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Identification of enriched sequences from a cDNA subtraction – hybridization procedure

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The subtractive-hybridization procedure (see e.g. (1)) is a powerful procedure for obtaining cDNA populations which are enriched for up- and down-regulated gene products. The method is based on the elimination of the common fragments within the cDNA populations being compared and is straight forward (1). Unfortunately, the analysis of the outcome is based on a screening hybridization assay and, hence, is indirect and time-consuming since individual molecules have to be cloned and analyzed.

Another useful procedure for the isolation of specific gene products is the differential display method as developed by Liang and Pardee (2), recently optimized (3) and automated (4). In this procedure differential gene expression, in cells or tissues, is visualized by differences in the intensities of cDNA fragments after electrophoretic separation on a sequencing gel. After conversion into a cDNA using reverse transcriptase all gene products can be amplified as distinct, primer-dependent groups of cDNA by choosing a distinct oligo-dT-NN primer and a set of (short) random primers which facilitates identification (2). Although this procedure allows in principle for the identification of all mRNAs it can not be used for the isolation of under-represented gene products.

Here we report that the combination of both procedures, i.e. enriching a cDNA population by the subtractive-hybridization method (1) and identifying the enriched fragments by the differential display method (2) on a polyacrylamide gel yields a very powerful procedure that allows the identification of cDNAs that are enriched as little as 3-fold in the test fraction.

In our laboratory we are studying the altered gene expression in a rat liver after a 70% hepatectomy. cDNA populations have been prepared from a normal and a regenerating (partially hepatectomized) rat liver (5). The subtractive-hybridization method as described by Wang and Brown (1), with minor modifications, was used to obtain the up- and down-regulated gene products. Both cDNA populations were tagged with their own unique set of primers/adapters, CTCTTGCTTGAATTCGG-ACTA-3′ and AATTCAGGCCAAGTCGGCCGG-3′ for the normal and regenerating liver cDNA, respectively. In this way the necessity of linker removal, as described in the original procedure (1), at any point during the method was eliminated. The cDNA needed for each round of subtraction was amplified with Taq DNA polymerase (94°C for 1 min, 58°C for 90 s, and 72°C for 2 min with 30 cycles, followed by a final 5-min elongation-step at 72°C). The respective primers could not be interchanged since no product was detectable after a complete amplification cycle with a mismatched primer (not shown). In order to identify the up- and down-regulated gene products after a completed subtractive-hybridization procedure, we adapted the display method from Liang and Pardee (2) with modifications. The two cDNA populations obtained after each round of subtractive-hybridization were amplified using Taq polymerase and the distinct primers in the presence of 0.5 μM [α-^{35}S]dATP (1200 Ci/mmol) (final volume: 25 μl). The experimental conditions were exactly the same as those used to generate the

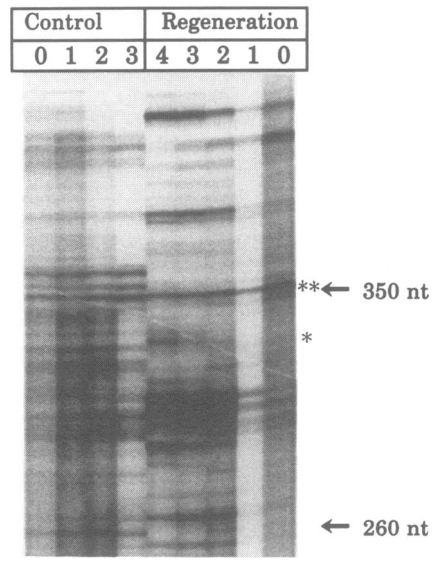

Figure 1. Display of gene products enriched in a regenerating rat liver. cDNA fragments from a regenerating and a control liver were separated on a sequencing gel. Visualized fragments were derived from these cDNA populations before and after one, two, three and four rounds of subtractive hybridization (lanes 0, 1, 2, 3 and 4, respectively). Symbol * indicates example of up-regulated gene product whereas ** represents a common, not affected cDNA. The arrows indicate the position of Sau3AI-restricted (^{32}P-end labelled) fragments of phage lambda as molecular-weight standard. The cDNA fragments were amplified in the presence of [α-^{35}S]-labelled dATP and autoradiography was performed by overnight exposure to an X-ray film.

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larger quantities of the cDNA populations needed for the actual subtraction procedure, except that the concentration of non-radioactive dATP was lowered from 200 μM to 20 μM to obtain a sufficiently high specific activity, while maintaining a normal amplification pattern (not shown). Samples containing comparable amounts of amplified starting material and material after each of the 1st through 4th rounds of subtraction were directly analyzed on a 6% DNA-sequencing polyacrylamide gel (Figure 1). Two main observations were made and will be described, viz. 1) the enrichment and 2) the comparison of enriched products in a regenerating and control rat liver.

The efficiency of the subtractive-hybridization procedure can be analyzed without the need of a laborious cloning and colony-hybridization screening. In Figure 1 the various 'Regeneration cDNA' fragments as found after 0–4 rounds of subtraction with 'Control cDNA' fragments, are compared on a polyacryamide gel. The fragments for which the intensity, i.e. the relative amount of the cDNA band (^{35}S-labelled) increases (see Figure 1, Regeneration lanes; example indicated by symbol *) with each round of subtraction, represent the up-regulated gene products. Also shown in the Figure are cDNA fragments of which the prevalence decreases as a result of the subtraction procedure or remains unaffected, even after 4 consecutive subtractive-hybridization cycles (bands with no change in staining intensity, example given by **). These latter cDNAs would obscure the colony screening procedure. Additional rounds of subtractive hybridization would be required to resolve them, i.e. enrichment or elimination.

Figure 1 also shows a direct comparison of the enriched cDNA fragments from a regenerating (Regeneration lane 4) and a Control rat liver (Control lane 3) allowing the identification of up- and down-regulated gene products, visible as enriched fragments which are unique for Regeneration or Control. Bands of equal size and staining intensity after multiple subtractive-hybridization cycles (cf lanes Regeneration 4 and Control 3), represent common gene products and are omitted from further characterization experiments. Based on the display analysis, several cDNA fragments were extracted from the polyacrylamide gel, amplified and sequenced. Among the up-regulated 'Regeneration – 4' cDNA fragments analyzed, were α_2-macroglobulin, albumin, mitochondrial DNA, hemopexin and vitamin D-binding protein, i.e. all gene products associated with the regeneration of liver. In addition, several mRNAs for which no similarity was found with that available from the public domain data banks were among the products identified.

In summary we have demonstrated that enrichment of up- and down-regulated rat liver gene products by a subtractive-hybridization procedure can be analyzed efficiently on a display gel and moreover that differences among the products can be isolated and characterized without a screening hybridization method. The novel, enriched gene products that were found in the regenerating rat liver are currently being analyzed in a *in vitro* expression/transfection system.

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