ORIGINAL ARTICLES

# Heterogeneity in residual function of MeCP2 carrying missense mutations in the methyl CpG binding domain

**S Kudo, Y Nomura, M Segawa, N Fujita, M Nakao, C Schanen, M Tamura**

*J Med Genet* 2003;**40**:487–493

See end of article for authors' affiliations

Correspondence to: Dr S Kudo, Hokkaido Institute of Public Health, Kita-19, Nishi-12, Kita-ku, Sapporo 060-0819, Japan; kudos@iph.pref.hokkaido.jp

Revised version received 7 March 2003
Accepted for publication 11 March 2003

Rett syndrome is a neurodevelopmental disorder with severe mental retardation caused by mutations in the *MECP2* gene. Mutations in the *MECP2* gene are also associated with other genetic disorders, including X linked mental retardation in males. Missense mutations identified so far are present primarily in the methyl CpG binding domain (MBD) of *MECP2*. Here, the functional significance of 28 MBD missense mutations identified in patients were analysed by transient expression of the mutant proteins in cultured cells. The effects of mutations were evaluated by analysis of the affinity of MeCP2 to pericentromeric heterochromatin in mouse L929 cells and on transcriptional repressive activity of MeCP2 in *Drosophila* SL2 cells. These analyses showed that approximately one-third (9/28) of MBD missense mutations showed strong impairment of MeCP2 function. The mutation of the R111 residue, which directly interacts with the methyl group of methyl cytosine, completely abolished MeCP2 function and mutations affecting β-sheets and a hairpin loop have substantial functional consequences. In contrast, mutations that showed marginal or mild impairment of the function fell in unstructured regions with no DNA interaction. Since each of these mutations is known to be pathogenic, the mutations may indicate residues that are important for specific functions of MeCP2 in neurones.

Rett syndrome (MIM 312750), an X linked dominant neurodevelopmental disorder, is one of the most common causes of mental retardation in young females, occurring with an incidence of 1/10 000 to 1/15 000.[1] Rett syndrome is characterised by severe neurological dysfunction such as dementia, autistic features, loss of acquired motor skills and speech, stereotypical hand movements, and seizures. The gene responsible for this disorder was identified as *MECP2*,[2] which encodes a 486 amino acid protein that binds to methylated DNA.[3] DNA methylation at cytosine residues in CpG dinucleotides plays an important role in X chromosome inactivation, genomic imprinting, developmental regulation of gene expression, inactivation of retroposons, and repression of transcriptional noise in mammals.[3] MeCP2 protein possesses a highly conserved 70 amino acid methyl CpG binding domain (MBD) that can bind to DNA containing a single methylated CpG base pair.[3] Nuclear magnetic resonance (NMR) solution analysis of the MBD showed a novel wedge shaped structure with four antiparallel β-sheets and a C-terminal helical region.[4 5] MeCP2 also contains a transcriptional repression domain (TRD) of approximately 100 amino acid residues, which interacts with the Sin3A histone deacetylase complex.[6 7] Therefore MeCP2 facilitates the formation of transcriptionally inactive, condensed chromatin by binding to methylated CpG through its MBD and recruiting histone deacetylase through the TRD.

Accumulating data indicate that approximately 80% of Rett syndrome patients carry an identifiable *MECP2* mutation.[8] Association of MeCP2 with Rett syndrome is supported by the observation that mice carrying targeted mutations in the *mecp2* gene develop symptoms characteristic of Rett syndrome patients.[9–11] Moreover, these rodent models indicate that the phenotypic presentation of MeCP2 dysfunction is influenced by genetic background and residual function of the mutant protein as well as by the relative levels of expression of the mutant protein, based on the more severe phenotype in male mice.[9–11] Perhaps not surprisingly based on these observations, mutations in *MECP2* have also been identified in patients with disorders phenotypically distinct from Rett syndrome, including severe neonatal onset encephalopathy and X linked recessive mental retardation (for a recent review, see Hammer *et al*[12]). Molecular dissection of the mechanisms underlying the phenotypic variability in humans is not a simple task, particularly in females where individual variation in X chromosome inactivation patterns obscures detection of direct correlation between phenotypic expression of specific mutations. In males harbouring *MECP2* mutations, however, the disparate phenotypes suggest that different mutations in *MECP2* are not functionally equivalent. In order to investigate the functional significance of missense mutations seen in patients with MeCP2 related disorders, we have developed cell culture based assays that allow examination of chromatin binding and transcriptional repression. We have used these assays to characterise several common MBD mutations that cause Rett syndrome[13] and two mutations that have been identified in males with forms of X linked recessive mental retardation.[14] In this study, we analysed 22 additional missense mutations in the MBD using both assay systems, and compared them to the four most common missense mutations in the MBD in Rett syndrome and two mutations identified in patients with X linked mental retardation characterised previously.[13 14] Based on the results of these functional assays and the recently reported solution structure of MBD complexed with DNA,[15] we describe the structure-function relationship of the MBD of MeCP2. In addition, we discuss the relationship between the functional significance of the mutations and the phenotypic variability of patients with MeCP2 related mental disorders.

## MATERIALS AND METHODS

### Cells and culture conditions

L929 cells (mouse fibroblast cell line, American Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/l glutamine, 500 U of penicillin per ml, and 100 μg of streptomycin per ml in a humidified 5% $CO_2$ atmosphere. The *Drosophila* Schneider cell line 2 (SL2) derived from *Drosophila* embryos (kindly provided by Dr R M Evans) was grown in Schneider's *Drosophila* medium (Invitrogen) with the same supplements as described above.

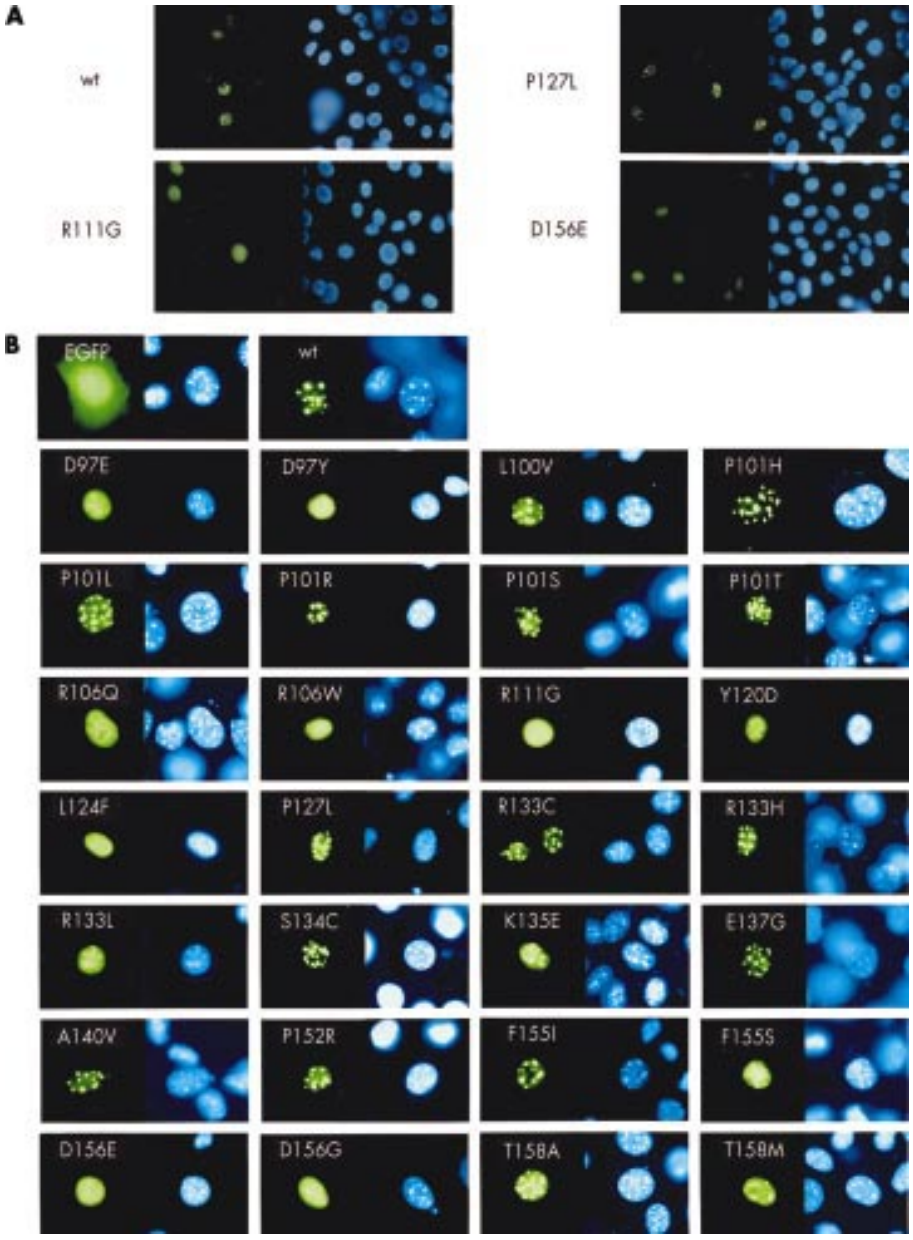


**Figure 1** Effects of MBD missense mutations on the affinity of MeCP2 to mouse pericentromeric heterochromatin. (A) Representative low magnified views of L929 cells transfected with wt or mutant expression plasmids. Localisations of GFP fusion proteins (left panel) were visualised by fluorescence microscopy and the nuclei were counterstained with DAPI (right panel). (B) Higher magnified views of fluorescent staining L929 cells showing the intranuclear localisations of GFP mutant proteins.

### Expression constructs

Expression plasmids encoding MeCP2 mutants bearing MBD missense mutations identified in patients[2 16–32] were constructed as follows. MeCP2 mutant cDNAs were generated by site directed mutagenesis using PCR with mismatched primers and a full length MeCP2 cDNA template as described previously.[13] Synthesised mutant DNA was cloned into the enhanced fluorescence vector pEGFP-C1 (Clontech) and the *Drosophila* expression vector pAc5.1/V5-His (Invitrogen). All constructs were verified by sequencing.

### Heterochromatin affinity analysis

GFP fusion expression constructs were introduced into L929 cells using Superfect (Qiagen). Two days later, transfected cells were fixed with 3.7% formaldehyde for 10 minutes, permeabilised with 0.5 % Triton X-100 for 20 minutes, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) at a final concentration of 1 μg /ml. The cells were observed under an Olympus fluorescence microscope using the appropriate optical filter. Microscopic images were collected with a CCD camera and a Macintosh G4 computer, and imported into Adobe Photoshop 6.0 for figure presentation.

### Luciferase assay

Transient expression analysis using *Drosophila* SL2 cells was performed as follows. The SNRPN-luciferase reporter construct (pGL-SNRPN) and the leukosialin-luciferase reporter construct (pGL-LS5)[13] were treated with *Sss*I (CpG) methylase (New England Biolabs) in the presence or absence of 5 mmol/l S-adenosylmethionine as described previously.[33] A total of 1.4 $\times 10^5$ SL2 cells were grown in 0.7 ml of Schneider's *Drosophila* medium in a 24 well plate. A total of 0.4 μg of the luciferase reporter construct was cotransfected with 0.2 μg of an Sp1 expression plasmid, pPacSp1, 0. 01 μg of pAc5.1-pRL, and 0.1 μg of a *Drosophila* expression plasmid bearing genes encoding MeCP2 mutants into SL2 cells by the calcium phosphate

**Table 1** Effects of missense mutations in the MBD on transcriptional repressive activity

| | Relative transcriptional activity (%)* | | | |
|---|---|---|---|---|
| | pGL-SNRPN | | pGL-LS5 | |
| Mutation | Methylated | Unmethylated | Methylated | Unmethylated |
| Vector | 100 | 100 | 100 | 100 |
| wt | 3.2 ± 0.3 | 56.1 ± 4.3 | 14.2 ± 1.6 | 59.6 ± 6.5 |
| D97E | 11.9 ± 2.4 | 85.3 ± 5.6 | 36.8 ± 2.1 | 102 ± 13.1 |
| D97Y | 15.1 ± 1.9 | 90.8 ± 10.5 | 35.3 ± 3.1 | 107 ± 6.4 |
| L100V | 3.8 ± 0.5 | 65.1 ± 3.2 | 15.7 ± 0.8 | 86.9 ± 7.1 |
| P101H | 2.4 ± 0.3 | 25.8 ± 3.3 | 10.1 ± 1.2 | 77.7 ± 5.8 |
| P101L | 3.8 ± 0.5 | 58.7 ± 8.1 | 12.9 ± 2.5 | 75.3 ± 3.8 |
| P101R | 1.9 ± 0.2 | 39.3 ± 4.9 | 12.1 ± 0.9 | 72.9 ± 4.5 |
| P101S | 1.8 ± 0.3 | 28.4 ± 3.6 | 13.9 ± 2.6 | 80.8 ± 6.6 |
| P101T | 2.2 ± 0.1 | 29.2 ± 4.1 | 14.2 ± 0.2 | 63.2 ± 8.1 |
| R106Q | 57.7 ± 4.8 | 126 ± 9.6 | 85.3 ± 9.8 | 117 ± 10.5 |
| R106W | 61.2 ± 7.6 | 137 ± 12.5 | 78.6 ± 5.1 | 126 ± 15.1 |
| R111G | 127 ± 11.2 | 119 ± 8.8 | 139 ± 10.8 | 123 ± 7.2 |
| Y120D | 13.7 ± 2.3 | 87.2 ± 10.6 | 33.1 ± 1.6 | 106 ± 11.8 |
| L124F | 22.4 ± 3.6 | 98.6 ± 3.7 | 34.8 ± 2.9 | 119 ± 13.9 |
| P127L | 2.3 ± 0.1 | 26.4 ± 4.1 | 13.1 ± 1.5 | 74.1 ± 4.8 |
| R133C | 2.1 ± 0.2 | 23.8 ± 3.7 | 14.4 ± 1.6 | 57.6 ± 8.1 |
| R133H | 2.3 ± 0.1 | 30.4 ± 1.8 | 13.9 ± 1.8 | 100 ± 7.9 |
| R133L | 13.6 ± 2.2 | 64.3 ± 5.6 | 42.3 ± 3.9 | 97.7 ± 8.8 |
| S134C | 2.3 ± 0.3 | 28.1 ± 2.1 | 11.2 ± 2.8 | 74.2 ± 9.5 |
| K135E | 12.9 ± 1.8 | 90.8 ± 8.4 | 27.2 ± 4.1 | 121 ± 10.6 |
| E137G | 19.2 ± 2.5 | 96.1 ± 7.9 | 31.8 ± 2.8 | 78.7 ± 6.2 |
| A140V | 2.6 ± 0.3 | 23.9 ± 4.6 | 11.7 ± 0.8 | 60.8 ± 5.1 |
| P152R | 6.1 ± 0.9 | 67.3 ± 8.7 | 16.4 ± 2.9 | 84.1 ± 6.6 |
| F155I | 3.1 ± 0.2 | 59.2 ± 7.5 | 16.6 ± 0.8 | 86.7 ± 10.2 |
| F155S | 57.8 ± 7.8 | 120 ± 8.8 | 55.3 ± 3.6 | 90.1 ± 4.9 |
| D156E | 38.3 ± 5.9 | 105 ± 7.5 | 50.7 ± 7.6 | 107 ± 8.1 |
| D156G | 37.1 ± 0.6 | 109 ± 11.8 | 49.2 ± 3.1 | 119 ± 10.9 |
| T158A | 5.8 ± 0.7 | 75.9 ± 6.2 | 17.9 ± 2.7 | 85.4 ± 8.8 |
| T158M | 17.3 ± 2.1 | 69.3 ± 4.1 | 32.1 ± 2.9 | 81.3 ± 6.7 |

*Relative transcriptional activities (%) compared with that of only pAc5.1/V5-His vector are presented.

method. After 48 hours, cells were lysed in 100 µl of lysis buffer and 10 µl of lysate was assayed for firefly and Renilla luciferase activities using the Dual-Luciferase reporter assay system (Promega). All transient transfection assays were carried out at least three times independently.

## RESULTS

### Effect of MBD missense mutations on affinity for mouse heterochromatin

Accumulating data on *MECP2* mutations indicate that missense mutations are present primarily in the MBD.[8] To determine the functional significance of these mutations, we examined the effects of a total of 28 MBD missense mutations[2 16–32] on heterochromatin affinity. This assay takes advantage of a unique feature of murine cells, which contain large foci of densely methylated pericentromeric constitutive heterochromatin within the nucleus that attract exogenously expressed MeCP2 protein.[34 35] The relative ability of the mutant protein to bind heterochromatin can be qualitatively assessed by direct visualisation by using a GFP-MeCP2 fusion protein.[13] Loss of MBD function leads to a decrease in the intensity of focal staining and an increase in staining throughout the nucleus. L929 cells were transfected with plasmids encoding GFP fusions of wild type or mutant proteins and intranuclear localisations of proteins were examined. In this analysis the same pattern was observed in almost all fluorescence stained cells expressing the same mutant form (fig 1A). In addition, there were no obvious alternations in nuclear morphology for cells overexpressing wild type and mutant proteins. The results of this analysis are summarised in fig 1B. Heterochromatin binding by the D97E, D97Y, R106Q, R106W, R111G, L124F, F155S, D156E, and D156G mutants was severely impaired, as indicated by the absence of clear focal staining and the diffusion of the GFP signal throughout the nucleus. An intermediate pattern was observed in the Y120D, R133L, K135E, T158A, and T158M mutants, which showed focal staining co-localising with DAPI staining apparent against a background of diffuse nuclear GFP signal, indicating that their affinity for heterochromatin is partially retained. Among the latter mutations, T158A exhibited more distinct foci in the nucleus, whereas the other mutations exhibited faint foci throughout the stained nucleus. Missense mutations at R133 and F155 showed differential effects depending on the amino acid substitution. Substitution of arginine with leucine at residue 133 (R133L) substantially reduced focal staining while changes from arginine to cysteine (R133C) or histidine (R133H) did not visibly affect MeCP2 localisation. Similarly, a change from phenylalanine to serine (F155S) led to diffuse nuclear staining consistent with impaired heterochromatin binding by the mutant protein while the change from phenylalanine to isoleucine (F155I) at F155 did not impair heterochromatin binding. By contrast, differing substitutions at D97, P101, R106, and D156 had more consistent effects on heterochromatin binding patterns. Several mutant GFP fusion proteins accumulated in heterochromatic regions comparable to the wild type protein, producing distinct foci co-localising with strong DAPI staining. Five different mutations at residue P101 showed a clear focal staining in the nucleus, indicating that assembly of the mutant protein into heterochromatin was minimally affected by these mutations at this site. Similarly the L100V, P127L, R133C, R133H, S134C, E137G, A140V, P152R, and F155I mutant proteins produced heterochromatin staining patterns that were indistinguishable from the wild type protein. Notably, the E137G and A140V are mutations identified in male patients with X linked mental retardation,[16 17] and

**Table 2** Summary of the effects of missense mutations in the MBD

| Mutation | Transcriptional repressive activity in SL2 cells | Nuclear staining in L929 cells | Phenotype | Reference |
|---|---|---|---|---|
| wt | Strong | Focal | Normal | |
| D97E | Moderate | Diffuse | RTT | 20 |
| D97Y | Moderate | Diffuse | RTT | 27 |
| L100V | Strong | Focal | RTT | 31 |
| P101H | Strong | Focal | RTT | 21 |
| P101L | Strong | Focal | RTT | 21 |
| P101R | Strong | Focal | RTT | 25 |
| P101S | Strong | Focal | RTT | 22 |
| P101T | Strong | Focal | RTT | 21 |
| R106Q | Weak | Diffuse | RTT | 26 |
| R106W | Weak | Diffuse | RTT | 2 |
| R111G | Weak | Diffuse | RTT | 29 |
| Y120D | Moderate | Focal + diffuse | RTT | 28 |
| L124F | Moderate | Diffuse | RTT | 24 |
| P127L | Strong | Focal | RTT | 30 |
| R133C | Strong | Focal | RTT | 2 |
| R133H | Strong | Focal | RTT | 27 |
| R133L | Moderate | Focal + diffuse | RTT | 23 |
| S134C | Strong | Focal | RTT | 19 ,21 |
| K135E | Moderate | Focal + diffuse | RTT | 29 |
| E137G | Moderate | Focal | X linked mental retardation | 17 |
| A140V | Strong | Focal | X linked mental retardation | 16, 17 |
| P152R | Strong | Focal | RTT | 19 |
| F155I | Strong | Focal + diffuse | RTT | 20 |
| F155S | Weak | Diffuse | RTT | 2 |
| D156E | Weak | Diffuse | RTT | 18 |
| D156G | Weak | Diffuse | RTT | 29 |
| T158A | Strong | Focal + diffuse | RTT | 22 |
| T158M | Moderate | Focal + diffuse | RTT<br>Encephalopathy in boy | 2, 32 |

our previous study indicated that these mutants retain considerable MeCP2 function.[14]

### Effect of MBD missense mutations on transcriptional repressive activity in *Drosophila* cells

To determine the effects of MBD missense mutations on transcriptional repressive activity of MeCP2, we undertook transient expression analysis using *Drosophila* SL2 cells. *Drosophila* cells are suitable for such analysis because they have very low background levels of methyl CpG binding activity and low levels of DNA methyltransferase activity and genomic methylation.[36] Our previous studies showing that MeCP2 expressed in SL2 cells substantially repressed Sp1 activated transcription from a methylated promoter indicated that transcriptional repressive activity of MeCP2 could be reconstituted in SL2 cells.[37] In this study, we used two reporter constructs containing the luciferase reporter driven by the promoter of the human imprinted *SNRPN* gene (pGL-*SNRPN*) and the human leukosialin gene (pGL-LS5). The SNRPN promoter provides high transcriptional activity and is highly sensitive to MeCP2 transcriptional repression.[13] The leukosialin promoter provides high transcriptional activity but is less sensitive to MeCP2 transcriptional repression.[13] *Drosophila* expression constructs encoding wild type or mutant MeCP2 were cotransfected into SL2 cells with the luciferase reporter plasmid and an Sp1 expression vector, as well as the pAc5.1-pRL control plasmid for normalisation. Exogenous expression of Sp1 is required for transcriptional activation of a reporter gene since SL2 cells lack Sp1.[38] The effects of mutants on the pGL-SNRPN reporter plasmid are summarised in table 1. Wild type MeCP2 strongly reduced Sp1 activated transcription from the methylated SNRPN promoter. Transcriptional activity was reduced approximately 30-fold in assays using 0.1 μg of expression vector. Cells expressing the R106Q, R106W, R111G, F155S, D156E, and D156G mutant proteins, however, showed significant diminution of transcriptional repression, particularly for the R111G mutation, which completely abolished MeCP2 repressive activity on Sp1 activated transcription from both methylated and unmethylated promoters. Intermediate impairment of repressive activity was observed in D97E, D97Y, Y120D, L124F, R133L, K135E, E137G, and T158M mutations, which showed a 5 to 10 fold reduction of transcription from the methylated SNRPN promoter. On the other hand, each of the five P101 mutations had little effect on transcriptional repressive activity of MeCP2, exhibiting substantial reduction of Sp1 activated transcription at levels comparable to wild type protein. Similar and substantial levels of repression were observed in L100V, P127L, R133C, R133H, S134C, A140V, P152R, F155I, and T158A mutants. When the leukosialin promoter was used as the reporter, the transcriptional repressive level of mutants was lower than with the SNRPN reporter, although similar effects on repressive activity were observed when the promoter was methylated (table 1). Interestingly, the P101H, P101S, P101T, P127L, R133C, R133H, S134C, and A140V mutants exhibited higher transcriptional repressive activities on Sp1 activated transcription from the unmethylated SNRPN promoter compared with that of wild type protein, reducing luciferase activities by approximately 3-4-fold, while the wild type protein reduced transcription by only about 40% using the same amount of expression plasmids. On the other hand, significant reduction of transcriptional activities from the unmethylated leukosialin promoter was not observed in these mutations. In this study, we also examined expression levels of wild type and mutant MeCP2 proteins by indirect immunofluorescence and western blot analysis and found that proteins expressed from these plasmids were produced in the nucleus at similar levels following transfection (data not shown). In addition, we tested the effects of the level of Sp1 expression on the transcriptional repressive potential of mutant proteins by transfecting half of the regular amount of an Sp1 expression plasmid. Under this low level of Sp1 expression, we observed the similar effects of missense mutations on the transcriptional repressive activities (data not shown). Table 2 summarises transcriptional repression

analyses using *Drosophila* cells and heterochromatin affinity analysis using the mouse cell system. Comparison of the results for both assays for each mutant form indicates that the results of these two methods are largely correlated.

In summary, we introduced a total of 28 mutations at 17 amino acid positions within the MBD. Nine mutations showed strong impairment of function in both assays. For two residues, the effect varied depending on the specific amino acid that was introduced, and at five positions the mutations had minimal to no effect on the function of the protein in these systems.

## DISCUSSION

The function of *MECP2* mutations was analysed by transfecting mouse L929 cells and *Drosophila* SL2 cells with mutant forms of MeCP2 to investigate the effects of mutations on the affinity to pericentromeric heterochromatin and transcriptional repressive activity, respectively. In this study, we also found that the effects of MBD missense mutations on heterochromatin affinity in mouse cells generally correlate with transcriptional repression of Sp1 activated transcription in *Drosophila* cells (table 2). The effects of these missense mutations can be addressed from a structure-function relationship using the model of the MBD developed by NMR solution analysis of human MBD1 bound to methylated DNA.[4 5 15] This model predicts a novel wedge shaped structure with four antiparallel β-sheets and a helical region. Since the solution structure of the MBD of MBD1 is almost identical to that of MeCP2,[4 5] we used structural data available for MBD1 to infer the location of mutation sites in the secondary structure of the MBD of MeCP2.

The two methyl groups of a methylated CpG base pair are recognised by a hydrophobic surface comprising five residues: three of them (K109, R111, Y123) interact with one methyl group and two of them (R133, S134) interact with the other (fig 2). In this study, we found that R111G produced a complete loss of MeCP2 function. Heterochromatin affinity analysis showed diffuse staining throughout the nucleus with this mutant and transient expression of the mutant in *Drosophila* cells completely failed to repress Sp1 activated transcription from both methylated and unmethylated promoters. It has been also proposed that R111 interacts with D121 and that this interaction enables the arginine side chain to bind to the DNA.[39] Thus, arginine at amino acid 111 of MeCP2, which is invariant among MBD family members in many species including plants and mammals,[40 41] is crucial for a stable interaction with methylated DNA. Thus, a substitution of arginine by glycine at this site results in an almost total loss of function of the MBD. These results are also consistent with previous observations that MBD protein bearing this mutation completely lacked affinity for methylated DNA.[39] By contrast, R133 and S134, which bind to a methyl group of a complementary methyl cytosine in CpG dinucleotide (fig 2), appear less critical for MBD function. Although R133 and S134 are highly conserved, substitutions at these sites are seen in MBD family proteins in *Arabidopsis*.[40 41] R133C, R133H, and S134C mutations had little effect on heterochromatin binding and transcriptional repressive potential. Previous studies showed that the R133C mutation strongly abolished the methyl DNA binding activity.[39 42] It is not clear whether the R133C mutant regains binding affinity for methylated DNA through interaction with other factors expressed in transiently transfected cells. Conversion of R133 to leucine (R133L) produced intermediate impairment of MBD function in both analyses, suggesting that this mutation may induce structural changes or diminish the capacity of the protein to bind methylated cytosine. It seems likely that the interaction of the methyl group with R133 and S134 might be weaker than the interaction of the methyl group with the R111 residue.

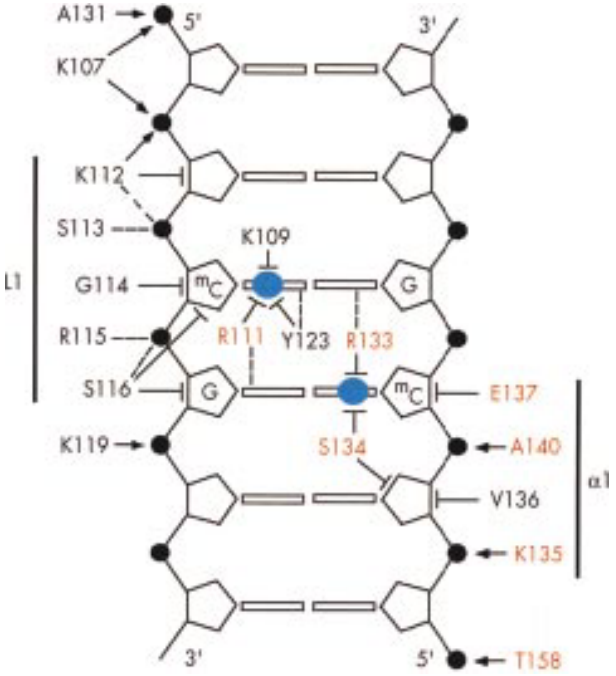


**Figure 2** Mutations at methyl CpG recognition sites. Interaction of methylated DNA with amino acid residues of the MBD are schematically presented on the basis of the NMR analysis of the MBD.[15] Deoxyribose moieties are represented as pentagons, DNA bases as open boxes, phosphates as black circles. The 5-methyl group of 5-methyl cytosine is shown as blue circles. Ionic interaction between residues of the MBD and DNA is indicated by arrows. Hydrophobic interactions are indicated by perpendicular lines, and hydrogen bonds are indicated by dashed lines. Residues seen in missense mutations found in Rett syndrome patients are shown as red letters.

Mutations of residues K135, E137, A140, and T158, which are predicted to interact with the DNA sugar phosphate backbone from NMR studies of the MBD of MBD1[15] (fig 2), show subtle to intermediate impairment of MBD function. The A140V mutation retained MBD function almost equivalent to wild type, although higher transcriptional repressive activity of Sp1 activated transcription from an unmethylated promoter was observed. Other mutations, K135E, E137G, T158A, and T158M, exhibited intermediate impairment in at least one of the two functional assays. Our functional analyses indicated that T158M had intermediate affinity to heterochromatin and moderate effects on transcriptional repressive activity. These results are consistent with reports that the T158M mutant exhibited only slightly lower binding affinity for methylated DNA compared with that of the wild type protein.[39 42]

Missense mutations in residues found in β-sheets and a hairpin loop strongly affect MBD function. D97E and D97Y in the β1-sheet show intermediate impairment of the transcriptional repressive activity and strong impairment of heterochromatin affinity. R106Q and R106W in the β2-sheet strongly impair MBD function. Previous NMR structural analysis showed that the R106W mutation leads to misfolding of MBD.[39] Furthermore, Y120D and L124F mutations in the β3-sheet show intermediate and strong impairment of the MBD function, respectively. Other mutations at sites of residues F155, D156 and T158 in a hairpin loop exhibit variable functional effects. In particular, substitution of phenylalanine with isoleucine at amino acid 155 (F155I) showed a subtle effect, whereas the substitution with serine (F155S) at the same site showed substantial loss of function. A previous structural study also showed that the F155S mutation disrupted proper MBD folding.[39] Missense mutations of the D156 residue, which is predicted to form a negatively

charged surface opposite to the DNA interface,[15] showed substantial effects on function, possibly resulting from a conformational change in the MBD.

Curiously, several mutant forms, including substitutions of L100, P101, P127, and P152, retained the capacity to bind heterochromatin and repress transcription in these assays. Notably each of these mutations involved residues that are outside the β/α strands and loop structures that are critical for DNA binding. Nonetheless, the retention of function in these assays appears to contradict the fact that they are known to be pathological mutations in humans and underlines the difficulty in developing model systems. In this case, the apparent paradox may result from differences in the chromatin structure of mitotically active L929 cells and postmitotic neurones, both in terms of histone composition and modification (for a recent review, see Ahmad and Henikoff[43]). It is becoming increasingly evident that histone modifications play critical roles in the transcriptional competency of genomic DNA and, as such, it is likely that MeCP2 binding is directly or indirectly affected by the nucleosomal context of the methylated CpG dinucleotide. These residues may be important for conferring specificity of interaction between MeCP2 and methylated DNA associated with nucleosomal proteins bearing specific modifications, which would not have been discerned by these studies. Interestingly, mutations such as P101H, P101S, P101T, P127L, R133C, R133H, S134C, and A140V exhibited higher transcriptional repressive activities on Sp1 activated transcription from the unmethylated SNRPN promoter compared with that of the wild type protein. These effects may depend on the context of the genes since such effects were not observed using the unmethylated leukosialin promoter reporter. It is possible that higher affinity binding of mutants to unmethylated genes leads to the aberrant gene expression by binding of the mutant protein to inappropriate unmethylated targets.

It is difficult to compare the level of residual function of MeCP2 in these in vitro analyses with phenotypes reported to be associated with specific mutations, in part because the phenotypic outcome is heavily influenced by X chromosome inactivation patterns. In addition, postnatal environment appears to affect the phenotypes of patients even if they have the same mutation. Mutations that showed intermediate to severe impression have been described in females presenting with classical and atypical Rett syndrome and males presenting with the neonatal onset encephalopathy. For example, a recent report described the T158M mutation in brothers of a patient with classical Rett syndrome.[32] One brother with this mutation died within a year of birth with severe encephalopathy. The milder presentations that have been reported including females with atypical Rett syndrome, such as the preserved speech variant, or other forms of X linked mental retardation in males have been associated with mutations that showed preservation of function in these assays. This is illustrated by the R133C and S134C mutations, which displayed conserved heterochromatin binding and transcriptional repressive activities in these assays, and are often associated with relatively mild clinical features in the patients, with many maintaining the ability to speak.[44–47] The R133H mutation exhibited near normal MBD function in our assays, but this mutation was also identified in a male patient with classical Rett syndrome.[48] Since it was reported that this male patient had somatic mosaicism, the influence of this mutation is much stronger than those of A140V and E137G mutations, which were identified in males with mental retardation and showed near normal MBD function.[16 17] This correlation is not straightforward, however, since mutations showing near normal function can also cause typical Rett syndrome. Therefore, it is conceivable that the gene expression is tightly regulated in the neuronal cells and even a subtle change of the gene regulation as observed in transcriptional regulatory potentials of some MBD missense mutants might bring about a significant influence on the neuronal function. Although many factors, such as other methyl CpG binding proteins, skewed X chromosome inactivation, and postnatal environment could be involved in the establishment of patient manifestations, our data provide the basis for understanding the genotype-phenotype correlation of MeCP2 related mental retardation.

## ACKNOWLEDGEMENTS

We thank Drs Masahiro Shirakawa, Kei Fujinaga, and Takeo Kubota for helpful discussions and Drs Ellen E Lamar and Glen Hill for critical reading of the manuscript. This work was supported by grants from the Ministry of Health and Welfare, the Yuasa Memorial Foundation, and the National Institute of Health, HD 37874 (CS).

**Authors' affiliations**
**S Kudo, M Tamura,** Hokkaido Institute of Public Health, Sapporo 060-0819, Japan
**Y Nomura, M Segawa,** Segawa Neurological Clinic for Children, Tokyo 101-0062, Japan
**N Fujita, M Nakao,** Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan
**C Schanen,** Nemours Research, Alfred I duPont Hospital for Children, Wilmington, DE19803, USA

## REFERENCES

1 **Hagberg B**, Aicardi J, Dias K, Ramos, O. A progressive syndrome of autism, dementia, ataxia and loss of purposeful hand use in girls: Rett's syndrome; report of 35 cases. *Ann Neurol* 1983;**14**:471-9.
2 **Amir RE**, Van den Veyver IB, Wan M, Tran CQ, Francke U. Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* 1999;**23**:185-8.
3 **Bird A**, Wolffe AP. Methylation-induced repression-belts, braces, and chromatin. *Cell* 1999;**99**:451-4.
4 **Wakefield RID**, Smith BO, Nan X, Free A, Soteriou A, Uhrin D, Bird AP, Barlow PN. The solution structure of the domain from MeCP2 that binds to methylated DNA. *J Mol Biol* 1999;**291**:1055-65.
5 **Ohki I**, Shimotake N, Fujita N, Nakao M, Shirakawa M. Solution structure of the methyl-CpG-binding domain of the methylation-dependent transcriptional repressor MBD1. *EMBO J* 1999;**18**:6653-61.
6 **Nan X**, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 1998;**393**:386-9.
7 **Jones PL**, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* 1998;**19**:187-9.
8 **Shahbazian MD**, Zoghbi HY. Molecular genetics of Rett syndrome and clinical spectrum of MECP2 mutations. *Curr Opin Neurol* 2000;**14**:171-6.
9 **Guy J**, Hendrich B, Holmes M, Martin JE, Bird A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet* 2001;**27**:322-6.
10 **Chen RZ**, Akbarian S, Tudor M, Jaenisch R. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet* 2001;**27**:327-31.
11 **Shahbazian MD**, Young JI, Yuva-Paylor LA, Spencer CM, Antalffy BA, Noebels JL, Armstrong DL, Paylor R, Zoghbi HY. Mice with truncated MeCP2 recapitulate Rett syndrome features and display hyperacetylation of histone H3. *Neuron* 2002;**35**:243-54.
12 **Hammer S**, Dorrani N, Dragich J, Kudo S, Schanen C. The phenotypic consequences of MECP2 mutations extend beyond Rett syndrome. *Ment Retard Dev Disabil Res Rev* 2002;**8**:94-8.
13 **Kudo S**, Nomura Y, Segawa M, Fujita N, Nakao M, Dragich J, Schanen C, Tamura M. Functional analyses of MeCP2 mutations associated with Rett syndrome using transient expression systems. *Brain Dev* 2001;**23**:S165-73.
14 **Kudo S**, Nomura Y, Segawa M, Fujita N, Nakao M, Hammer S, Schanen C, Terai I, Tamura M. Functional characterization of MeCP2 mutations found in male patients with X linked mental retardation. *J Med Genet* 2002;**39**:132-6.
15 **Ohki I**, Shimotake N, Fujita N, Jee JG, Ikegami T, Nakao M, Shirakawa M. Solution structure of the methyl-CpG-binding domain of human MBD1 in complex with methylated DNA. *Cell* 2001;**105**:487-97.
16 **Orrico A**, Lam C-W, Galli L, Dotti MT, Hayek G, Tong S-F, Poon PMK, Zappella M, Federico A, Sorrentino V. MECP2 mutation in male patients with non-specific X-linked mental retardation. *FEBS Lett* 2000;**481**:285-8.
17 **Couvert P**, Bienvenu T, Aquaviva C, Poirier K, Moraine C, Gendrot C, Verloes A, Andres C, Le Fevre AC, Souville I, Steffann J, des Portes V, Ropers HH, Yntema HG, Fryns J-P, Briault S, Chelly J, Cherif B. MECP2 is highly mutated in X-linked mental retardation. *Hum Mol Genet* 2001;**10**:941-6.
18 **Amir RE**, Zoghbi H. Rett syndrome: Methyl-CpG-binding protein 2 mutations and phenotype-genotype correlations. *Am J Med Genet* 2000;**97**:147-52.
19 **Huppke P**, Laccone F, Kramer N, Engel W, Hanefeld F. Rett syndrome: analysis of MECP2 and clinical characterization of 31 patients. *Hum Mol Genet* 2000;**9**:1369-75.

20 **Xiang F**, Buervenich S, Nicolao P, Bailey ME, Zhang Z, Anvret M. Mutation screening in Rett syndrome patients. *J Med Genet* 2000;**37**:250-5.
21 **Cheadle JP**, Gill H, Fleming N, Maynard J, Kerr A, Leonard H, Krawczak M, Cooper DN, Lynch S, Thomas N, Hughes H, Hulten M, Ravine D, Sampson JR, Clarke A. Long-read sequence analysis of the MECP2 gene in Rett syndrome patients: correlation of disease severity with mutation type and location. *Hum Mol Genet* 2000;**9**:1119-29.
22 **Vacca M**, Filippini F, Budillon A, Rossi V, Mercadante G, Manzati E, Gualandi F, Bigoni S, Trabanelli C, Pini G, Calzolari E, Ferlini A, Meloni I, Hayek G, Zappella M, Renieri A, D'Urso M, D'Esposito M, MacDonald F, Kerr A, Dhanjal S, Hulten M. Mutation analysis of the MECP2 gene in British and Italian Rett syndrome females. *J Mol Med* 2001;**78**:648-55.
23 **De Bona C**, Zappella M, Hayek G, Meloni I, Vitelli F, Bruttini M, Cusano R, Loffredo P, Longo I, Renieri A. Preserved speech variant is allelic of classic Rett syndrome. *Eur J Hum Genet* 2000;**8**:325-30.
24 **Obata K**, Matsuishi T, Yamashita Y, Fukuda T, Kuwajima K, Horiuchi I, Nagamitsu S, Iwanaga R, Kimura A, Omori I, Endo S, Mori K, Kondo I. Mutation analysis of the methyl-CpG binding protein 2 gene (*MECP2*) in patients with Rett syndrome. *J Med Genet* 2000;**37**:608-10.
25 **Hampson K**, Woods CG, Latif F, Webb T. Mutations in the MECP2 gene in a cohort of girls with Rett syndrome. *J Med Genet* 2000;**37**:610-12.
26 **Buyse IM**, Fang P, Hoon KT, Amir RE, Zoghbi HY, Roa BB. Diagnostic testing for Rett syndrome by DHPLC and direct sequencing analysis of the MECP2 gene: identification of several novel mutations and polymorphisms. *Am J Hum Genet* 2000;**67**:1428-36.
27 **Hoffbuhr K**, Devaney JM, LaFleur B, Sirianni N, Scacheri C, Giron J, Schuette J, Innis J, Marino M, Philippart M, Narayanan V, Umansky R, Kronn D, Hoffman EP, Naidu S. MeCP2 mutations in children with and without the phenotype of Rett syndrome. *Neurology* 2001;**56**:1486-95.
28 **Inui K**, Akagi M, Ono J, Tsukamoto H, Shimono K, Mano T, Imai K, Yamada M, Muramatsu T, Sakai N, Okada S. Mutational analysis of MECP2 in Japanese patients with atypical Rett syndrome. *Brain Dev* 2001;**23**:212-5.
29 **Laccone F**, Huppke P, Hanefeld F, Meins M. Mutation spectrum in patients with Rett syndrome in the German population: evidence of hot spot regions. *Hum Mutat* 2001;**17**:183-90.
30 **Nielsen JB**, Henriksen KF, Hansen C, Silahtaroglu A, Schwartz M, Tommerup N. MECP2 mutations in Danish patients with Rett syndrome: high frequency of mutations but no consistent correlations with clinical severity or with the X chromosome inactivation pattern. *Eur J Hum Genet* 2001;**9**:178-84.
31 **Chae JH**, Hwang YS, Kim KJ. Mutation analysis of MECP2 and clinical characterization in Korean patients with Rett syndrome. *J Child Neurol* 2002;**17**:33-6.
32 **Villard L**, Cardoso AK, Chelly PJ, Tardieu PM, Fontes M. Two affected boys in a Rett syndrome family: clinical and molecular findings. *Neurology* 2000;**55**:1188-93.
33 **Kudo S**, Fukuda M. Tissue-specific transcriptional regulation of human leukosialin (CD43) gene is achieved by DNA methylation. *J Biol Chem* 1995;**270**:13298-302.
34 **Lewis JD**, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, Bird A. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 1992;**69**:905-14.
35 **Nan X**, Tate P, Li E, Bird A. DNA methylation specifies chromosomal localization of MeCP2. *Mol Cell Biol* 1996;**16**:414-21.
36 **Lyko F**. DNA methylation learns to fly. *Trends in Genet* 2001;**17**:169-72.
37 **Kudo S**. Methyl-CpG-binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated. *Mol Cell Biol* 1998;**18**:5492-9.
38 **Courey AJ**, Tjian R. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell,* 1988;**55**:887-98.
39 **Free A**, Wakefield RID, Smith BO, Dryden DTF, Barlow PN, Bird A. DNA recognition by the methyl-CpG binding domain of MeCP2. *J Biol Chem* 2000;**276**:3353-60.
40 **Hendrich B**, Bird A. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol* 1998;**18**:6538-47.
41 **Wade PA**, Wolffe AP. ReCoGnizing methylated DNA. *Nat Struct Biol* 2001;**8**:575-7.
42 **Ballestar E**, Yusufzai TM, Wolffe AP. Effects of Rett syndrome mutations of the methyl-CpG binding domain of the transcriptional repressor MeCP2 on selectivity for association with methylated DNA. *Biochemistry* 2000;**39**:7100-6.
43 **Ahmad K**, Henikoff S. Epigenetic consequences of nucleosome dynamics. *Cell* 2002;**111**:281-4.
44 **Uchino J**, Suzuki M, Hoshino K, Nomura Y, Segawa M. Development of language in Rett syndrome. *Brain Dev* 2001;**23**:S233-5.
45 **Yamashita Y**, Kondo I, Fukuda T, Morishima R, Kusaga A, Iwanaga R, Matsuishi T. Mutation analysis of the methyl-CpG-binding protein 2 gene (MECP2) in Rett patients with preserved speech. *Brain Dev* 2001;**23**:S157-60.
46 **Monros E**, Armstrong J, Aibar E, Poo P, Canos I, Pineda M. Rett syndrome in Spain: mutation analysis and clinical correlations. *Brain Dev* 2001;**23**:S251-3.
47 **Zappella M**, Meloni I, Longo I, Hayek G, Renieri A. Preserved speech variants of the Rett syndrome: molecular and clinical analysis. *Am J Med Genet* 2001;**104**:14-22.
48 **Armstrong J**, Poo P, Pineda M, Aibar E, Gean E, Catala V, Monros E. Classic Rett syndrome in a boy as a result of somatic mosaicism for a MECP2 mutation. *Ann Neurol* 2001;**50**:692.