Construction of Libraries for Methylation Sites by In-gel Competitive Reassociation (IGCR)

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Abstract

The in-gel competitive reassociation (IGCR) procedure was successfully applied to construct a comprehensive library enriched in DNA fragments containing C5mCGG sequences from mouse liver and brain genomic DNA. For IGCR, methylation-insensitive restriction enzyme (Msp I) digests were used as target DNA and methylation-sensitive restriction enzyme (Hpa II) digests as competitor DNA. Southern blot analysis indicated that 60 to 70% of the clones in the library were derived from the methylated sites and overall enrichment was 200- to 1000-fold. IGCR was further applied to construct a library for the sites differentially methylated between brain and liver DNA. In the library, approximately 20% of the Hpa II sites exhibited different degrees of methylation between these tissues.

Key words: differential cloning; subtraction; methylation; Hpa II; library

1. Introduction

DNA methylation is known to be associated with a wide variety of biological phenomena. Methylation in the CpG island in the promoter region is implicated in the regulation of gene expression. Furthermore, it was proposed that methylation of CpG dinucleotides often causes base transition from C to T, resulting in C to T drift in the base composition of genes, a transition observed during aging of diploid cells and proliferation of tumors. Alteration of methylation patterns of genomic DNA during development and aging has also been reported in mouse intracisternal A particle genes, c-myc oncogene, spermatogenesis-specific genes and others. Methylation moieties in the mammalian genome decrease with aging. Furthermore, DNA methylation of certain genes such as Igf2, SNRPN, and Igf2r is related to paternal or maternal inheritance of their expression, suggesting that DNA methylation is, directly or indirectly, involved in the mechanism of genomic imprinting.

Several groups have devised methods to analyze and eventually clone methylation sites from eukaryotic genomic DNA. Restriction landmark genomic scanning (RLGS) method allows rapid scanning of differentially methylated sites. Clark et al. used sodium bisulfite to modify cytosine residues other than 5-methylcytosine, converting cytosine to uracil, which were then analyzed on sequencing gels. Fragments containing CpG islands were preferentially cloned by denaturing gradient gel electrophoresis, a technique referred to as segregation of partly melted molecules (SPM).

Previously, we reported a differential cloning procedure of genomic DNA, in-gel competitive reassociation (IGCR), to enrich and clone restriction DNA fragments that show differences in their size between two DNA preparations (target and reference DNA), and this method has proven to be quite useful for cloning altered DNA sequences present in complex eukaryotic genomes. We demonstrated that single-copy genomic DNA fragments in mouse genomic DNA could be enriched up to 105-fold by IGCR, and the technique was also applied to construct a library enriched in recombinant sites present in human extrachromosomal DNA and libraries of polymorphic DNA between mouse strains.

In this paper, we show that IGCR can be used to construct libraries enriched in DNA fragments with methylated sites.

2. Materials and Methods

2.1. Materials

Restriction enzymes, Klenow fragment and T4 DNA ligase were purchased from New England Biolabs, and bio-11-dCTP and bio-11-dUTP were from Sigma. Other materials used for the IGCR method are described in detail elsewhere. PCR primers for the 539-bp Msp I- and Bfa I-fragment from 3174 were GCAACTG-GACAATCAGAAAG and GAAGCAGCATCAGTGAC-GAC, which were used to amplify the region between nucleotides 3401 and 3805 (GenBank accession no. PX1CG).
2.2. Construction of the library of methylation sites

IGCR was performed as described previously\textsuperscript{22,23} with slight modifications for the cloning of methylated sites. Genomic DNA from mouse brain and liver were used for this purpose. Target DNA, from which DNA fragments with methylated CCGG sequences were to be isolated, was digested with methylation-insensitive \textit{Msp} I plus \textit{Bfa} I (4 units/\mu g DNA, respectively), and end-filled in the mixture containing 20 \mu M of each dNTP (except dCTP) and 36 \mu M bio-11-dCTP with 5 units of Klenow fragment for 1 hr at 25\degree C. \textit{Bfa} I was used to increase the complexity of DNA fragments. Reference DNA (competitor DNA) from the same source as the target DNA, was digested with methylation-sensitive \textit{Hpa} II plus \textit{Bfa} I (2 units/\mu g DNA, respectively), and subsequently treated with bacterial alkaline phosphatase (0.3 units/\mu g DNA). After IGCR of the mixture of target (200 ng) and reference DNA (20 \mu g) followed by adaptor ligation, fragments labeled with biotin were adsorbed on avidin-coated tubes to eliminate fragments with \textit{Bfa} I sites on both ends. PCR was then performed to amplify the DNA fragments adsorbed on the tubes. Since target DNA fragments with unmethylated CCGG sites were subtracted by the same fragments in the reference DNA created by \textit{Hpa} II and \textit{Bfa} I digestion, only those fragments containing methylated CCGG sites were enriched after PCR amplification. The amplified DNA was digested with \textit{Not} I and cloned into the \textit{Not} I site of pBluescript SK(-).

2.3. Enrichment of differentially methylated \textit{Hpa} II sites between brain and liver genomic DNA

Mouse brain genomic DNA, used as target DNA, was treated with \textit{Hpa} II, followed by bacterial alkaline phosphatase (0.06 unit/\mu g DNA) treatment. This DNA sample was purified and digested with \textit{Bfa} I and biotinylated with 36 \mu M bio-11-dUTP and 20 \mu M dATP. Mouse liver genomic DNA (reference DNA) was prepared by digestion with \textit{Bfa} I and \textit{Hpa} II, followed by bacterial alkaline phosphatase treatment (0.3 unit/\mu g DNA). A mixture of the target (200 ng) and the reference DNA (20 \mu g) was then subjected to IGCR and subsequent avidin selection. In this scheme, the fragments with \textit{Bfa} I sites on both ends and a methylated \textit{Hpa} II site in the middle should be specifically enriched. After a second cycle of IGCR, the sample was digested with \textit{Not} I and cloned into the \textit{Not} I site of pBluescript SK(-). Clones containing an \textit{Hpa} II site in the cloned fragments were selected and used for Southern blot analysis.

3. Results and Discussion

3.1. Methylation sites in mouse brain and liver genomic DNA

We first examined the degree of enrichment of DNA fragments with the methylated CCGG sequence among those carrying methylated and unmethylated CCGG sequences. An exogenous marker (539 bp \textit{Msp} I and \textit{Bfa} I

\begin{figure}[h]
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\caption{Enrichment of a marker DNA by IGCR. A: Enrichment of \textit{\alpha}X174 DNA among \textit{Msp} I-fragments from mouse genomic DNA. IGCR procedure was performed with \textit{Msp} I+\textit{Bfa} I-digested liver (for lanes 1 to 4) or brain (for lanes 5 to 8) DNA as a target against \textit{Hpa} II+\textit{Bfa} I-digested brain or liver DNA as a reference, respectively. Twenty-six picograms of \textit{Msp} I+\textit{Bfa} I-digested \textit{\alpha}X174 was mixed with 2.5 \mu g of target DNA before IGCR, and enrichment of the 539-bp \textit{Msp} I-\textit{Bfa} I fragment was monitored by PCR. After the first (lanes 1, 2, 5 and 6) and the second (3, 4, 7 and 8) cycles of IGCR, 0.1 \mu g aliquots of the products (control and IGCR samples after avidin selection) were examined by PCR (18 cycles of 1 min at 94\degree C, 2 min at 50\degree C and 3 min at 72\degree C, with 10 min extension at 72\degree C at the end). The 405-bp PCR products are arrowed in the figure. B: Overall enrichment of the marker DNA. Target DNA fragments used in the first cycle and the IGCR products (liver DNA, lanes 1 to 5; brain DNA, lanes 6 to 10) were examined by PCR.}
\end{figure}
Figure 2. Southern blot analysis of the clones in the library of methylated sites in mouse DNA. Five clones (A to E: pML4, pML5, pML6, pML9 and pML10) from liver DNA and one clone (F: pMB13) from brain DNA were used for hybridization with Msp I+Bfa I-digested liver (lane 1), brain (lane 3) DNA and Hpa II+Bfa I-digested liver (lane 2) and brain (lane 4) DNA.

fragments from φX174 DNA), which was added only to the target sample, was enriched approximately 20-fold in the libraries constructed from brain or liver DNA (Fig. 1A). The overall enrichment of the marker among all DNA fragments including those with Bfa I sites on both ends after a single cycle of IGCR was approximately 200-fold for liver DNA and 1000-fold for brain DNA (Fig. 1B). This indicates that fragments with at least one Hpa II site at the ends occurred roughly once every 10 to 50 fragments, and most were created by Bfa I alone. This frequency was confirmed with a control sample which was not subjected to IGCR (data not shown). Since there was no enrichment of the methylated DNA fragments in the second IGCR cycle for both samples (Fig. 1A, lanes 3, 4, 7 and 8), which suggested that the enrichment had reached a plateau after the first IGCR cycle, we analyzed the clones in the library obtained after the first IGCR cycle.

Figure 2 shows the results of Southern blot analysis of the clones in the liver (pML4, pML5, pML6, pML9 and pML10) and brain (pMB13) libraries. Each of the six clones shown here was apparently derived from genomic DNA exhibiting certain degrees of methylation (resistant to Hpa II). The figure also indicates that repetitive as well as single-copy sequences with the methylation sites were effectively enriched. Of 26 clones analyzed, none showed methylation patterns which were different between liver and brain, indicating that the majority of the methylation sites were common to the DNA of both tissues. Table 1 summarizes the characteristics of the clones in the brain
and liver libraries. Among 13 clones selected randomly from these libraries, nine (69%) and eight (62%) from liver and brain DNA, respectively, represented methylated sequences. Another aspect of the libraries was that methylated repetitive sequences were also effectively enriched (33% of methylated sequences in the liver DNA library and 38% in the brain DNA library). Both libraries exhibited complexity of approximately $3.5 \times 10^4$, which was calculated from the frequencies of the occurrence of single-copy genomic DNA fragments in the libraries (data not shown).

### Table 1. Summary of the enrichment of methylated sites by IGCR.

| Tissue | State of Methylation | Copy No. | Clone No. | Total |
|--------|----------------------|----------|-----------|-------|
| Liver  | M                    | Single   | 6 (46%)  | 9 (69%) |
|        | NM                   | Multiple | 3 (23%)  | 4 (31%) |
| Brain  | M                    | Single   | 5 (39%)  | 8 (62%) |
|        | NM                   | Multiple | 3 (23%)  | 5 (38%) |

a) Determined by resistance to Hpa II digestion. M, methylated; NM, not methylated or not determined due to high repetitiveness.

b) Copy no. in the genome based on Southern blot analysis.

3.2. Sites differentially methylated between brain and liver genomic DNA

A library of clones exhibiting different degrees of methylation between brain and liver DNA was also constructed by IGCR. For this, we used mouse brain genomic DNA as target DNA and liver DNA as reference DNA. Approximately 20% (4 of 20) of the clones in the library exhibited 50% differences in the degree of methylation. Figure 3 shows Southern blots of the brain and liver DNA probed with the clones that showed differences in methylation between these tissues. Differences in the degree of methylation between these tissues were generally 10% to 50%. Since none of the 26 clones from the libraries of both brain and liver DNA showed apparent differences (see Fig. 2), it is quite clear that differentially methylated sites between the two tissues were enriched by IGCR.

3.3. Strategy for cloning methylation sites from genomic DNA

We have described characteristics of the libraries of methylated Hpa II sites in brain and liver genomic DNA. These libraries were constructed by subtracting the unmethylated DNA fragments by competitive reassociation in the gels. We incorporated several modifications into the original IGCR protocol for the enrichment of methylated DNA fragments. For example, we codigested DNA with Msp I (Hpa II) and Bfa I, the latter to further create smaller DNA fragments by cutting at CTAG sequence. Since CG dinucleotides are under-represented in man-
malian genome. Hpa II or Msp I digestion alone would not create appropriate size of DNA fragments for PCR amplification. The in-gel competitive reassociation has made this type of modification possible, since it allows processing of even highly complex mixtures of DNA fragments. We also introduced the avidin selection step to enrich biotinylated Hpa II sites as the majority of the fragments had Hpa I sites at both ends. As a result of these modifications, just one cycle of IGCR resulted in 200- to 1000-fold enrichment of DNA fragments with specific methylated sites (Fig. 1 and Table 1), having reached a plateau.

Southern blot analysis of genomic DNA probed with clones from the libraries showed that these clones were derived from the genomic DNA that had been methylated by 50 to 100% (Fig. 2). Furthermore, methylated repetitive sequences seemed to be enriched as effectively as unique sequences, since approximately 35% of the clones were derived from repetitive sequences (Table 1).

We also showed that the IGCR procedure can be used to construct libraries enriched in clones showing different degrees of methylation between two specific tissues. Approximately 20% of the clones with Hpa II sites exhibited differences between brain and liver. Since the occurrence of differentially methylated sites among tissues is very infrequent, it was quite laborious to enrich and eventually to clone differentially methylated sites. In fact, most of the procedures used to enrich methylation sites described previously involve construction of libraries for all the methylation sites. Our strategy using IGCR described here provides a much more efficient and versatile way to clone methylation sites, which should facilitate studies of the mechanism of tissue-specific methylation as well as those of methylation during development, differentiation and aging.

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