Title
Transcriptional repression by methyl-CpG-binding protein 2

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Chair

University of California, San Diego

2011
DEDICATION

For their love and support, I dedicate this thesis
to my parents and Cassandra Martell
EPIGRAPH

Men fear thought as they fear nothing else on earth -- more than ruin -- more even than death.... Thought is subversive and revolutionary, destructive and terrible, thought is merciless to privilege, established institutions, and comfortable habit. Thought looks into the pit of hell and is not afraid. Thought is great and swift and free, the light of the world, and the chief glory of man.

-Bertrand Russell
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Thank you to all the members of the Kadonaga lab for your support. I have enjoyed working with all of you. Thank you to Timur for bringing the MeCP2 project to the lab.

To my friends and family, I am very grateful for your all of your love and support. To my mother and father, thank you for all you have done for me and for always having faith in me.

Finally, I would like to thank Cassandra Martell for her unfailing love and encouragement. In all things, you have been a continuing source of support.
Methyl-CpG-binding protein 2 (MeCP2) is a founding member of the methyl-CpG-binding protein family and is required for normal development. Mutations in MeCP2 are the primary implicated cause of the X-linked neurodevelopmental disorder known as Rett Syndrome. Currently, the
mechanism by which MeCP2 selects and represses target genes is not well understood. We have sought to understand the function of MeCP2 by the biochemical analysis of CpG-methylation-specific transcriptional repression by purified recombinant human MeCP2. This work includes the development of an experimental system for the determination of the effect of the location and density of methyl-CpG dinucleotides on transcriptional repression. It has been found, for instance, that methylation in the core promoter region is sufficient but not necessary for transcriptional repression by MeCP2. These studies will enable the detailed analysis of the mechanism by which MeCP2 represses gene transcription.
I:

Introduction
DNA methylation is an epigenetic modification that is critical for many cellular processes like X chromosome inactivation, genomic imprinting, transcription, and chromatin structure (11). It has also been found to play an important role in cancer. For example, the genomes of tumor cells tend to be hypomethylated compared to normal cells (5).

DNA methylation plays an important role in transcriptional regulation in mammals; the majority of CpGs in mammals being methylated, CpG islands being a notable exception (2). Generally, DNA methylation is strongly correlated with transcriptional repression. Genes having a large amount of methylated CpGs tend to be repressed. CpG methylation involves the replacement of a hydrogen atom by a methyl group at position 5 of the cytosine ring. This process is maintained and catalyzed by a class of proteins known as methyltransferases which are required for embryonic development (15, 17, 22).

The information encoded by methylated CpGs needs to be effectively interpreted and translated into the appropriate regulatory response. The MBD (methyl-CpG-binding domain) family of proteins lies at the interface of this interaction. Methyl-CpG-binding protein 2 is an original member of the methyl-CpG-binding protein family (18). MeCP2 was originally identified by its ability to bind to methylated DNA. Mutations in MeCP2 have been shown to cause the neurodevelopmental disorder known as Rett syndrome (1). In line with this observation, MeCP2 has been found in very high concentration in the neurons...
of the post natal brain (6). Roughly 80% of sporadic cases of Rett syndrome are caused by mutations in MeCP2 (3). MeCP2 is found on the long arm of the X chromosome, and the disease is inherited in an X-linked dominant pattern (1). Rett syndrome almost exclusively affects females (1). This observation suggests that the complete absence of functional MeCP2 has lethal consequences very early in development. Rett syndrome is considered an autism spectrum disorder because it shares some similar clinical manifestations of autism including seizures, loss of speech, and stereotypic hand movements (27). Rett Syndrome is characterized by normal development for the first 6-18 months of life followed by developmental regression (7).

MeCP2 is a 53 kDa monomeric protein which has a TRD (transcription repression domain) and a MBD (methyl-CpG-binding domain). Both have been well studied. MeCP2 binds to methylated CpG dinucleotides via the MBD and represses transcription through the TRD (19, 20). However, the true nature of these interactions and how MeCP2 selects target genes to be repressed is not completely understood. The fact that the majority of CpG dinucleotides in mammals are methylated had caused initial speculation that MeCP2 acts as a global repressor of transcription, but this hypothesis does not appear to be accurate. Several studies have found MeCP2 to repress only a relatively small selection of target genes (4, 13, 25). In vivo repression of transcription has been shown to involve recruitment of Sin3a-HDAC (histone
deacetylases) complexes (9, 21). However, transcriptional repression by MeCP2 is not solely dependent on HDAC complexes because a substantial fraction of MeCP2-mediated repression is resistant to the HDAC inhibitor, trichostatin A (14). The mechanism of HDAC-independent transcriptional repression is unknown. Additionally, MeCP2 has been found to interact with many proteins including DNA and RNA modifying enzymes (9, 12, 26). Further investigation into the mechanism of transcriptional repression by MeCP2 and how MeCP2 selects target genes is needed. Furthermore, it could provide insights into new effective treatments for Rett syndrome.
II:

Results
Preparation of Hela nuclear extract and purification of recombinant MeCP2

To investigate transcriptional repression by MeCP2 in vitro, it was first necessary to generate HeLa nuclear extract and wild-type recombinant MeCP2. A HeLa nuclear extracts was prepared and then tested to find the optimal concentration that had the highest transcription signal. This was done by comparing the HeLa nuclear extract with a previous extract of known strength. In vitro transcription analysis found that the newly prepared extract was approximately three times stronger than the previous standard (Figure 1). Recombinant wild-type MeCP2-chitin binding domain fusion protein was expressed in *E. coli*. The fusion protein was purified using a chitin resin, and the CBD (chitin binding domain) was removed by intein cleavage. SDS-PAGE was used to quantify and to determine the purity of the protein (Figure 1.1).

Repression of in vitro transcription by MeCP2 requires CpG methylation

In vitro transcription reactions with HeLa nuclear extract were carried out in the presence or absence of wild-type recombinant MeCP2. Both fully-methylated DNA templates and unmethylated templates were tested. The CpG-methylated templates were prepared by using the M.Sss I methylase. The addition of MeCP2 to reactions with unmethylated DNA template did not have a significant effect on transcription (Figure 2.) However, the addition of MeCP2 to reactions with fully methylated DNA template led to almost complete repression of transcription (Figure 2).
MeCP2 inhibits pre-initiation complex formation

We next investigated the mechanism by which MeCP2 is able to repress transcription in vitro. To this end, in vitro transcription reactions were carried out in the presence of trichostatin A (TSA). TSA is a broad range HDAC inhibitor that is able to inhibit both classes of HDACs (23). The presence of TSA had no effect on the level of transcriptional repression by MeCP2 on a fully methylated DNA template (data not shown). We set out to determine the mechanism of HDAC-independent transcriptional repression.

The transcription from the basal machinery can be divided into 3 steps: pre-initiation complex (PIC) formation, initiation, and elongation. The distinct steps of transcription can be isolated by the ordered addition of factors. Also, by adding the detergent Sarkosyl to the reaction after PIC formation, transcription can be limited to one round (Figure 3). Having only one round of transcription greatly simplifies the experiment. The addition of MeCP2 before HeLa nuclear extract at time point A, allowing MeCP2 to affect all steps of transcription, caused complete repression of transcription. However, addition of MeCP2 at later time points C and D only allowed MeCP2 to affect initiation and elongation respectively (Figure 3). No significant repression of transcription was seen from adding MeCP2 at time points C and D, thus indicating that MeCP2 was not affecting those steps of transcription. Therefore, the repression seen at time point A must be due to inhibition of PIC formation and not the other stages of transcription. At time point B, MeCP2 was added at the
same time as HeLa nuclear extract which led to 10 fold repression of transcription (Figure 3).

**Repression of in vitro transcription by MeCP2 does not require CpG methylation in the core promoter**

A possible explanation for how MeCP2 inhibits PIC formation is that MeCP2 is able to cause steric inhibition by binding to the core promoter and occluding basal transcription factor binding sites. To test this hypothesis, the SCPX-0CpG promoter was created. SCPX-0CpG is the same as the previous promoter that was used, but it lacks the presence of CpG dinucleotides. Specifically, this core promoter has a 100 bp CpG-deficient window centered at the transcription start site. The pUC119 plasmid backbone is still methylated. The number of base pairs occupied by bound full length MeCP2 is not known, but binding of just the MBD causes a DNase I footprint that protects approximately 12 bp surrounding the methylated CpG dinucleotide (19). Given these data, it is unlikely that MeCP2 bound to the plasmid vector outside of the core promoter region will be able to occlude the binding of the basal transcription factors within the 100 bp CpG-free window. In vitro transcription analysis showed that MeCP2 is able to repress transcription of the SCPX-0CpG plasmid by almost 10 fold, even though there are no methyl-CpG dinucleotides in the core promoter. Therefore, the inhibition of PIC formation seen previously is not due solely to effects of steric hindrance, and by MeCP2.
Six methylated CpGs in the core promoter are sufficient for repression by MeCP2

How MeCP2 selects target genes remains the most important and unanswered questions about MeCP2. The answer to this question would provide important insights into our understanding of Rett syndrome. The repression of a CpG-free core promoter by MeCP2 caused us to question the role of CpG positioning on the repression of transcription by MeCP2. It is known that the majority of CpGs in the mammalian genome are methylated (3), which is compounded by the fact that MeCP2 can bind to methyl-CpGs in any sequence context (13, 16). We were therefore interested in the role that CpG positioning plays in transcriptional repression by MeCP2.

To this end, we employed a CpG-free plasmid that would allow us to place CpGs at different positions of the plasmid relative to the core promoter. Using this CpG-free plasmid, we assembled a construct with six CpGs in the core promoter and one with no CpGs in the core promoter, with both having no CpGs in the plasmid. Unfortunately, this system was complicated by the presence of two Matrix Attachment Regions (MARs) in the CpG-free plasmid. Chicken MeCP2 was originally named ARBP (attachment region binding protein) for its ability to bind to MARs (8, 24). Rat MeCP2 shares a 125 amino acid stretch that is 96.7% identical with the MAR binding domain of ARBP (24). To eliminate this potential complication, both MARS were removed from the vector by using the restriction enzyme Eco RI. Two Eco RI sites flank the
region of the plasmid that contains both MARs. The result was a linear transcription construct that is approximately 2,000 bp shorter than the constructs containing both MARs. The removal of the MARs enables the effects from our experiments to be due solely to the methylation status of the DNA. In vitro transcription reactions were performed on the plasmid containing only six CpGs in the core promoter in the presence or absence of MeCP2. The addition of MeCP2 resulted in five-fold repression of transcription, indicating that methylation in the core promoter alone, although not as severe, is sufficient for repression by MeCP2 in vitro.

**MeCP2 cannot repress transcription from a methyltransferase treated CpG-free vector**

For the purpose of verifying the methyl-CpG specific nature of our system, a plasmid containing zero CpGs in both the core promoter and the plasmid backbone was treated with M. Sss I methylase. The MARs were subsequently removed as previously described above, and then an in vitro transcription reaction was carried out in the presence or absence of MeCP2. We found that the addition of MeCP2 had no effect on the level of transcription, which is an important control because it verifies that repression of in vitro transcription is methyl-CpG dependent and is not due to some sort of artifact caused by treatment with M. Sss I methylase.
Figure 1: Preparation and quantification of HeLa nuclear extract strength by in vitro transcription analysis and purification of recombinant wild-type MeCP2

A. The SCPX promoter was transcribed with increasing amounts of HeLa nuclear extract and compared to a previously tested standard condition to determine the optimal concentration for subsequent experiments.

B. SDS-PAGE of purified recombinant wild-type MeCP2 stained with Coomassie Blue.
<table>
<thead>
<tr>
<th>HeLa Nuclear Extract (ul)</th>
<th>6 ul of standard</th>
<th>8 ul of unknown strength</th>
<th>6 ul of unknown strength</th>
<th>4 ul of unknown strength</th>
<th>2 ul of unknown strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Activity</td>
<td>(100)</td>
<td>55</td>
<td>35</td>
<td>40</td>
<td>110</td>
</tr>
</tbody>
</table>
Figure 1: Preparation and quantification, continued

B

MW (kDa)

WT MeCP2
Figure 2: Transcriptional repression by MeCP2 is methyl-CpG dependent

In vitro transcription analysis of methylated and unmethylated pUC-SCPX templates titrated with MeCP2 reveals that the addition of MeCP2 to the reaction results in repression of the methylated pUC-SCPX, but does not significantly repress the unmethylated pUC-SCPX. Full methylation of the plasmid was achieved by treatment with M. Sss I methylase and verified by bisulfite sequencing.
Figure 3: MeCP2 inhibits pre-initiation complex formation

A. Schematic diagram of the steps of transcription and their relation to the steps in the in vitro transcription experiment.

B. In vitro transcription reaction of methylated pUC-SCPX with MeCP2 added at various time points of the reaction. Significant repression is seen only when MeCP2 is added with or before the HeLa nuclear extract. This experiment was performed by Dr. Joshua Theisen.
Figure 4: Methylation in the core promoter is not necessary for repression of transcription by MeCP2

In vitro transcription reactions were carried out with methylated and unmethylated pUC-SCPX-0CpG. pUC-SCPX-0CpG contains no CpGs in the core promoter, but has CpGs in the plasmid backbone. Upon addition of MeCP2, only methylated pUC-SCPX-0CpG is repressed.
A diagram showing the effect of MeCP2 (pmol) on the relative activity of unmethylated and methylated DNA. The x-axis represents MeCP2 concentrations in pmol, with values 0, 3.3, 6.6, and 10. The y-axis represents relative activity, with values (100), 132, 70, 68, 99, 65, 24, and 12.
Figure 5: Methylation in the core promoter is sufficient for repression by Mecp2 and a CpG-free plasmid is not repressed by MeCP2

The 0-Me-CpG construct contains no CpGs, and the 6-Me-CpG construct contains six CpGs in the core promoter. Both were treated with M. Sss I methylase. Constructs were subjected to in vitro transcription analysis in the presence or absence of MeCP2. MeCP2 is unable to repress transcription from the 0-Me-CpG construct, but causes five-fold repression from the 6-Me-CpG construct.
III:

Discussion
In this work, we investigated the mechanism of repression by MeCP2, and began to explore the role of methyl-CpG positioning in repression by MeCP2. This study used an in vitro methyl-CpG-dependent system of repression by MeCP2. Furthermore, we observed that the HDAC-independent mechanism of repression by MeCP2 involves the inhibition of PIC formation. We have found that methyl-CpGs in the core promoter, while sufficient, are not necessary for transcriptional repression by MeCP2. Lastly, through the use of a CpG-free plasmid, we have laid a foundation from which to study the positioning of methyl-CpGs more rigorously in the future.

In our investigation into the repression of transcription by MeCP2 in vitro, we have found that transcriptional repression by MeCP2 is methyl-CpG dependent (Figure 1). This repression was previously shown by studies in our lab to not be dependent on HDACs (data not shown). Thus, we have established an in vitro transcription system by which to study HDAC-independent repression of MeCP2. Subsequently, we have found this HDAC-independent mechanism of transcription to act through inhibition of PIC formation (Figure 3). Given that we have found MeCP2 to inhibit PIC formation, it was possible that the presence of MeCP2 binding to the methylated promoter was occupying binding sites for the basal transcription factors, thereby displacing them from the promoter. However, in vitro transcription reactions using a plasmid that only contained methyl-CpGs in the backbone, but not in the core promoter, still resulted in repression upon the
addition of MeCP2 (Figure 4). This indicated that inhibition of PIC formation by MeCP2 was not due solely to steric hindrance, and that methylation in the core promoter in not required for repression by MeCP2.

The means by which MeCP2 selects target genes remains to be determined. The identification of valid target genes of MeCP2 is critical to our understanding of Rett syndrome. In this investigation, we have begun to examine the role that methyl-CpG position plays in the selection of target genes by MeCP2. Initially, our effort was complicated by the presence of 2 MARs, which MeCP2 may interact with, in the CpG-free plasmid derived from pCpG-Luc. These MARs were subsequently removed, but the fact that MeCP2 does have a MAR binding activity suggests that MeCP2 may play a role in chromatin structure and organization; further study should be done to investigate this potential activity.

The CpG-free DNA template provides the basis for a systematic investigation into the effects of methyl-CpG positioning. We found that MeCP2 is unable to repress in vitro transcription in the absence of methyl-CpGs. Also, we have shown that six methyl-CpGs in the core promoter are sufficient for repression of in vitro transcription (figure 5). Taken together, our evidence has shown that more methyl-CpGs in the plasmid generally lead to more repression by MeCP2. We plan to investigate the effect of methyl-CpGs upstream, downstream, and 2,000 bp away from the transcription start site in the future.
Our investigation has found a new mechanism of repression by MeCP2, and began to explore the role of methyl-CpG positioning in repression by MeCP2. These insights have increased our understanding of MeCP2 function and will help in the identification of more MeCP2 targets genes in the future, which will hopefully aid in the development of treatments for Rett syndrome.
Chapter: IV

Materials and Methods
Preparation of Hela nuclear extract

The HeLa nuclear extract used in the in vitro transcription reactions was prepared as follows. All steps were carried out at 4 °C. HeLa-S3 cell pellets from 12L of culture (approximately 50 ml of HeLa-S3 cells, from the National Cell Culture Center) were washed with 150 ml PBS + 5 mM MgCl2. The cells were then pelleted by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 150 ml PBS + 5 mM MgCl2 and then pelleted again by centrifugation at 3000 rpm for 10 min. The final pellet was resuspended in 60 mL Buffer H [10 mM Tris-HCl (pH 7.9), 10 mM KCl, 750 μM spermidine, 150 μM spermine, 0.1 mM EDTA, 0.1 mM EGTA]. Cells were incubated on ice for 20 min and then lysed by 20 strokes with a 40 mL Dounce using the B (loose) pestle. Nuclei were pelleted by centrifugation in an SS-34 rotor at 8500 rpm for 10 min. Pelleted nuclei were resuspended in 60 mL Buffer H and pelleted a second time by centrifugation in an SS-34 rotor at 8500 rpm for 10 min. Pelleted nuclei were resuspended in 80 mL Buffer AB [15 mM HEPES (pH 7.6), 110 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 1 mM benzamidine-HCl, 0.2 mM PMSF, 1 mM sodiumbisulfite]. Clumps of nuclei were dispersed by 3 strokes with a 40 mL Dounce using the B (loose) pestle. Another 60 mL of Buffer AB was added to the dispersed nuclei, bringing the total volume to approximately 140 mL. Nuclei were then lysed by addition of 1/10th volume (approximately 11 mL) of 4 M ammonium sulfate and incubation for 20 min on a rotating wheel. Nuclear debris was pelleted by
ultracentrifugation in a Ti45 rotor at 35,000 rpm for 60 min. The supernatant was transferred to a 250 mL beaker and 0.3 g of pulverized ammonium sulfate was added per 1 mL of supernatant. The solution was stirred for 15 min and then precipitated proteins were pelleted by centrifugation in an SS-34 rotor at 15,000 rpm for 20 min. Protein pellets were resuspended in 5 mL HEG Buffer [25 mM HEPES (pH 7.6), 0.1 mM EDTA, 10% (v/v) glycerol] containing 0.1 M KCl, 1 mM DTT, 1 mM benzamidine-HCl, 0.2 mM PMSF and 1 mM sodium bisulfite. Proteins were dispersed by 10 strokes with a 15 mL Dounce using the A (tight) pestle. Insoluble debris was pelleted by centrifugation in an SS-34 rotor at 10,000 rpm for 10 min. The supernatant was dialyzed three times for 50 min against 2 L HEG containing 0.1 M KCl, 1 mM DTT, 1 mM benzamidine-HCl, 0.2 mM PMSF and 1 mM sodium bisulfite. Insoluble debris was pelleted by centrifugation in an SS-34 rotor at 10,000 rpm for 10 min. The supernatant was then aliquoted, flash frozen and stored at –80 °C.

Expression and purification of recombinant wild-type MeCP2

Recombinant wild-type MeCP2 proteins were expressed and purified as described in (27), with the exception of the sonication step, which was altered to decrease temperature. In short, chitin binding domain (CBD) fusion proteins were expressed in bacteria overnight at 16 °C. The bacteria were lysed by sonication and the cell contents were bound to chitin beads (New England Biolabs). The fusion proteins were cleaved from the beads by overnight incubation in buffer containing 50 mM DTT. Column eluate was
dialyzed twice for 1 hour against KSB [25 mM HEPES (pH 7.6), 400 mM potassium acetate, 6 mM MgCl₂, 20% (v/v) glycerol, 1 mM DTT, 1 mM benzamidine-HCl, 0.2 mM PMSF]. The purity and concentration of the proteins was estimated by SDS-PAGE and compared to bovine serum albumin standards.

**In vitro transcription analysis with HeLa nuclear extract:**

In vitro transcription reactions were carried out as follows. For reactions with pUC119 templates, 250 ng of template and 1000 ng of unmethylated pUC119 competitor were included in each reaction. For reactions involving pCpG-Txn templates with both MARs removed, 250 ng of template, 500 ng of unmethylated pUC119 competitor and 250 ng of methylated pUC119 competitor were added to the reaction. MeCP2 was incubated at 30 °C with template and competitor DNA for 20 min in a total volume of 36 ul in a buffer containing 20 mM HEPES (pH 8.0), 69 mM potassium acetate, 8 mM MgCl₂, 3.5% (w/v) polyvinyl alcohol, 4.2 mM ATP and 3.5% (v/v) glycerol. Afterwards, 10 ul of HeLa nuclear extract diluted in HEG containing 0.1 M KCl was added. The reaction was then incubated at 30 °C for 90 min in a total volume of 46 ul in buffer containing 21 mM HEPES (pH 8.0), 22 mM KCl, 54 mM potassium acetate, 6.5 mM MgCl₂, 2.7% (w/v) polyvinyl alcohol, 3.3 mM ATP and 5% (v/v) glycerol. Following this incubation, 4 ul of 5 mM rNTP was added and the reaction was incubated at 30 °C for 20 min in a final volume of 50 ul in buffer containing a final concentration of 20 mM HEPES pH 8.0), 20 mM KCl,
50 mM potassium acetate, 6 mM MgCl₂, 2.5% (w/v) polyvinyl alcohol, 3.4 mM ATP, 0.4 mM CTP, GTP and TTP, and 4.5% (v/v) glycerol. In reactions with Sarkosyl, buffer volumes were adjusted to allow the addition of 2 µL of 5% (v/v) Sarkosyl for a final concentration of 0.2% (v/v) Sarkosyl. Reactions were stopped by the addition of 100 µL of Stop Buffer [20 mM EDTA, 200 mM sodium chloride, 1% (w/v) sodium dodecyl sulfate, 0.3 mg/mL glycogen] and 5 µL of 2.5 mg/mL proteinase K and incubated for 20 min at 37 °C. Transcripts were subjected to primer extension analysis as previously described in (10) using 5′-³²P-labeled M13 reverse sequencing primer (AGCGGATAAACAATTTCACACAGGA) for pUC119 constructs or 5′-³²P-labeled pCpG-PE1 primer (GGAAAGAGAAGAAGGTTAGTACATTGT) for pCpG-Txn constructs with both MARs removed. Quantification of reverse transcription products was done with a PhosphorImager (GE Health Sciences). Experiments were carried out a minimum of three independent times to ensure reproducibility.

DNA Templates

DNA templates used within vitro transcription reactions were prepared by ligating the SCPX (GCGTGTACCGTGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCCTCAGATCGCCTGGAGACGTCGAGCCGTGCTCCATAGAAGACAC) or SCPX-0CpG (GCTTGTACTATGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCCTCAGATCGCCTGGAGACCTCAAGCCTCCATAGAAGACAC) promoters into the Pst I and
Xba I sites of the multiple cloning region of pUC119 or the MCSp of the pCpG-Txn plasmid. In order to remove the two MAR sequences in the plasmid, pCpG-Txn constructs were digested with Eco RI followed by phenol/chloroform extraction, ethanol precipitation, and resuspended in water.

**pCpG-Txn plasmid**

The pCpG-Txn was generated by Dr. Joshua Theisen as follows. The pCpG-Txn plasmid was derived from the pCpG-Luc plasmid (Invivogen, San Diego, CA). pCpG-Luc was digested with Pst I to remove the mCMV enhancer and hEF1 promoter. The resulting plasmid was then digested with Pci I, blunted with Klenow and religated to destroy the Pci I restriction site. The resulting plasmid was then modified by the insertion of double-stranded oligonucleotides into the Pst I and Xba I sites. This destroyed the existing multiple cloning site and generated a new proximal multiple cloning site (MCSp). The resulting plasmid was then modified by the insertion of double-stranded oligonucleotides into the Sph I site. This created a distal multiple cloning site (MCSd).
References


