Differential subtraction chain, a method for identifying differences in genomic DNA and mRNA

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ABSTRACT

Identifying the genetic differences between two organisms or cell types has been a major goal in modern biomedical research. Recently, we developed a novel methodology that can rapidly identify the differences between two populations of DNA. This method, termed ‘differential subtraction chain’ (DSC), is based on a novel ‘negative amplification’ strategy that converts (amplifiable) tester sequences to counterpart (unamplifiable) driver sequences. The result is a double exponential elimination of amplifiable sequences in the testers, while preserving the sequences in the testers that have no counterpart in the drivers. We applied this methodology to the genome of a glioblastoma cell line. A homozygous deletion was rapidly identified. We extended this technique to identifying the unique sequences in mRNA. Two CDC25 transgene fragments were quickly identified in a cdc25B transgenic mouse. We also applied this methodology to systems with profound differences in mRNA expression. In a ‘prostate epithelia subtracting blood cells’ DSC reaction, a sample of unique gene fragments which are absent in the prostate but present in the blood were identified. Lastly, we detected rare (1 virus/100 cells) Herpes simplex virus type 2 (HSV-2) sequences in a tissue culture, indicating good sensitivity of this methodology. Overall, DSC represents a fast, efficient and sensitive method for identifying differences in genomic DNA and mRNA and can be easily applied in a variety of biological systems.

INTRODUCTION

Cloning the DNA sequence that corresponds to a genomic defect has been essential to our understanding of the genetic cause of a disease. Identifying the mRNA species that are turned on or off in specific tissues or after specific events is very useful in studies that involve embryo development, cell growth, differentiation and aging. If a unique sequence is present or a common sequence is missed in a tumor tissue when it is compared with its normal counterpart, it can be used as a tumor marker. Finding unique DNA fragments in infectious tissue helps to identify novel infectious agents (1). Numerous methods designed to identify differences in sequences have been reported. Many of them involve physical separation between testes and drivers (2–10). In the past 5 years, a PCR-based technique, called representational difference analysis (RDA), employing a differential enrichment approach to identify and enrich the differences between tested DNA samples has been developed (11). However, this method is very complicated and labor intensive. mRNA differential display (12) and RNA fingerprinting (13) by randomly primed PCR represent potentially faster and easier techniques to identify differential expression genes. However, high background and false positive results are frequently associated with these methods. These techniques also tend to be biased toward abundantly expressed sequences. Recently, a new PCR-based cDNA subtraction technique, termed suppression subtractive hybridization (SSH), was described (14). This technique used suppression PCR to preferentially amplify differential tester sequences to generate a cDNA probe library. Although this technique can dramatically enrich some differential DNA fragments, only one cycle of hybridization is permitted. Understandably, a significant background may be present.

In this report, we describe a novel PCR-based sequential subtractive hybridization method that rapidly isolates unique DNA sequences present between two tissue or cell types. This method, referred to as differential subtraction chain (DSC), employs a ‘negative amplification’ strategy to identify and to enrich the differences between two populations of DNA. This strategy produces fast and efficient isolation of unique tester sequences with minimal background. We have applied this method to identify genomic and mRNA differences in a variety of model systems. At the genomic level, DSC was applied to a human glioblastoma cell line and a genetic alteration in the tumor genome was rapidly identified. To detect the differences in mRNA expression, we applied DSC to a cdc25B transgenic mouse and rapidly identified the transgene cDNA fragments. In addition, we applied DSC to detect the differences in mRNA expression between a human prostate cell line and matched...
blood cells. Several novel along with two known unique gene fragments were identified.

MATERIALS AND METHODS

Materials

Breast tissue of MMTV-cdc25B transgenic mice were kindly provided by I. Bernard Weinstein (Columbia University). Glioblastoma cell line GL15 and matched blood cells as well as breast cancer cell lines were obtained through the Columbia University Tumor Bank. Normal primary prostate epithelial cultures were produced from organ donors as described by Krill et al. (15) and matched blood cells were obtained through the Western Pennsylvania Tissue Bank. Herpes simplex virus-infected cell cultures and Hep-2 cells were kindly provided by Laurel Aurelian (University of Maryland). Oligonucleotides for primers/adaptors were HPLC purified and were purchased from Operon Tech (CA).

Genomic DSC

Genomic DNA of glioblastoma cell line GL15 and matched blood cells was purified by a previously described procedure (16). One to five micrograms of genomic DNA were digested with BglII (glioblastoma cell line GL15) or HindIII (HSV-2-infected Hep-2 cells) for 3–6 h. The digestion products were purified with a Qiaquick purification kit (Qiagen, CA), which was performed according to the manufacturer’s recommendations. The procedure for ampiclon generation was as described previously (11). Briefly, the purified restriction products were mixed with adaptor/primer (HindIII set I, AGCACCTCTGACCTCT, CACCCGA/AGCTTTGCAGCTGGA; HindIII set II, ACCGACGTGACTATCCGAAACA/AGCTTTGCTATG; BglII set I, AGCACCTCTGACCTCT, CACCCGA/AGCTTTGCAGCTGGA; BglII set II, ACCGACGTGACTATCCGAAACA/AGCTTTGCTATG) in a total volume of 20 μl TE buffer. The mixture was heated to 72°C for 3 min and cooled to 4°C in 10 min (in a PCR machine). The annealed products were ligated with Ready-to-Go ligation mixture (Pharmacia, NE) at 16°C for 30 min, which was performed according to the manufacturer’s recommendations. The ligation products were purified with a QiAquick PCR purification kit and subject to PCR amplification using Taq polymerase (Perkin Elmer) under the following conditions: 94°C for 1 min, 68°C for 3 min for 30–35 cycles. For cDNA DSC, hybridization time was reduced to 10–14 h in each round. Northern blot hybridizations were performed according to a previously described procedure (18). The primer sets for the JBL series were as follows: JBL1a, ATCCGACACGGACGATGTAJTGTTGTTGA; JBL1b, GCCGTACTACCTGGACGGACGGAG; JBL2a, TCTCTACCTGGACGGACGGACGGAG; JBL3a, GTGACGGACTAACGAGTATCG; JBL4a, GACGGAGCTACCTCGAGTTACGAC; JBL4b, GACGGAGCTACCTCGAGTTACGAC; JBL5a, GTCCTTAGCCATCAGATACGAC; JBL5b, GTCCTTAGCCATCAGATACGAC; JBL6a, AGCCCAAGCTGGGGTGAGT; JBL6b, AGCCCAAGCTGGGGTGAGT; JBL7a, GTCCTTAGCCATCAGATACGAC; JBL7b, GTCCTTAGCCATCAGATACGAC; JBL8a, GTCCTTAGCCATCAGATACGAC; JBL8b, GTCCTTAGCCATCAGATACGAC; JBL9a, GTCCTTAGCCATCAGATACGAC; JBL9b, GTCCTTAGCCATCAGATACGAC; JBL10a, GTCCTTAGCCATCAGATACGAC; JBL10b, GTCCTTAGCCATCAGATACGAC.

RESULTS

Principles of DSC

Identification of genomic deletions or novel infectious agents has usually been accomplished by subtractive hybridization. However, due to the high complexity of eukaryotic genomes and the low enrichment of target sequences, this method was found inefficient for subtracting genomic DNAs from higher eukaryotes. This limits the application of the method to the identification of relatively long and relatively abundant
sequences. To reduce the complexity of genomic sequences in hybridization, we adopted the concept of genomic representational sampling proposed by Lisitsyn et al., in which short fragments of endonuclease-digested genomic DNAs are ligated with adaptors and amplified to produce ‘amplicons’ (11; Fig. 1A). Presumably, the genomic representation by amplicons would reduce the complexity of sequences by 80- to 100-fold and dramatically reduce the time needed to perform hybridization (11). However, once the amplicons were generated, we abandoned the differential enrichment approach. Instead, we developed a ‘negative amplification’ strategy to eliminate homologous sequences present in two different DNA pools. The principle of our strategy is based on the assumption that the maximum efficiency of a subtractive process depends on the starting tester/driver ratio. The survival number(s) of tester after each round of DSC follows the probability of tester to self-anneal in the preceding round, which is 1/2 before round 1 of DSC, 1/4 after round 1, 1/8 after round 2, 1/16 after round 3, 1/32 after round 4,…, and 1/2ⁿ after n rounds. Therefore, the survival copy(s) of tester can be predicted by the equation \( Y_e = y \cdot \frac{1}{A} \), where \( Y_e \) is the probable copy number(s) of survived tester after n round(s) of DSC, \( y \) is the total copy number(s) of a tester species and its relevant driver and \( A \) is the probability of the tester(s) self-annealing before the first round of DSC. Similarly, the number(s) of tester converted to driver in each round of DSC follows the probability of tester cross-annealing with driver, which is 1/2 before round 1 of DSC, 3/4 after round 1 of DSC, 15/16 after round 2, 65/64 after round 3, 65535/65536 after round 4,…, and 1 – 1/2ⁿ after n rounds. The copies of driver that survive each round of DSC can be predicted by the equation \( Y_o = y (1 - A) \cdot \frac{1}{A} \), where \( Y_o \) denotes total copy number(s) of driver after n round(s) of DSC. The probability for complete elimination of a tester species can be assessed (1 – \( \frac{Y_e}{Y_o} \)). (C) Subtraction efficiency of DSC on the \( \lambda \) amplicon. Five nanograms of 610 bp \( \lambda \) amplicon (\( \lambda_a \)) were subtracted by 3.9 (lanes 1–5) or 39 \( \mu \)g (lanes 6–10) of total \( \lambda \) DNA (HindIII restricted). The DSC products were amplified by PCR after 0 (lanes 1 and 6), 1 (lanes 2 and 7), 2 (lanes 4 and 9), 3 (lanes 5 and 10) rounds of DSC. (D) Subtraction efficiency of DSC in human genomic amplicons. One hundred nanograms of human liver genomic amplicon testers were subtracted in a DSC reaction in the presence of 10 \( \mu \)g of liver amplicon drivers from the same tissue. The DSC products were amplified by PCR after 0 (lane 1), 1 (lane 2), 2 (lane 3) and 3 (lane 4) rounds of DSC. (E) Identifying the presence of \( \lambda \) DNA sequence between two DNA pools using DSC. Fifty picograms of \( \lambda \) DNA were mixed with 1 \( \mu \)g of human liver genomic DNA to generate mixed amplicon testers. One hundred nanograms of these testers were subtracted by 10 \( \mu \)g of liver genomic amplicon drivers. The DSC products were amplified by PCR after 0 (lane 1), 1 (lane 2), 2 (lane 3) and 3 (lane 4) rounds of DSC. (F) Southern blot hybridization of (E) with 32 P-labeled \( \lambda \) DNA probe.
the contrast ratio of the DNA that is used to subtract (driver, non-adaptor-tagged amplicon) to the DNA being subtracted (tester, adaptor-tagged amplicon). In subtractive hybridization (2–10) or RDA (11), a high ratio between the drivers and testers is achieved through repeated addition of drivers and sequential dilution of testers in each cycle of subtractive hybridization. Unfortunately, this may result in the loss of rare sequences as well as an increase in time spent and in number of steps in the procedure. In DSC a high ratio between drivers and testers is obtained through a process that converts testers to homologous drivers after each subtractive round. This 'negative enrichment' process is accomplished by removing the primers from both ends of the testers with mung bean nuclease after they cross-annealed to drivers (Fig. 1A). In the subsequent round of subtractive hybridization, the testers without primers will become drivers and serve to convert more testers from the tester population. The result is a double exponential decrease in the copy number(s) of tester species that have homologous counterparts in the driver population and a corresponding increase in the copy number(s) of the driver species (see Fig. 1B for a mathematical evaluation). We term this process 'negative amplification'. Presumably, if $10^6$ copies of a target sequence present in one population is to be eliminated (Y/100), then the presence of 99-fold ($y = 10^{11}$, $A = 1/100$) copies of the corresponding driver it would take 3 ($n = 2.46$) rounds of negative amplification to achieve complete elimination of this tester. This negative amplification process achieves two goals. (i) By complete removal of the primers from the unwanted sequences, it eliminates any possible amplification while preserving the rare DNA sequences. This will improve the subtraction sensitivity and efficiency and produce cleaner products (less false positive results). (ii) By bypassing the need for positive enrichment processes between hybridizations, it dramatically simplifies and speeds up the subtractive process.

To demonstrate the subtraction efficiency of DSC, a 560 bp fragment of HindIII-digested λ DNA was ligated with an adaptor/primer and amplified to generate a tester (λa). More than $7.7 \times 10^9$ (5 ng) copies of this amplified fragment were used in a DSC subtraction in the presence of 10- or 100-fold of the corresponding DNA fragment of λ DNA (driver). As shown in Figure 1C, complete elimination of the tester was achieved after four rounds of DSC when 10-fold copies of driver were applied, while only three rounds of DSC were required to reach a similar result when 100-fold copies of driver were used.

These results fit well with the mathematical model shown in Figure 1B: when 10-fold copies of driver over tester were applied, ~395 copies of tester survived after three rounds of DSC. The minimum number of copies of tester that can survive four rounds of DSC is 4.18 $\times 10^{12}$ (when $T_d = 1/4$, $y_d = 1$ and $y_0 = 1/5$), where $y_0$ denotes tester copy number before the first round of DSC, then $y_d = 1/5^{1/4}$. When 100-fold copies of driver were applied, the minimum copies of tester that were needed to survive three rounds of DSC were 1.07 $\times 10^{12}$. Clearly, complete subtraction of the λ amplicon should be achieved within four rounds of DSC if 10-fold driver is used and within three rounds if 100-fold is used. To evaluate the utility of DSC in human genome subtraction, amplicons generated from genomic DNA of a human liver (100 ng) were used as testers and were subtracted by 100-fold copies of drivers generated from the same tissue. No tester survived two rounds of DSC (Fig. 1D), reflecting lower copies of individual tester species. The ability of DSC to identify differences between two pools of DNA was evaluated by mixing minute quantities of λ DNA with human liver genomic DNA to generate mixed testers and subtracting the mixture in the presence of 100-fold copies of liver drivers. After two rounds of DSC, only the λ amplicon was amplified (Fig. 1E and F).
A is the probability of tester self-annealing before the first round of DSC. Because of the enormous normalization ability of DSC, subtraction of a tester species ranging from $1.03 \times 10^7$ to $1.07 \times 10^{14}$ in copy number will synchronize when 100-fold copies of driver are applied, i.e. a tester with a copy number within this range will survive the second round of DSC but not the third round. To demonstrate this normalization function of DSC, serially diluted $\lambda a$ tester was mixed with 100-fold copies of $\lambda a$ driver in DSC reactions. As demonstrated in Figure 2, $\lambda a$ tester starting with 50 ng ($7.7 \times 10^7$), 5 ng ($7.7 \times 10^6$) and 500 pg ($7.7 \times 10^4$) survived two rounds of DSC reaction but not three rounds, while $\lambda a$ tester starting with 1 pg ($1.54 \times 10^4$) did not survive the second round of DSC. These results clearly confirm our mathematical prediction and indicate that DSC subtracts effectively regardless of variations in abundance of individual tester species.

To demonstrate the sensitivity of DSC to detect differences in rare sequences, Hep-2 cell cultures were infected with low or moderate multiplicity of infection units (m.o.i.) of Herpes simplex virus type 2 (HSV-2) (0.01 or 5 m.o.i., respectively).

Shortly after infection, DNA was extracted from the cultures. As demonstrated in Figure 3, after two rounds of DSC, two DNA fragments from both low and moderate multiplicity of infection were identified, which were confirmed to be HSV-2 sequences by sequencing. In addition, by mixing serially diluted artificial $\lambda a$ tester with hepatic cell genomic testers and subtracting with hepatic cell drivers, DSC scored positive on the $\lambda a$ tester starting with less than 100 copies (data not shown).

**Application of DSC to detect genomic homozygous deletion**

To test the ability of DSC to identify genetic defects in human diseases, a human glioblastoma cell line GL15 with a known homozygous deletion encompassing p16/p15 (CDKNA/CDKNB) was used as our test model. Amplicons of GL15 and matched blood DNA were generated by BglII digestion. To identify DNA deleted in the tumor, the normal amplicons were used as testers and the tumor amplicons were used as the drivers. As shown in Figure 4A, after three cycles of subtraction a distinctive band pattern was identified. To test whether these

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**Figure 4.** Identification of genomic DNA sequences missing in glioblastoma cell line GL15. (A) DSC products of BglII (lanes 1–4) testers from matched normal cells when 100-fold of respective glioblastoma cell drivers were applied, obtained after 0 (lane 1), 1 (lane 2), 2 (lane 3) and 3 (lane 4) rounds of DSC. (B) Southern blot hybridization of amplicons with DSC products. Round 3 DSC product BglII amplicons were used as probes to hybridize to respective amplicons generated from normal (N) or tumor (T) tissue. (C) Enrichment of LI sequence from normal amplicons by DSC. Lanes 1–4 represent Southern blot hybridization on DSC products of BglII testers from rounds 0 (lane 1), 1 (lane 2), 2 (lane 3) and 3 (lane 4) of DSC, with the PCR products of the LI probe (upper panel) and C15 (lower panel) clones as probes. (D) Homozygous deletion of the LI probe in GL15. Lanes 1–3 represent PCR products of primers specific for exon 2 of the p16 gene from GL15 cells (lane 1, T), normal tissue (lane 2, N) and BAC clone 59I23 (lane 3, B). Lanes 4–6 represent PCR products of primers specific for the LI probe, from BAC clone 59I23 (lane 4), normal tissue (lane 5) and GL15 cells (lane 6). Lanes 7–10 represent Southern blot hybridization on BglII-digested genomic DNA from normal (lanes 7 and 9) and GL15 (lanes 8 and 10) cells, with LI as probe.
homozgyous deletions of p16 were also deficient in L1. L1 could be amplified in all lines containing p16.

The majority of DSC products are not homozgyously deleted, but appear to be missing from the GL15 tumor amplicons. As has been noted by Lisitsyn and Wigler (11), many of the products of effective subtraction are due to restriction length polymorphisms that are deleted by loss of heterozygosity. Additionally, point mutations that affect BgII sites can lead to effective subtraction.

**Application of DSC to detect differences in mRNA expression**

With slight modification, DSC can be used to identify mRNA sequences that are uniquely present in one organism compared with another in 2–3 days. The human-derived MMTV-cdc25B (19) transgenic mouse was chosen as a model system to test the usefulness of DSC in identifying unique mRNA sequences. To generate cDNA amplicons, a 4 bp cutter restriction enzyme DpnII that produces a 4 bp 5’-overhang was selected. On average, this enzyme will cut every 256 bp. Presumably, most of the cDNA will be cut at least twice so that amplicon fragments can be generated (20). As shown in Figure 5A, excessive DpnII-restricted cDNA amplicons from wild-type mouse were used as drivers to subtract the amplicons from the cdc25B transgenic mouse. After three rounds of DSC subtraction, two distinct (650 and 350 bp) DNA fragments were amplified. Southern blot hybridization with these DSC products confirmed that they were only present in transgenic cDNA amplicons (Fig. 5B). These two DSC products were subsequently cloned and sequenced and confirmed to be fragments of cdc25B cDNA. These cloned DSC products were then used as a probe to perform northern blot hybridization on mRNA extracted from wild-type and cdc25B transgenic mouse. After three rounds of DSC subtraction, two distinct (650 and 350 bp) DNA fragments were amplified. Southern blot hybridization with these DSC products confirmed that they were only present in transgenic cDNA amplicons (Fig. 5B). These two DSC products were subsequently cloned and sequenced and confirmed to be fragments of cdc25B cDNA. These cloned DSC products were then used as a probe to perform northern blot hybridization on mRNA extracted from wild-type and cdc25B transgenic mouse (Fig. 5C). The results confirmed that these probes are unique to the transgenic mouse.

To test the utility of DSC in systems with profound differences in mRNA expression, cDNA amplicons from a primary human prostatic epithelium culture (N277) were used as drivers to subtract counterparts from the blood cells of the same individual. As shown in Figure 6A, after three rounds of DSC, multiple amplified tester fragments emerged. To verify whether these amplified DSC products were only present in the normal genomic amplicons, round 3 DSC products were used as a probe to perform Southern blot analyses on amplicons from GL15 and its paired normal DNA. Several distinct bands that corresponded to the DSC products were identified in the normal amplicons (Fig. 4B), while no distinct hybridization bands were seen in the tumor counterpart, indicating that these DSC products were not represented in tumor amplicons. Ten clones of DSC products after round 3 were randomly selected and sequenced. Primers specific for four unique DSC product sequences were made to examine whether these DSC products represented homozygous deletion in tumor cells. One of the four DSC products examined was found to be homozygously deleted in the tumor genome (Fig. 4D). This product, designated L1, was present in a bacterial artificial chromosome clone (59I23) that contains p16, arguing that L1 and p16 are within the same deletion. L1 was greatly enriched during DSC subtraction, in contrast to de-enrichment of probe C15, which is shared by both normal and tumor amplicons (Fig. 4C). In addition, a panel of 25 breast cancer cell lines was screened with L1 primers (data not shown). Three of the four lines harboring amplified DSC products were only present in the normal genomic amplicons, round 3 DSC products were used as a probe to hybridize to total mRNA extracted from cdc25B transgenic (lane 1) and wild-type mice (lane 2). The lower panel represents hybridization with probes from glycerol 3-phosphate dehydrogenase. Similar results were obtained with the 350 bp DSC product.

**Table 1. Differentially expressed gene fragments of blood cells from prostate**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene sequence</th>
<th>No. of clones</th>
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<td>BDP4</td>
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BDP, blood differential from prostate gene fragment.
products represent fragments of genes present only in blood cells, the round 3 DSC products were used as probes to perform Southern blot hybridization on amplicons from blood cells and prostate epithelium. An intense hybridization reaction was seen with the blood cell amplicons, while a negative reaction was found with the prostate epithelial counterpart (Fig. 6B).

The mixture of round 3 DSC products was subsequently cloned. Twenty colonies were randomly picked and sequenced. As shown in Table 1, 10 species of unique sequences were identified. Two of these were the T cell receptor ε and α chains. Surprisingly, the rest of the fragments represent previously unknown sequences. To further investigate the uniqueness of these fragments in the blood cells, RT–PCR on mRNA from prostate epithelium and blood cells was performed using primers specific to these sequences. As shown in Figure 6D, no PCR product was identified in mRNA from prostate epithelium, while PCR products with the appropriate sizes were seen in the blood cell counterpart.

**DISCUSSION**

The DSC method described in this report offers a good alternative to RDA and conventional subtractive hybridization as a method for identifying unique DNA sequences, because DSC represents a considerably easier (75% fewer steps) and faster procedure (2–3 days compared with weeks or months). DSC also offers excellent subtraction efficiency. This is because DSC tolerates low starting driver/tester ratios and the starting quantity of rare testers can be increased dramatically. Furthermore, the DSC subtraction series are performed without tester dilution. When uniquely expressed mRNA species are desired, DSC offers several technical advantages over differential display and RNA fingerprinting (12,13). These include: a clean background;
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