Chapter 5

MUTATION OF THE M.BAMHI AND SCREENING

Inactive mutants of BamHI methylase were generated both by PCR method as well as by chemical mutagenesis. Both the methods were employed to avoid any inherent bias of either method.

5.1 Generation of mutants by PCR (forced error method)

Polymerase chain reaction is very versatile technique and is widely used in various ways e.g. amplification, mutation detection, sequencing, mutagenesis, fingerprinting etc. To generate mutants we took advantage of the error caused by Taq Polymerase in presence of imbalanced nucleotide concentration. One of the constraints in our mutagenesis procedure was that we needed a limited number of mutation in the gene (1 or 2 mutation in 1.76 Kb). This required us to optimise the method.

In this method the gene was amplified under the conditions given below in presence of one of the nucleotide in excess as compared to three other. The four reaction, one each with excess A or C or G or T was set.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>10X <em>Taq</em> polymerase buffer (Promega)</td>
<td>10 μl</td>
</tr>
<tr>
<td>3' primer (100 pmole/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>5'primer (100 pmole/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>2 mM dNTP mix (Pharmacia)</td>
<td>10 μl</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>10 μl</td>
</tr>
<tr>
<td>Forcing nucleotide (2.5 mM)</td>
<td>4.0 μl (A, C, G, or T)</td>
</tr>
<tr>
<td>H2O to make volume</td>
<td>100 μl</td>
</tr>
<tr>
<td><em>Taq</em> polymerase (Promega, 5.0 U/μl)</td>
<td>0.5 μl (2.5 U)</td>
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Cycling condition was same as in section 4.2. The PCR products were pooled and gel purified and then cloned as in section 4.4 and screened as in section 5.3.

The ligated product was transformed in *E. coli* DH10B and plated on tet plates. Isolated single colonies picked up from plate and were grown in 5 ml LB and DNA isolated by mini prep method. DNA from each colony was digested with *EcoRI* (4U/μg) and *BamHI* (20U/μg) separately and was run on a gel. The *EcoRI* digestion confirmed the presence of insert and digestibility of the DNA, while *BamHI* digestion if occurred showed that the clone was inactive. All those colonies whose DNA was not digested by *BamHI* were discarded. From each plate 20-40 colonies were screened. The inactive mutants obtained were labelled as A2, A9, MM2, MM3, MM4, 4.1, 4.2, 4.3, 8.1, 8.2, 8.3, 9.1, 9.2, 9.3, 9.4. These mutants were finally screened and selected by heteroduplex analysis and heteroduplex chemical cleavage method as described later.

### 5.2 Random mutagenesis by chemical method

We followed the method of Myers *et al.* (1985). The mutagenizing agents used were Formic acid, Nitrous acid, Potassium permanganate and Hydroxylamine. We also used UV radiation to generate mutants but in our hands we could get completely inactive mutants only with hydroxylamine. The conditions used are given below.

The wild type *BamHI* methylase clone pMAP6.0 DNA (purified by cleared lysate method) 8.0 μg in 80 μl T10E1 was mixed with 520 μl 100 mM sodium phosphate buffer pH 6.0, hydroxylamine 400μl of 1.0 M and 2μl of 0.5 M EDTA. The mix incubated at 37°C for 23, 36, 47 and 63 hrs. followed by precipitation with 2.5 volume ethanol and 0.1 volume 3M sodium acetate pH 5.2. DNA pellet obtained by centrifugation at 14000rpm for 15 minutes at 4°C. Pellet was washed once with 70% ethanol and resuspended in T10E1 reprecipitated again as above to remove any traces of hydroxylamine. Mutated DNA was then digested with *EcoRI* and the insert released was purified and cloned as in section 4.4. This sub cloning was necessary because the vector also gets mutated during mutagenesis, which might interfere with the further propagation of the plasmid. For each time point (23, 24, 36, 47, 63 hrs) product one ligation was set up. The ligated product of mutant DNA was transformed in *E.coli* DH10B.
and plated on tetracycline plates. From each plate of respective treated time points, 48-96 colonies were screened. The screening was done by digesting mini prep DNA from each colony with EcoRI and BamHI separately. BamHI, if linearises the plasmid indicated inactive clone while EcoRI digestion checked the insert and digestibility of the DNA preparation. The mutants thus obtained were labelled as 2367, 2376, 2407, 2408, 2410, 2414, 2415, 2422, 3635, 3643, 3646, 4701, 6302, 6303, 6311 where first two digits signifies the hrs. of treatment with hydroxylamine. The mutants selected for further study were 6302 and 6303 designated as pMAP6M2 and pMAP6M3 hereafter.

5.3 Screening of the inactive mutants

The individual colonies of mutants generated in section 5.1 and 5.2 was inoculated in 5.0 ml LB and DNA was prepared by LiCl boiling rapid preparation method (see section 9.5.1) and checked with EcoRI and BamHI digestions. Only those clones which were fully inactive were selected and further characterised by heteroduplex analysis and heteroduplex chemical cleavage analysis. Final confirmation of exact position and nature of the mutation was obtained by dideoxy sequencing method. The results are shown in figure 5.1 & 5.2.

5.4 Heteroduplex analysis for detection of mutation

Heteroduplex is the double stranded DNA formed by annealing the two homologous strands one from the wild type gene and the other from the mutant having a base change or small insertion or deletion compared to the wild type sequence. Heteroduplex analysis is one of the easiest method to detect the mutation. This method rely on the electrophoretic mobility change due to a bubble or bulge or kink generated by mismatched or unmatched bases in the heteroduplex. The mismatched base may be rotated into the grooves or flipped out side the duplex, or the duplex may be bent at the point of mismatch. Thus when electrophoresed in a non denaturing gel, duplex with mismatches move differently from those with no mismatches. We used 6-15% step gradient native PAGE for better resolution. Gel composition and preparation method is described below.
Figure 5.1 Screening of inactive mutants.
Odd numbered lanes; digestions with EcoRI, 1.76kb insert is released. Even numbered lanes; digestions with BamHI, no cleavage except in lane 6 (panel a) and lane 2 (panel b). In lane 2 complete cleavage (totally inactive mutant) and in lane 6 incomplete cleavage is seen.
Figure 5.2 Screening of inactive mutants.
Odd numbered lanes; digestion with *Bam*HI, linearized plasmid showing no protection due to inactive methylase. Even numbered lanes; uncut plasmid same as in the odd numbered lanes.
Gel conditions: 6-15% step gradient(native PAGE)

<table>
<thead>
<tr>
<th>Components</th>
<th>6%(15 ml)</th>
<th>10%(10 ml.)</th>
<th>15%(15ml)</th>
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<tbody>
<tr>
<td>50% Glycerol</td>
<td>3.0 ml</td>
<td>2.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>10X TBE</td>
<td>1.5 ml</td>
<td>1.0 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.0 ml</td>
<td>3.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>(29:1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>7.5 ml</td>
<td>3.5 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>APS 10%</td>
<td>200 µl</td>
<td>100 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>7 µl</td>
<td>4 µl</td>
<td>5 µl</td>
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The 15% solution was poured first then 10% followed by 6% in a pre assembled 20x20x1 cm gel apparatus and allowed to polymerize for 1 hour.

**Heteroduplex formation:** Heteroduplex analysis was done as per the protocol used by White et al., 1993. Equal volume of labelled wild type 1.76 kb PCR product amplified from wild type clone was mixed with unlabelled 1.76 kb PCR product amplified from the mutant clone and digested with TaqI and BstNI(5 units each µg DNA) in 1XTaqI buffer at 60°C for two hours. Annealing was done by heating at 95°C for 10 minutes and allowing it to cool slowly up to room temperature. The heteroduplex DNA thus obtained was loaded on the gel mentioned above alongside homoduplex from wild type and mutant DNA and electrophoresed in 0.5x TBE at 4°C for 3000 volt hour. The gel was run at 200 V for 10 mins., 150V for 1hr and finally voltage was reduced to 120 V till the run completed. After the run was complete the gel was dried and exposed to X-ray film.

The figure 5.3 shows the heteroduplex band in between 618 and 269 bp homoduplex bands. This band can arise from one of the three fragments of sizes 766, 618 or 269 bp obtained by double digestion with Taq I+BstNI.
Figure 5.3  Heteroduplex analysis of mutants.
Panel A Lane 1 and 3: homoduplexes from pMAP6(wt) and pMAPM4.2 (mutant) respectively, Lane 2 heteroduplex from wt and the mutant. Homoduplex bands marked A, B, D & E. Heteroduplex band marked C
Panel B Lane 1 heteroduplex from pMAPM3 and pMAP6, Lane 2 & 3 homoduplex from pMAPM3 and pMAP6 respectively. Homoduplex bands marked A,B,D & E. Heteroduplex band marked C.
5.5 Heteroduplex chemical cleavage method for mutation detection

The chemical cleavage of mismatch (ccm) method (Cotton et al., 1988) allows the mutation sites to be detected even in kilobase length pieces of nucleic acid. Chemical cleavage is based on the principle that mismatch or unmatched residues are more reactive to modifications by hydroxylamine. Hydroxylamine modifies C, subsequent cleavage of modified base is obtained by Piperidine cleavage. Cleavage products are resolved by denaturing PAGE. The sequence context surrounding the mismatch is important in determining whether a particular site will be modified or not. The method is more reliable as compared to other available methods as well as it gives the approximate position of mutation.

For CCM the following steps were followed.
1. Gel purification of the probe
2. Double digestion of PCR amplified gene with TaqI and BstNI
3. Heteroduplex formation
4. Modification by Hydroxylamine
5. Cleavage by piperidine
6. Separation of cleavage products on denaturing PAGE

Probe: The BamHI methylase gene (1.76kb) of both wild type and mutant were amplified. The wild type gene was amplified in presence of $^{32}$Pα dATP to radio label the PCR product throughout. The PCR products were gel purified by Promega PCR purification system.

Digestion: Amplified DNA was digested with TaqI and BstNI to get fragments of sizes 766, 618,269 and 129bp which are within the range of analysis. The PCR product was digested in 1X TaqI buffer containing 5.0 unit of TaqI and 5.0 units of BstNI at 60°C for 2 hours followed by ethanol precipitation.

Heteroduplex: The double digested wild type (labelled) and mutant (unlabelled) DNA were mixed in equal ratio, heated at 95°C for 10 minutes in 1X annealing buffer [0.6M NaCl, 6mM Tris(pH 7.5),and 7mM MgCl$_2$] and allowed to
cool slowly till it reached room temperature and then chilled on ice. DNA was precipitated by ethanol, washed with 70% ethanol, air dried and used in the subsequent step for modification.

**Hydroxylamine modification:** To the dried DNA (heteroduplex) dissolved in 6.0 µl H₂O, 20 µl hydroxylamine [2.5 M, freshly prepared by dissolving 1.39 g hydroxylamine hydrochloride in 1.6 ml H₂O and pH adjusted to 6.0 with di-ethylamine] was added and incubated at 37°C for 3 hours. The reaction was stopped by adding stop solution 200µl [0.3M sodium acetate (pH 5.2) 0.1M EDTA, 25µg/ml tRNA] and 750µl ethanol. DNA precipitated at -20°C for 30 minutes then spun at 14000 rpm at 4°C for 30 minutes. Pellet was washed with 70% ethanol and air dried, the next step was Piperidine cleavage.

**Piperidine cleavage:** The modified dried DNA pellet was resuspended in 50µl freshly diluted piperidine (1M), heated the samples at 90°C for 30 minutes. Chilled the samples on ice and added 50µl stop solution and 300µl ethanol and precipitated by ethanol as above. Precipitation was repeated to remove traces of piperidine and pellet resuspended in formamide loading dye (1000cpm/µl).

**Electrophoresis:** Samples denatured at 95°C for 5 minute and chilled on ice and loaded on 5% denaturing urea PAGE. The gel was pre run for 30 minutes and the plate temperature was maintained at 50°C. After the run was over, gel was fixed in 10% methanol 10% acetic acid for 20 minutes and dried at 60°C for 6 hours and exposed to X-ray film for two days.

Results of chemical cleavage are shown in figure 5.4. Lane 1 and lane 2 both show two fragments of approximate sizes of 430 and 320 bp which is from the fragment 746 bp. These DNAs therefore, have mutation at similar position. In lane 3 we could not detect any cleavage. Lane 4 is the wild type gene as control also gave some background bands. Lane 5 and 6 have more bands and hence suggest more than one mutation. In lane 5 (pMAP6.0M9.3) after cleavage the fragments generated are of
Figure 5.4 Heteroduplex chemical cleavage analysis of mutants. Lane 1 pMAP6M2, Lane 2 pMAP6M3, Lane 3 pMAP6M4.2, Lane 4 pMAP6, Lane 5 pMAP6M9.3, Lane 6 pMAP6M8.3, Lane 7 100 bp ladder marker
approximate sizes 500,430, 160,140 which may come from cleavage of either 766 bp or 616 bp fragments or both. In lane 6 (pMAP6.0M8.3), the cleavage products obtained are of sizes 510, 325, 265, 40, and 30 bp which again may be the cleavage products from both the 766 and 618 bp fragments.

5.6 Expression of Mutants

Protein expression in *E. coli*, under the control of its own promoter, of the mutants was checked. *E. coli* DH10B cells having mutant methylase were grown to late log phase, cells were harvested and washed with 1X PBS and resuspended in 40 μl of 1X methylase extraction buffer. Resuspended cells were incubated on ice for 5 min followed by addition of 40 μl 2X protein sample buffer and incubated at 90°C for 5 min followed by sonication for 10 seconds at 4°C. The lysed cells were centrifuged in a microfuge at 15000 rpm at 4°C for 20 min and supernatant was collected in a new tube. Lysate 20-25 μl was checked on 10% SDS PAGE as described in Materials and Methods (Figure 5.5). The extra band of the methylase obtained is of expected size. All the 5 mutants checked were expressing at low level similar to wild type. The *E. coli* containing only the vector was used as control.

5.7 In Vivo assay of inactive mutants

Inactive methylase mutants were transformed in *E. coli* JH139 containing *dinD::lacZ* which upon DNA damage give blue colour on X-gal plates. This in-vivo assay gives an indication whether there is any DNA damage due to mer response caused by methylation. Results shown in figure 5.6 & 5.7 also support that mutants were indeed completely inactive as there was no blue colour produced over background.
Figure 5.5 Expression of mutants in E.coli under the control of its own promoter. The expressed protein band marked with arrow.
Lane 1 DH10B with only vector pACYC184, Lane 2 DH10B with pMAP6 (wild type) Lane 3 Molecular weight marker, Lane 4 DH10B with pMAP6M2, Lane 5 DH10B with pMAP6M3, Lane 6 DH10B with pMAP6M4.2, Lane 7 DH10B with pMAP6M9.3, Lane 8 DH10B with pMAP6M8.3
Figure 5.6 SOS response of inactive methylase mutants, in vivo assay of enzyme activity (see text for details)
A- Cells only, C- Cells with only vector, B, D, E, F, G and H- inactive mutants.
Figure 5.7 SOS response of inactive methylase mutants