libraries that one wants to compare, one has practically unlimited amounts of material for subtractions and the subtractions can easily be done both ways (to find genes that are turned on or genes that are turned off). It takes only 3 days to prepare a subtracted plasmid library starting from circular ssDNA.

The reported method avoids some potentially problematic steps common to other methods. First, the use of DNA/DNA- instead of RNA/DNA-based subtractions avoids problems connected to the omnipresence of RNases.[1,7,8] Second, photobiotinylation of RNA[5] or DNA[8] is not necessary. Third, the subtracted molecules are already in a very useful vector (pBluescript), eliminating the technical difficulties and the loss

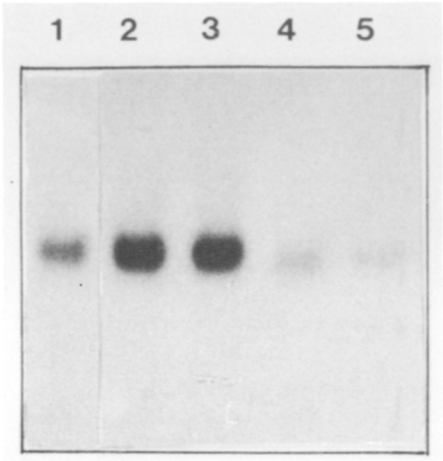

FIGURE 5 A 2.5-kb mRNA (probe made from S8 in Fig. 3) induced after 2 hr of treatment with 0.2 mM 8-CPT-cAMP (lane *2*) and 0.2 mM 8-CPT-cAMP + 37 μg/ml cycloheximide (lane *3*). The other lanes serve as controls: (Lane *1*) RNA from untreated cells; (lane *4*) cycloheximide alone (37 μg/ml); (lane *5*) the phosphatase inhibitor okadaic acid (1 μM). Total RNA (25 μ) was applied to each lane of a denaturing 1.5% agarose gel. Membrane exposure time was 3 days.

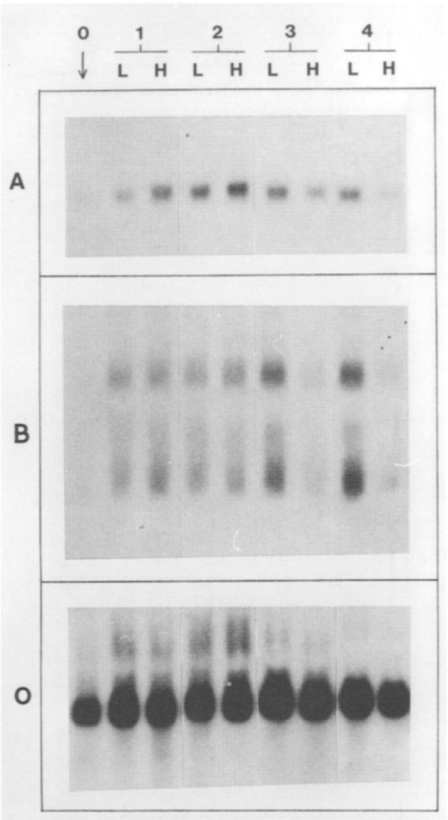

FIGURE 6 A 2.6-kb mRNA (*A*, probe made from S7 in Fig. 3) and 2.2- + 1.3-kb mRNAs (*B*, probe made from clone F1-2 in Table 1) induced by incubating the cells in 5 μM 8-CPT cAMP (lanes *L*) or 200 μM 8-CPT-cAMP (lanes *H*). Notice different time kinetics of induction at low and high cAMP concentrations. Hours of cAMP treatment are indicated on top. Hybridization of the same membrane with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe is shown below (*C*). Total RNA (25 μg) was applied to each lane of a denaturing 1.5% agarose gel. Membrane exposure times were 4 days (*A*), 48 hr (*B*), and 16 hr (*C*). Single-stranded F1-2 probe, corresponding to the sense-strand of the cloned sequence, recognized four weakly expressed bands that are not seen in *B*.

that can be associated with subcloning of subtracted cDNA[1] or PCR-amplified subtracted molecules.[5,10] Fourth, the presented method can be done with readily available reagents, standarized for high-efficiency cDNA cloning, and there is no need to make the directional libraries in several types of vectors, e.g., vectors with reversed cloning sites.[8] Fifth, extensive and laborious screening of subtracted colonies is not necessary because the number of false positives does not represent a major problem using the described procedure (see below).[1,5,8] Finally, and maybe most important, potentially biased PCR amplifications of subtracted heterogeneous cDNA populations are avoided.[5,7,10]

There are, however, at least two steps in the presented protocol that can introduce a bias. The first is the phagemid excision of the pBluescript from the λ-ZAP virus. The excisional efficiency is not the same for different λ inserts. The bias thus introduced can be minimized by shortening the excision time from overnight to 3 hr (see ref. 11, where excision of entire λ-ZAP libraries is discussed). A second bias might be introduced by the use of asymmetric PCR. The efficiency of asymmetric PCR is often unpredictable for a given sequence. Different primer 1/primer 2 ratios must be tried to find optimal conditions.[12] The efficiency of asymmetric PCR on a heterogeneous population of molecules, as in this case, is even less predictable. This bias affects, however, only the specificity of the method (i.e., increase the number of false-positive clones in the plasmid library) but not, at least in theory, its sensitivity. This method thus has a major advantage compared to methods where the PCR-introduced bias directly affects the chance of discovering the true positives, e.g., methods based on PCR amplification of the DNA molecules remaining after subtraction.[5,7,10] In addition, for a given molecule an even low-efficiency asymmetric PCR might generate a sufficiently large excess of complementary biotinylated DNA to obtain 100% subtraction of the corresponding molecule. It is noteworthy that the only fragment that was amplified after two rounds of PCR done directly on the subtracted DNA (with T3 and T7 primers) was the 140-bp fragment corresponding to pBluescript without cDNA insert (corresponds to the 140 bp band seen in Fig. 2). Several different PCR conditions were tried, but it proved difficult to avoid the preferential amplification of the 140-bp fragment. Even a secondary PCR on DNA from the very faint high-molecular-weight smear (the same smear as seen in Fig. 2 but much fainter) done after excision from the gel and purification of the DNA by GeneClean, gave similar results (data not shown). Thus, it proved difficult to isolate subtracted sequences by doing PCR on the ss plasmids remaining after subtraction.

The screening of the subtracted colonies, being bacteria or phage-colonies, is a very time-consuming part of any subtraction. In this case an initial screening can be done by growing the transformed bacteria on X-Gal/IPTG plates.[1] Using the hybridization conditions described above, we routinely obtained one-third blue, one-third bluish-white, and one-third white-only colonies in our subtracted plasmid library. The fraction of nonrecombinant pBluescripts in our nonsubtracted ss plasmid preparations was 20%, i.e., the subtraction caused an apparent enrichment of nonrecombinant plasmids. This might be due to the lack of biotin-dUTP incorporation in some of the 140-bp fragments amplified from the nonrecombinant plasmids, representing the multiple cloning site only. There are only 19 T's in this sequence, which means that, provided the incorporation of biotin-16-dUTP is as efficient as dTTP, there will be an average of 4.75 biotin-molecules per multiple cloning site DNA. If, however, the incorporation of biotin-16-dUTP is somewhat less efficient than dTTP, some nonbiotinylated 140-bp fragments can be expected. Judged by an estimate of the yield on ethidium bromide-stained agarose gels, asymmetric PCR with biotin-dUTP gave

about 50% of the yield obtained without biotin-dUTP (data not shown). These nonbiotinylated molecules will protect nonrecombinant ss plasmids from being subtracted, thus increasing the number of false positives. Fortunately these false positives can easily be eliminated by doing blue/white screening on X-Gal/IPTG plates.

Several strategies can be employed in the final screening of the subtracted clones.[1,5,7] A direct screening on +/− Northern blots is the most sensitive. Probes are easily prepared by PCR using T3/T7 primers (see above). The presented subtractive method increased the likelihood of finding mRNAs clearly induced by cAMP (F1-2, S7, S8, S11, S15) or RNAs showing unusual expressional patterns (S3, S12, S13, S16). The latter finding is not surprising. Random cloning artefacts can be expected to be unique for a given library and will therefore not be subtracted. Note that four out of the 10 clones in Table 1 lack a poly(A) tail, and that one clone (S3) might have resulted from hybridization of complementary sequences during the cDNA cloning procedure. Clone S12 corresponds to a rat-specific sequence found in the middle of many introns. It may not be a cloning artifact because the clone is primed from a conserved stretch of 40 A's only interrupted by two C's and one G. It remains to be proven that this really is an expressed sequence. Clone S10, corresponding to the rat acidic ribosomal protein P0, is only weakly induced by cAMP and therefore not counted among the "true" subtractive clones.

In conclusion, a specificity of approximately 25% can be expected using the presented method. The subtraction apparently increases the likelihood of finding atypical clones lacking a poly(A) tail or showing bizarre expressional patterns. Some of these clones are surely clonal artifacts. The sensitivity is high enough to detect low-abundance mRNAs, and the use of PCR does not affect the method's sensitivity.

ACKNOWLEDGMENTS

The expert technical assistance of Erna Finsås was highly appreciated. I am indebted to Michel Lanotte and Stein Ove Døskeland for their encouragement and support, without which this work could never have been done, and to Anders Molven for his helpful discussion of this manuscript. This work was supported by the EC Science Program and the Blix foundation.

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Received September 14, 1992; accepted in revised form December 7, 1992.