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Identification and Functional Characterization of the p66/p68 Components of the MeCP1 Complex

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Methylation of cytosine at CpG dinucleotides is a common feature of many higher eukaryotic genomes. A major biological consequence of DNA methylation is gene silencing. Increasing evidence indicates that recruitment of histone deacetylase complexes by methyl-CpG-binding proteins is a major mechanism of methylated DNA silencing. We have previously reported that the MeCP1 protein complex represses transcription through preferential binding, remodeling, and deacetylation of methylated nucleosomes. To understand the molecular mechanism of the functioning of the MeCP1 complex, the individual components of the MeCP1 complex need to be characterized. In this paper, we report the identification and functional characterization of the p66 and p68 components of the MeCP1 complex. We provide evidence that the two components are different forms of the same zinc finger-containing protein. Analysis of the p66 homologs from different organisms revealed two highly conserved regions, CR1 and CR2. While CR1 is involved in the association of p66 with other MeCP1 components, CR2 plays an important role in targeting p66 and MBD3 to specific loci. Thus, our study not only completes the identification of the MeCP1 components but also reveals the potential function of p66 in MeCP1 complex targeting. The identification and characterization of all the MeCP1 components set the stage for reconstitution of the MeCP1 complex.

Dynamic changes in chromatin structure play important roles in DNA-templated processes, such as transcription, replication, recombination, and repair (11, 17). Thus far, at least two types of enzymatic activities have been identified that are capable of altering chromatin structure to allow protein factors access to nucleosomal DNA. One involves multiprotein complexes that utilize energy derived from ATP hydrolysis to "remodel" nucleosomes (16, 17); the other involves covalent modification, in particular acetylation/deacetylation, of core histone tails (13). The purification and functional characterization of the nucleosome remodeling and histone deacetylase (HDAC) complex, NuRD/Mi-2 complex, suggest that the two chromatin-modifying enzymatic activities could be coupled (15, 19, 22, 24).

NuRD/Mi-2 complex is a multiprotein complex that possesses both nucleosome remodeling and histone deacetylase activities (15, 19, 22, 24). This complex has been purified from both HeLa cells and *Xenopus laevis* eggs (18, 24). When the complex was purified from HeLa cells, seven major polypeptides were identified (24). In addition to the four-subunit histone deacetylase core, HDAC1/2 and RbAp46/p48, which is also present in the Sin3 histone deacetylase complex (23, 26), NuRD contains at least three more subunits: Mi2, MTA2, and MBD3 (24, 25). Mi2 is a SWI2/SNF2 type helicase/ATPase domain-containing protein that was first identified as a dermatomyositis-specific autoantigen (12) and has recently been demonstrated to be responsible for the remodeling activity of the NuRD complex (1, 20). MTA2 is a novel protein that is highly similar (65% identical) to the candidate metastasis-associated protein MTA1 (14, 25). Biochemical characterization of MTA2 indicates that it plays an important role in modulating the histone deacetylase activity of the NuRD complex (25). MBD3 is a methyl-CpG-binding domain-containing protein, similar to MBD2 (4). However, the function of MBD3 in the NuRD complex is not known (25). When the complex was purified from *Xenopus* egg extracts, it was composed of six subunits: the ATPase xMi2, the MTA1-like protein (xMTA2), the histone deacetylase xRpd3, the histone binding protein xRbAp48, the methyl-CpG-binding domain-containing protein xMBD3, and a zinc finger protein, p66 (18). Thus, most of the NuRD/Mi-2 components are conserved between humans and *Xenopus*.

The identification of a methyl-CpG-binding domain-containing protein MBD3 in the NuRD/Mi2 complex suggests that this complex may potentially be recruited to methylated DNA for transcriptional silencing. A gel-mobility-shift assay revealed that the highly purified seven-component NuRD complex does not show affinity binding to methylated DNA (25). However, the NuRD complex can be targeted to methylated DNA in vitro through an interaction with the methyl-CpG-binding protein MBD2 (25). This in vitro interaction was substantiated by the recent demonstration that the NuRD complex is able to associate with MBD2 in vivo to form the MeCP1 complex (3). The MeCP1 complex was initially described as a methyl-CpG-specific binding activity (7) and has been shown to include MBD2 and the histone deacetylase core, HDAC1/2 and RbAp46/p48 (9). Using a methyl-CpG-specific-binding assay and Western blot analysis, we have recently purified and defined the molecular composition of the MeCP1 complex to

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include MBD2, all seven NuRD components, and two novel polypeptides of 66 and 68 kDa (3). Importantly, the MeCP1 complex is able to repress transcription through preferential binding, remodeling, and deacetylation of methylated nucleosomes (3).

To analyze the MeCP1 complex further, we have now identified and characterized the two novel components of the MeCP1 complex, p66 and p68. We found that the two polypeptides represent different modified forms of the same protein that is conserved from *Caenorhabditis elegans* to humans. The protein contains two highly conserved regions, CR1 and CR2. We provide evidence that CR1 is involved in the association of p66/p68 with the MeCP1 complex, while CR2 is required for the localization of p66/p68 and MBD3 to specific loci. Our data support a potential function of p66/p68 in MeCP1 complex targeting.

MATERIALS AND METHODS

p66/p68 identification, cloning, and DNA constructs. The procedure for the purification of the MeCP1 complex has been described previously (3). The purified MeCP1 complex was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. The p66 and p68 bands were excised and subjected to in-gel tryptic digestion and mass spectrometric analysis as previously described (2). Mass spectrometric analysis of the protein bands corresponding to p66 and p68 revealed that they both have peptide masses that match a cDNA clone, KIAA1150, in the HUGE database (www.kazusa.or.jp/huge). 5′ rapid amplification of cDNA ends (5′ RACE) (Gibco BRL) extended the KIAA1150 cDNA 297 bp at the 5′ end. The longest cDNA for p66 contains 5,348 bp encoding 593 amino acids. All the p66 constructs were generated by PCR using the human p66 cDNA as a template. All clones were verified by DNA sequencing. The various deletions of Gal4-DNA binding domain-p66 constructs used in Fig. 4C were made by PCR amplification of the human p66 cDNA and cloning into the *Eco*RI and *Xba*I sites of vector pSG424. The various p66 fragments were released and cloned into pFlag-CMV2 and pBluescript to generate the Flag-tagged constructs used in Fig. 3B and Fig. 5B, and for in vitro translation used in Fig. 2B. The pEGFP-p66 constructs used in Fig. 5A were generated by the same method. The mouse p66 sequence was derived from assembly of overlapping mouse expressed sequence tag (EST) clones. All the constructs for glutathione *S*-transferase (GST) fusion proteins used in Fig. 2A and GFP-MBD3 used in Fig. 5 have been described previously (4, 9, 25, 26).

GST pulldown assay. Two micrograms of GST or GST fusion proteins was bound to 10 μl of glutathione-immobilized agarose beads (Sigma) and incubated with an equal amount of in vitro-translated full-length p66 or deletion mutant p66 in 300 μl of buffer B (50 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol) containing 150 mM KCl and 0.05% NP-40. After incubation at 4°C for 4 to 8 h, the beads were washed three times with buffer B containing 300 mM KCl and 0.05% NP-40 before being resolved by SDS-PAGE. When dry, the gels were exposed to X-ray film. In vitro translation was performed using the rabbit reticulocyte lysate kit according to the manufacturer's instructions (Promega).

Luciferase assay. About 1.5×10^5 293T cells were seeded in each well of a 12-well plate 1 day before transfection. A total of 300 ng of plasmid DNA, including 50 ng of pCH110 (Pharmacia) encoding β-galactosidase, was used for each transfection using the Qiagen Effectene Transfection Reagent. Twenty-four hours after transfection, cells were harvested, and luciferase and β-galactosidase activities were measured by using the luciferase and β-galactosidase assay systems (Promega). Transfection efficiency was normalized by using the β-galactosidase assay. For Tricostantin A (TSA) treatment, TSA was added to the culture medium (final concentration, 100 ng/ml) 24 h posttransfection, and cells were harvested after another 24 h of incubation.

Immunoprecipitation. Twenty-four hours after transfection, cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) before being lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, and 0.7 μg of pepstatin/ml). After incubation for 30 min at 4°C, the cell debris was removed by centrifugation at 14,000 rpm for 5 min in an Eppendorf centrifuge (Brinkmann Instruments, Inc.). About 1 mg of protein extracts was incubated with 20 μl of Flag-M2 agarose beads (Sigma) at 4°C for 4 h. After three washes with lysis buffer, the immunoprecipitated proteins were analyzed by Western blotting.

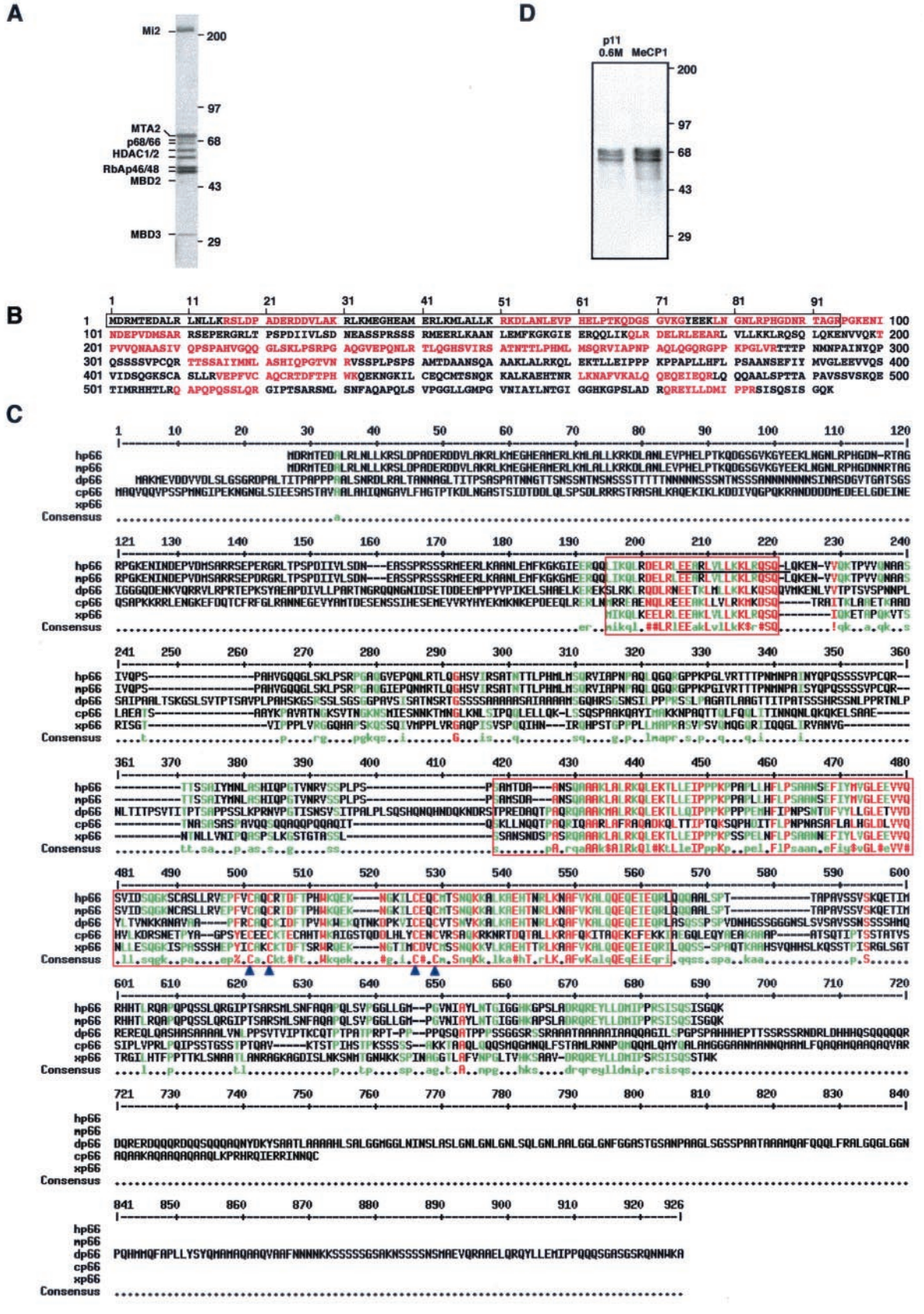
Immunofluorescent staining. Twenty-four hours before transfection with the Qiagen Effectene Transfection Reagent, about 10^5 NIH 3T3 cells were seeded in each well of a 12-well plate. Twenty-four hours after transfection, cells were washed twice with PBS and then fixed in 3% paraformaldehyde (in PBS) for 10 min at room temperature. After a wash with PBS, the cells were incubated with 0.2% Triton X-100 (in PBS) for 5 min at 4°C. Then the cells were blocked with 0.5% bovine serum albumin (in PBS) for 30 min and stained with anti-Flag M2 antibody (IBI; 1:1,000) for 1 h at room temperature. After three washes with PBS, the cells were first incubated with rhodamine-conjugated goat anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories) diluted 1:50 with 0.5% bovine serum albumin in PBS for 30 min and then stained with 1 μg of 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma)/ml for 10 s. The cells were then washed three times with PBS, mounted with DAKO fluorescent mounting medium, and visualized by a Zeiss immunofluorescence microscope.

RESULTS

p66 and p68 are different forms of a novel zinc finger protein. Using a methyl-CpG-specific-binding assay and Western blot analysis, we previously purified the MeCP1 complex (3). The complex contains 10 polypeptides, including MBD2, the 7 known NuRD components, and 2 unidentified polypeptides of 66 and 68 kDa (Fig. 1A). To identify the two proteins, the purified MeCP1 complex was resolved by SDS-PAGE. After Coomassie staining, the protein bands corresponding to p66 and p68 were excised and subjected to in-gel tryptic digestion. The resulting peptides were analyzed by mass spectrometry. This analysis revealed that p66 and p68 have identical mass spectra, which mostly match that of a conceptual tryptic digestion of a partial cDNA clone, KIAA1150 (GenBank accession number AB032976). Therefore, it is likely that p66 and p68 represent the same protein and that p68 is the modified form (see below). For simplicity, we refer to the protein as p66.

Because KIAA1150 is a partial cDNA clone, we performed 5′ RACE to isolate the full-length p66 cDNA. Sequencing of several clones derived from 5′ RACE resulted in extension of the KIAA1150 clone by 297 bp. The combination of KIAA1150 and the 5′ extension resulted in a cDNA of 5,348 bp encoding an open reading frame of 593 amino acids (Fig. 1B). Mass spectrometric analysis identified four peptides that match the conceptual translation of the 5′ RACE product (Fig. 1B), suggesting that KIAA1150 and the RACE product encode different regions of p66. In addition, we also verified the integrity of the cDNA by reverse transcription-PCR (data not shown). Several lines of evidence indicate that the deduced 593 amino acids represent full-length p66. First, the calculated molecular mass, 65.26 kDa, is almost identical to the estimated apparent molecular mass of 66 kDa (data not shown). Second, all the peptides of p66 and p68 that were identified by mass spectrometry analysis (18 peptides for each) are included within the 593 amino acids (Fig. 1B). Third, transfection of a mammalian expression vector encoding p66 into 293T cells generated a doublet comigrating with the native p66/p68 (data not shown). Fourth, the first methionine conforms to the Kozak initiator sequence (6).

Analysis of the entire coding sequence of p66 revealed a GATA-type zinc finger domain (Fig. 1B). A search of the GenBank databases with human p66 identified homologs in the mouse, *Xenopus*, *Drosophila melanogaster*, and *C. elegans* (Fig. 1C) which are 98.3, 46.4, 36.0, and 25.3% identical to human p66, respectively. Interestingly, the p66 homolog in


A
Mi2
200
97
MTA2
p68/66
68
HDAC1/2
RbAp46/48
MBD2
43
MBD3
29
D
p11
0.6M MeCP1
200
97
68
43
29
B
1 MDRMTEDALR LNLLKRSLDP ADERDDVLAK RLKMEGHEAM ERLKMLALLK RKDLANLEVP HELPTKQDGS GVKGYEEKLN GNLRPHGDNR TAGRPGKENI 100
101 NDEPVDMSAR RSEPERGRLT PSPDIIVLSD NEASSPRSSS RMEERLKAAN LEMFKGKGIE ERQQLIKQLR DELRLEEARL VLLKKLRQSQ LQKENVVQRT 200
201 PVVQNAASIV QPSPAHVGQQ GLSKLPSRPG AQGVEPQNLR TLQGHSVIRS ATNTTLPHML MSQRVIAPNP AQLQGQRGPP KPGLVRTTTP NMNPAINYQP 300
301 QSSSSVPCQR TTSSAIYMNL ASHIQPGTVN RVSSPLPSPS AMTDAANSQA AAKLALRKQL EKTLLEIPPP KPPAPLLHFL PSAANSEFIY MVGLEEVVQS 400
401 VIDSQGKSCA SLLRVEPFVC AQCRTDFTPH WKQEKNGKIL CEQCMTSNQK KALKAEHTNR LKNAFVKALQ QEQEIEQRLQ QQAALSPTTA PAVSSVSKQE 500
501 TIMRHHTLRQ APQPQSSLQR GIPTSARSML SNFAQAPQLS VPGGLLGMPG VNIAYLNTGI GGHKGPSLAD RQREYLLDMI PPRSISQSIS GQK
C
1 10 20 30 40 50 60 70 80 90 100 110 120
hp66 MDRMTEDALRLNLLKRSLDPADERDDVLAKRLKMEGHEAMERLKMLALLKRKDLANLEVPHELPTKQDGSGVKGYEEKLNGNLRPHGDN-RTAG
np66 MDRMTEDALRLNLLKRSLDPADERDDVLAKRLKMEGHEAMERLKMLALLKRKDLANLEVPHELPTKQDGSGVKGYEEKLNGNLRPHGDNNRTAG
dp66 MAKMEVDDVVDLSLGSGRDPALTITPAPPPAALSNRDLRALTANNAGLTITPSASPATNNGTTSNSSNTNSNSSSTTTTTNNNNNSSSMTNSSSANNNNNNNSINASDGVTGATSGS
cp66 MAQVQQVPSSPMNGIPEKNGNGLSIEESASTAVAALAHIQNGAVLFHGTPTKDLNGASTSIDTDDLQLSPSDLRRRSTRASALKAQEKIKLKDDIVQGPQKRANDDDDMEDEELGDEINE
xp66
Consensus .....a.....

121 130 140 150 160 170 180 190 200 210 220 230 240
hp66 RPGKENINDEPVDMSARRSEPERGRLTPSPDIIVLSDN---EASSPRSSSRMEERLKAANLEMFKGKGIEERQQLIKQLRDELRLEEARLVLLKKLRQSQ-LQKEN-VVQKTPVVQNAAS
np66 RPGKENINDEPVDMSARRSEPDRGRLTPSPDIIVLSDN---EASSPRSSSRMEERLKAANLEMFKGKGMEERQQLIKQLRDELRLEEARLVLLKKLRQSQ-LQKEN-VVQKTPVVQNAAS
dp66 IGGGQDENKVQRRVLRPRTEPKSYAEAPDIVLLPARTNGRQQNGNIDSETDDEEMPPYVPIKELSHAELKEREKSLRKLRQDLRNEETKLMLLKKLKQSQQVMKENLVVTPTSVSPNNPL
cp66 QSAPKKRRLENGKEFDQTCFRFGLRANNEGEVYAMTDESENSSIHESEMEVVRYHYEKMKNKEPDEEQLRERLNMRREAENQLREEEAKLLVLRKMKDSQ-----TRAITKLAMETKAAD
xp66 MIKQLKEELRLEEAKLVLLKKLRQSQ--------IQKETMPQKVTS
Consensus .....er..nikql.##LRlEEakLvlLkK$r#SQ.....!qk..a.qk..s

241 250 260 270 280 290 300 310 320 330 340 350 360
hp66 IVQPS----------PAHVGQQGLSKLPSRPGAGVEPQNLRTLQGHSVIRSATNTTLPHMLMSQRVIAPNPAQLQGQRGPPKPGLVRTTTPNMNPATNYQPQSSSSVPCQR---
np66 IVQPS----------PAHVGQQGLSKLPSRPGAGIEPQNMRTLQGHSVIRSATNTTLPHMLMSQRVIAPNPAQLQGQRGPPKPGIVRTTTPNMNPAISYQPQSSSSVPCQR---
dp66 SAIPAALTSKGSLSVTPTSAVPLPAHSKGSMSSLSGSGPRAVSISATNSRTGSSSSAAAAASAIAAAAMSGQHRSGSNSILPPPRSSLPAGATLAAGTTITPATSSSHRSSNLPPRTNLP
cp66 LAEATS----------AAYKPAVATNGKSVTNGKNSMIESNNKTMNGLKNLSIPQLLELLQK-LSSQSPAAKQAYIMAKKNPAQTTQLFQQLTTINNQNLQKQKELSAAE------
xp66 RISGT----------VIPPPLVAGGQHAPSKQSSQIVMPPLVRGAQPISVSPQQIHN---IRQHPSTGPPPLLAAPRASVPSVGMQGRIIQQGLIRVANVG----------
Consensus ....t.....p....rg....pgkqs..i.....G...is...q.....sq....g.p..lnapr.s.p..q...q.i.....i.....

361 370 380 390 400 410 420 430 440 450 460 470 480
hp66 ----------TTSSAIYMNLASHIQPGTVNRVSSPLPS----------PSAMTDA---ANSQAAAKLALRKQLEKTLLEIPPPKPPAPLLHFLPSAANSEFIYMVGLEEVVQ
np66 ----------TTSSAIYMNLASHIQPGTVNRVSSPLPS----------PSAMSDA---ANSQAAAKLALRKQLEKTLLEIPPPKPPAPLLHFLPSAANSEFIYMVGLEEVVQ
dp66 NLTITPSVTIITPTSAPPSSLKPRNVPGTISNSVSITPALPLSQSHQNQHNDQKNDRSTPREDAQTPAQRQAAAKMALRKQLEKTLLQIPPPKPPPENHFIPNPSNTDFVYLLGLETVVD
cp66 ----------TNASQSASPAVQQSQQAQQPQQAQIT----------QSKLLNQQTPAQRIQAARLAFRAQADKQLTTIPTQKSQPHDITFLPNPNASAFLALHGLDLVVQ
xp66 ----------NTNLLVNIPQHSPSLKGSTGTASSL----------SSANSNDSPRSRQAAAKLALRKQLEKTLLEIPPPKPSSPELNFLPSAANEFIYLVGLEEVVQ
Consensus ....tt.sa...p.as.s..g.....ss..........p.....pA.rqaAAk$AlRkQl#KtLleIPppKp..pel.FlPsaan.eFiy$vGL#eVV#

481 490 500 510 520 530 540 550 560 570 580 590 600
hp66 SVIDSQGKSCASLLRVEPFVCAQCRTDFTPHWKQEK-----NGKILCEQCMTSNQKKALKREHTNRLKNAFVKALQQEQEIEQRLQQQAALSPT----------TAPAVSSVSKQETIM
np66 SVIDSQGKNCASLLRVEPFVCAQCRTDFTPHWKQEK-----NGKILCEQCMTSNQKKALKREHTNRLKNAFVKALQQEQEIEQRLQQQAALSPT----------TAPAVSSVSKQETIM
dp66 YLTVNKKRNAVPAA-----PFRCAQCKIDFTPVWIWEKQTNKDPKVICEQCVTSWVKKALKREHTNRLKQAFVKALQQEQEIEQRLASQSSPSPVDNHGSSGGGNSLSVSAVSSNSSSSHHQ
cp66 HVLKDRSNETPYA--GPSYECEECKTECAHTWKAIGSTQDDLHLYCENCVRSAQKRKNRTDQTALLKRAFQKITAQEKEFEKKIAEGQLEQYAEAKAAP------ATSQTIPTSSTATVS
xp66 NLLESQGKISPASSSHEPYICAKCKTDFTSRWRQEK-----NGTIMCDVCMSSNQKKVLKREHTTRLKAAFVKALQQEQEIEQRILQQSS-SPAQTKAAHSVQHHSLKQSSTPISRGLSGT
Consensus .ll.sqgk..pa....ep%.Ca.Ckt.#ft..Wkqek....#g.i.C#.Cn.SnqKk.lka#hT.rLK.AFvKalqQEqEiEqri.qqss.spa..kaa.....p.S.....

601 610 620 630 640 650 660 670 680 690 700 710 720
hp66 RHHTLRQAPQPQSSLQRGIPTSARSMLSNFAQAPQLSVPGGLLGM--PGVNIAYLNTGIGGHKGPSLADRQREYLLDMIPPRSISQSISGQK
np66 RHHTLRQAPQPQSSLQRGIPTSARSMLSNFAQAPQLSVPGGLLGM--PGVNIAYLNTGIGGHKAPSLADRQREYLLDMIPPRSISQSISGQK
dp66 REREQLQRSHASAAAAALVNLPPSVTVIPTKCQTPTPAIPRPT-PP---PPQSQATPPPSSGGSRSSRAAATAAAAAIAAQQAGILSPGPSPAHHHEPTTSSRSSRNDRLDHHHQSQQQQQR
cp66 SIPLVPRLPQIPSSTGSSTPTQAV------KTSTPIHSTPKSSSSS---AKKTAAQLQQQSMQGMNQLFSTAMLRNNPQMQQMLQMYGLAMGGGAANMANNQMAMLFQAQAMQAAQAQVAR
xp66 TRGILHTFPPTTKLSNAATLANRAGKAGDISLNKSNMTGNWKKSPINAGGTLAFVNPGLTVHKSAAV-DRQREYLLDMIPSRSISQSSTWK
Consensus ....l...p.....tl.....p...tp....sp..ag.t.A..npg...hks....drqreylldmip.rsisqs.....

721 730 740 750 760 770 780 790 800 810 820 830 840
hp66
np66
dp66 DQRERDQQQRDQQSQQQAQNYDKYSAATLAAAAHLSALGGMGGLNINSLASLGNLGNLSQLGNLAALGGLGNFGGASTGSANPAAGLSGSSPAATAAAMQAFQQQLFRALGQGLGGN
cp66 AQAAKAQAAQAQAAQLKPRHRQIERRINNQC
xp66
Consensus .....

841 850 860 870 880 890 900 910 920 926
hp66
np66
dp66 PQHMMQFAPLLYSYQMAMAQAAQVAAFNNNNKKSSSSSGSAKNSSSSNSHAEVQRAAELQRQYLLEMIPPQQQSGASGSRQNNWKA
cp66
xp66
Consensus .....

Xenopus had been previously identified as a component of the *Xenopus* Mi-2 complex (18). However, the reported *Xenopus* p66 contains only 420 amino acids, which is significantly shorter than its human, mouse, fly, or worm homologs (Fig. 1C). Interestingly, a conceptual translation of the reported *Xenopus* p66 cDNA upstream of the initiation methionine with a frame shift extends the protein an additional 120 amino acids with significant homology to the N-terminal amino acid sequence of human p66 (data not shown). A search of an EST database identified a *Xenopus* EST clone (BG364338) that contains such a frame shift. Therefore, it is possible that the discrepancy between the *Xenopus* p66 and those from other organisms lay in a sequencing error. Sequence comparison of the p66 homologs from different organisms identified two highly conserved regions, including a region with a GATA-type zinc finger (Fig. 1C). It is interesting that, as with other MeCP1-specific components (Mi2, MTA2, MBD2, and MBD3), an apparent homolog of p66 was not found in *Saccharomyces cerevisiae*, suggesting that the MeCP1 complex is likely involved in multicellular processes.

To confirm that the cDNA we cloned indeed encodes the p66/p68 polypeptides present in the MeCP1 complex, a peptide corresponding to amino acids 176 to 191 of p66 was used to generate antibodies. Antibodies against this peptide specifically recognized the 66- and 68-kDa proteins present in the purified MeCP1 complex, as well as those present in the crude 0.6 M P11 fraction of the HeLa nuclear extracts (Fig. 1D). Therefore, we conclude that the p66 protein identified is a component of the MeCP1 complex. In agreement with our conclusion, a recent study demonstrated copurification of p66 with a NuRD-like protein complex (5).

Conserved region 1 (CR1) of p66 is required for its association with the MeCP1 complex. To gain insight into the function of p66 in the MeCP1 complex, we analyzed the ability of p66 to interact with each of the MeCP1 components. Since our efforts to express full-length p66 in a bacterial expression system failed, we expressed p66 in vitro using the rabbit reticulocyte lysate system and performed GST pulldown assays. Figure 2A demonstrates that p66 interacted with MBD3, as well as with MBD2 and RbAp46/p48, in this assay. However, the p66–MBD2 and p66–RbAp46/p48 interactions are weaker than that between p66 and MBD3. These interactions are specific, as evidenced by the fact that GST, HDAC1/2, and MTA2 failed to interact with p66 under the same conditions. The differences between the p66–MBD3 and p66–MBD2 interactions should be noted. Although MBD2 and MBD3 are related proteins, they have two major differences. While MBD2 has an N-terminal extension, MBD3 contains a glutamic acid stretch at its C terminus. Whether these differences account for their different affinities to p66 remains to be determined.

To characterize the p66/MBD3 interaction further, different deletion constructs of p66 were generated and were used to synthesize p66 mutant proteins for GST pulldown assays. The results shown in Fig. 2B indicated that interaction between MBD3 and p66 requires the presence of the CR1 region (compare lanes 6 and 9). Thus, we conclude that CR1 of p66 plays an important role in the p66–MBD3 association in vitro.

Having established the role of CR1 in p66–MBD3 association in vitro, we sought to determine whether a similar role for CR1 can be demonstrated in vivo. Therefore, N-terminal Flag-tagged full-length p66, as well as several p66 deletion mutants, was expressed in 293T cells. Protein extracts derived from the transfected cells were used in immunoprecipitation experiments using antibodies against Flag. The immunoprecipitates were divided into two parts and analyzed by Western blotting using antibodies against components of the MeCP1 complex (Fig. 3A) or Flag (Fig. 3B). The results shown in Fig. 3A indicate that while the MeCP1 components efficiently coimmunoprecipitated with Flag-p66 (Fig. 3A, lane 8), they failed to coimmunoprecipitate with Flag-tagged p66 mutants lacking CR1 (Fig. 3A, lanes 2 and 7). In contrast, all the Flag-tagged p66 mutants containing CR1 successfully coimmunoprecipitated with components of the MeCP1 complex (Fig. 3A, lanes 3 to 6). Since similar amounts of Flag-p66(1–165) and Flag-p66(1–190) (Fig. 3B, lanes 2 and 3) or Flag-p66(190–593) and Flag-p66 (Fig. 3B, lanes 7 and 8) were present in the immunoprecipitates, the most reasonable explanation for the above results is that CR1 of p66 is required for its association with other components of the MeCP1 complex in vivo.

As discussed above, mass spectrometry studies suggest that p68 is a modified form of p66. Consistent with this conclusion, we found that all the Flag-tagged constructs that include the N-terminal 165 amino acids generated a doublet recognized by Flag antibodies (Fig. 3B, lanes 2 to 5). However, transfection of a construct encoding Flag-p66(1–110) to 293T cells generated only a single protein band recognized by the anti-Flag antibody (data not shown). Collectively, these data suggest that the potential modification is likely to occur between amino acids 110 and 165. Inspection of the amino acid sequence between positions 110 and 165 identified a serine-rich region, raising the possibility that p66 might be modified by phosphorylation. However, phosphatase treatment of p66 has no effect on the modification (data not shown). The nature and functional significance of the modification are currently under investigation.

FIG. 1. p66 is a component of the human MeCP1 complex. (A) Silver staining of the purified MeCP1 complex. The identities of the polypeptides are indicated. p68 is a modified form of p66. (B) Amino acid sequence of human p66. The peptides that match the masses obtained from mass-spectrometric analysis are shown in red. Amino acid sequences derived from 5′ RACE are boxed. (C) Alignment of human p66 with its homologs from other organisms. Sequences used in the alignment include p66 from the human (AF411836), mouse (AF411837), *Xenopus* (AF171099), *Drosophila* (AE003547), and *C. elegans* (T19482). Alignment was performed using the MultAlin program (www.toulouse.inra.fr/multalin.html). Red and green represent 100% and more than 50% identity, respectively. The two highly conserved regions, CR1 and CR2, are marked by rectangles. Arrowheads indicate the zinc finger. The peptide used for antibody production is underlined. Symbols: !, I or V; $, L or M; %, F or Y; #, N, D, Q, E, B, or Z. (D) Western blot analysis using affinity-purified p66 antibodies. Samples used are crude nuclear extracts (0.6 M KCl elution from a phosphocellulose P11 column) and purified MeCP1 complex. Note that p66 can migrate as a doublet or triplet depending on gel conditions.

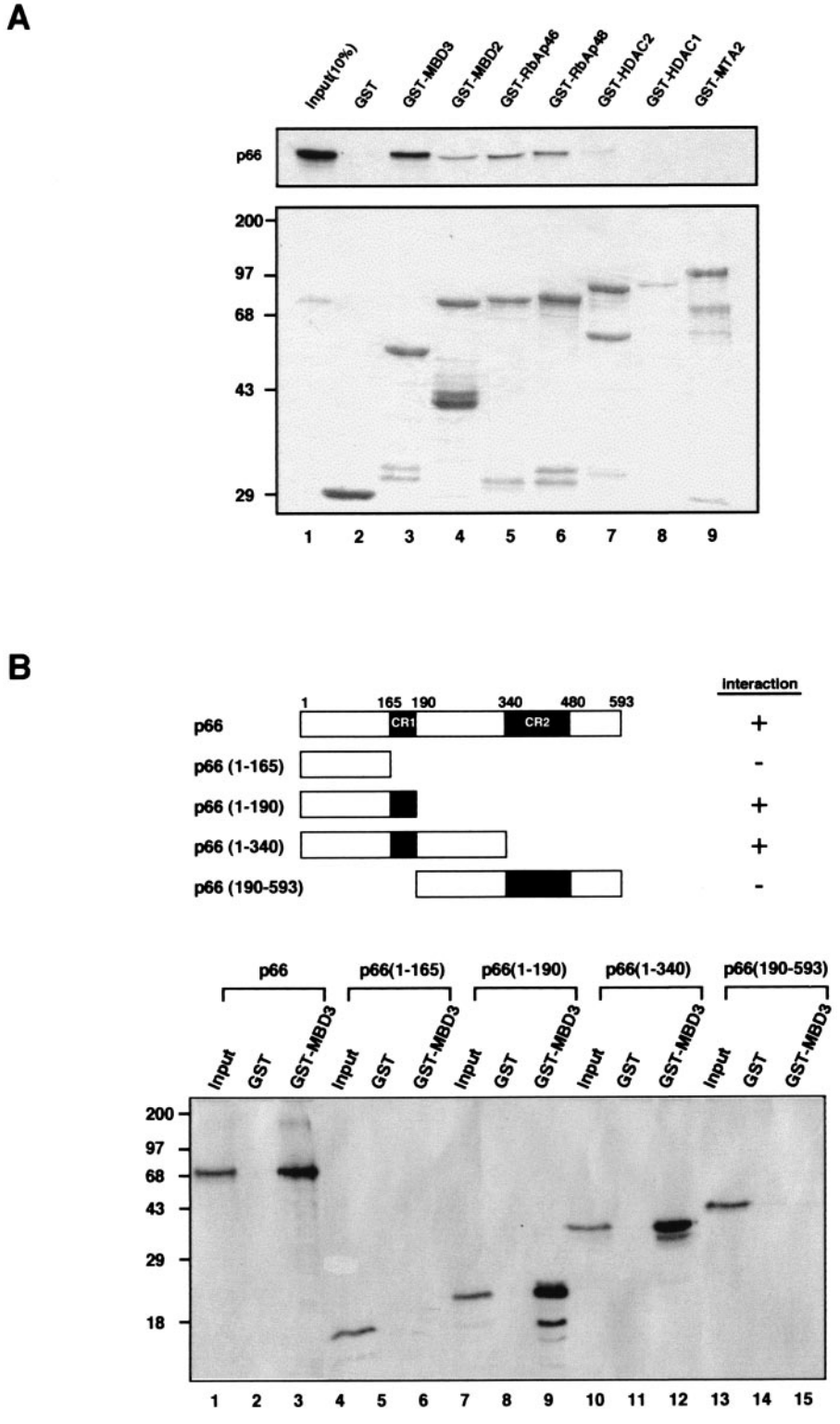


FIG. 2. CR1 is required for p66–MBD3 interaction in vitro. (A) p66 interacts with multiple components of the MeCP1 complex. In vitro-translated p66 protein was subjected to GST pulldown assays using the purified GST fusion proteins given above the gel. After electrophoresis, the gel was subjected to Coomassie blue staining (lower panel) and autoradiography (top panel). (B) CR1 is required for p66–MBD3 interaction. Shown at the top are diagrams of full-length p66 and p66 deletion mutants used in the GST pulldown assays. Plus and minus signs represent interaction and no interaction, respectively. Input lanes represent 10% of the total proteins used in the GST pulldown. Lanes 3, 6, 9, 12, and 15 show amounts of full-length p66 or p66 deletion mutants pulled down by GST-MBD3.

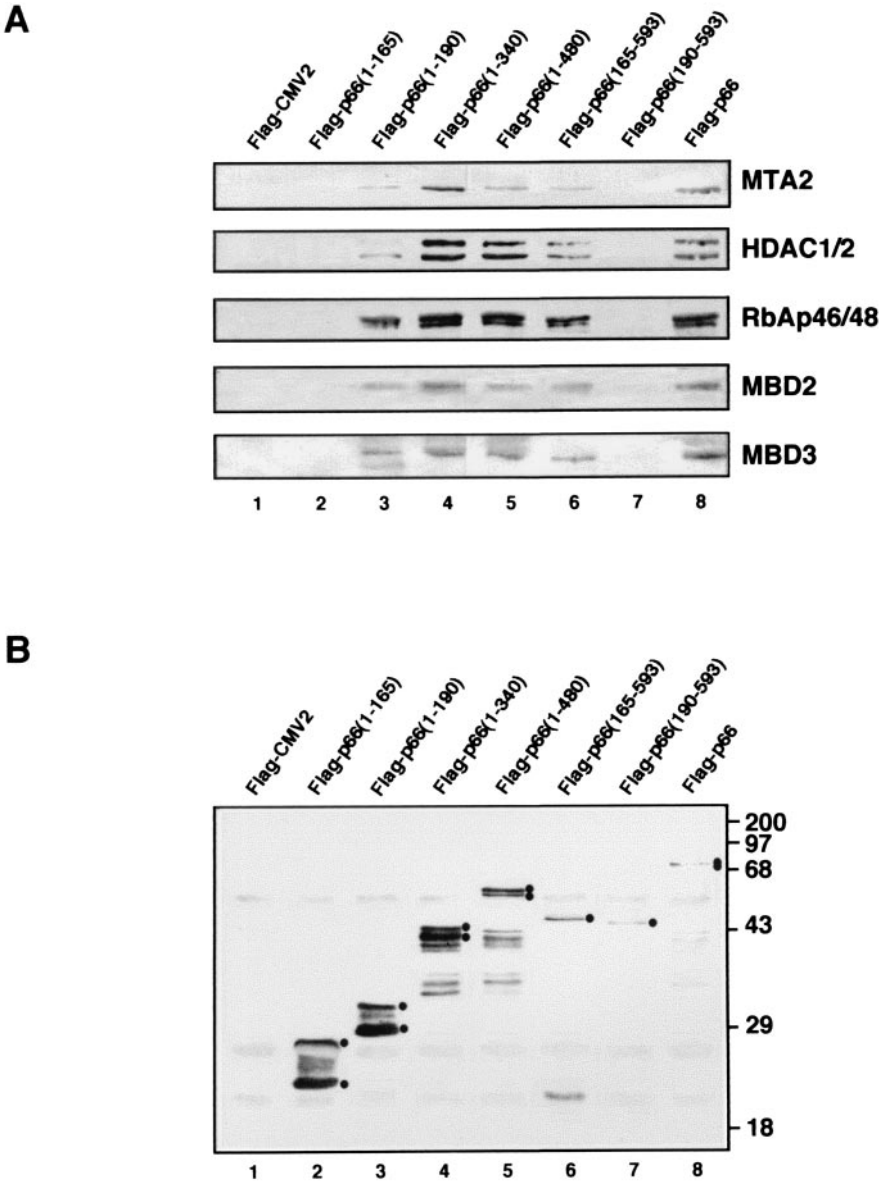


FIG. 3. CR1 is required in order for p66 to interact with other components of the MeCP1 complex in vivo. (A) Western blot analysis of different MeCP1 components. Cell lysates of 293T cells transfected with full-length Flag-tagged p66 and various deletion constructs were immunoprecipitated with an anti-Flag antibody. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose filters, and probed with the antibodies given at the right of the gel. The deletion constructs are given above the gel. (B) The same samples used for panel A were probed with an anti-Flag antibody to show that all the constructs are expressed and that the modification of p66 is within the N-terminal 165 amino acids. Dots indicate the corresponding p66 proteins.

Transcriptional repression by p66 requires CR1. The above data establish that p66 is a component of the MeCP1 complex and that CR1 is required for its incorporation into the MeCP1 complex. Previously, we demonstrated that targeting the MeCP1 complex to a reporter through MBD2 resulted in transcriptional repression (3, 9). Since p66 is a component of the MeCP1 complex, we speculated that targeting the MeCP1 complex to a reporter through p66 would also result in transcriptional repression. Accordingly, a reporter containing five Gal4-DNA binding sites upstream of the thymidine kinase (TK) promoter was cotransfected into 293T cells with a mammalian expression vector encoding Gal4-p66. Figure 4A demonstrates that targeting p66 to a promoter resulted in dose-dependent transcriptional repression.

Given the association of p66 with histone deacetylases (Fig. 1A and 3A), we expect that histone deacetylase activity is at least partially responsible for the repression activity of p66. To test this prediction, the transcriptional activity of p66 was analyzed in the presence and absence of a histone deacetylase inhibitor, TSA. Figure 4B demonstrates that the transcriptional repression activity of p66 is partially released in the presence of TSA. Therefore, p66-mediated transcriptional repression is at least partially dependent on histone deacetylation.

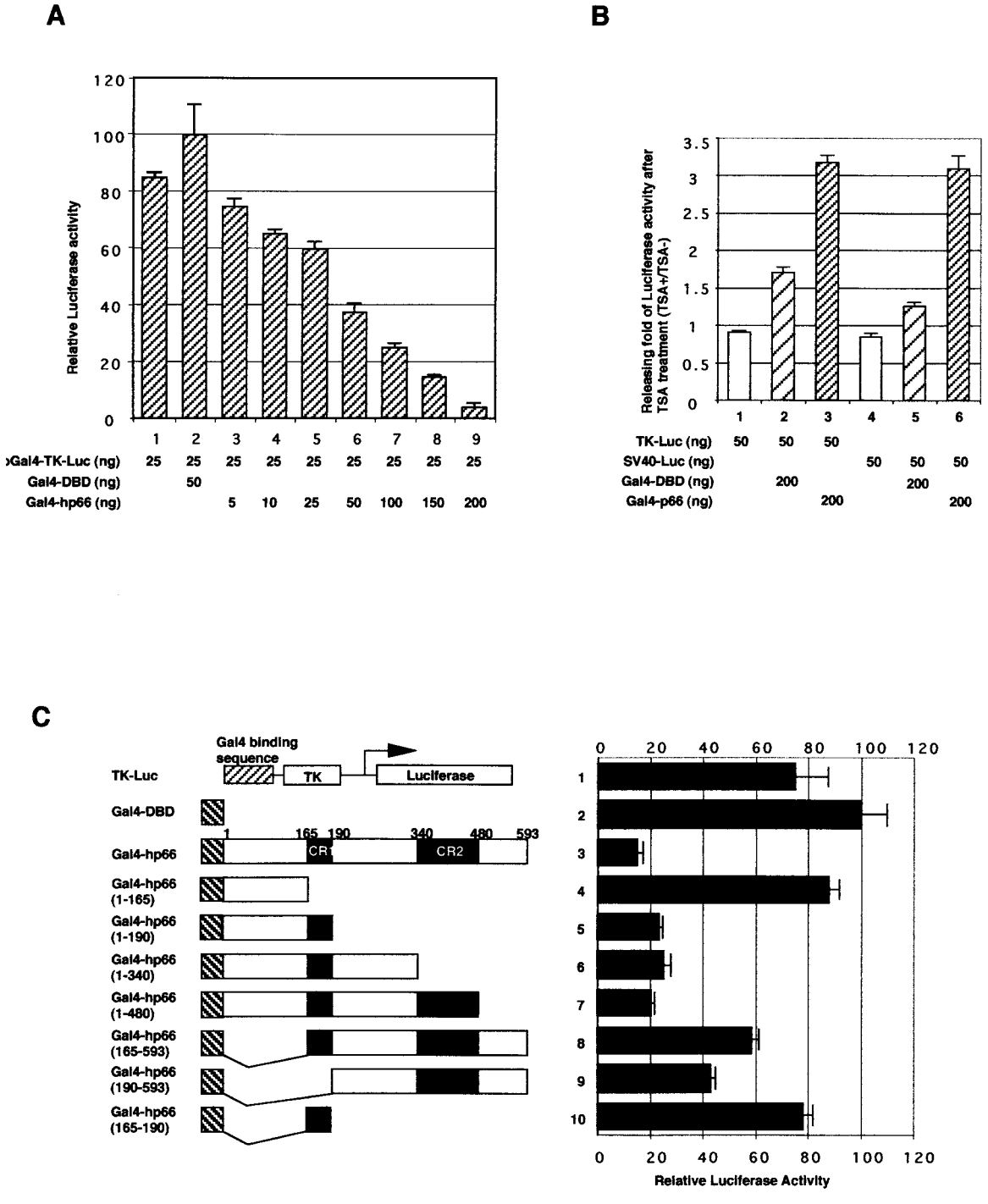


FIG. 4. Transcription repression by p66 requires CR1. (A) p66 represses transcription when tethered to a promoter. A reporter containing five Gal4 binding sites upstream of the TK promoter was cotransfected into 293T cells with a plasmid encoding Gal4-p66. The amount of DNA for each plasmid used in transfection is indicated. Transcription activity was determined by measuring luciferase activity. Transfection efficiencies were normalized using β-galactosidase assays. Data are averages of two independent experiments. Error bars, variations between two independent duplicated experiments. (B) The transcription repression activity of p66 is partially dependent on histone deacetylase activity. Data presented are ratios of luciferase activity in the presence or absence of the histone deacetylase inhibitor TSA (100 ng/ml). Two reporters with different promoters were analyzed. Error bars, variations between two independent duplicated experiments. (C) The repression activity of p66 depends on CR1. (Left) Diagrams of the reporter and Gal4–p66 deletion constructs. (Right) Luciferase activities of the different p66 deletion mutants relative to that of the Gal4-DNA binding domain (Gal4-DBD). Transfection efficiencies were normalized and data were averaged as described for panel A.

To analyze the p66-mediated transcriptional repression further, different regions of p66 were fused to the Gal4-DNA binding domain and tested for their abilities to repress a reporter gene containing Gal4-binding sites. The results shown in Fig. 4C indicate that p66-mediated transcriptional repression requires the presence of CR1 (compare rows 4 and 5). However, CR1 by itself does not seem to be sufficient for mediating efficient transcriptional repression. Efficient transcriptional repression by p66 requires the presence of CR1 and an extension 5′ to CR1 (Fig. 4C; compare rows 5 and 10). Since the transcriptional repression activity of p66 correlates with its ability to associate with the MeCP1 components (Fig. 3A), we conclude that the major mechanism of p66-mediated transcriptional repression involves the assembly and recruitment of the MeCP1 complex. Collectively, the above data suggest that CR1 is responsible for the incorporation of p66 into the MeCP1 complex.

The CR2 of p66 is involved in localization of p66 and MBD3 to specific loci. Having established the role of CR1, we turned our attention to another highly conserved region, CR2. This region contains a GATA-type zinc finger motif (C-X2-C-X17-C-X2-C). Previous studies have demonstrated that this type of zinc finger is involved in protein-protein or protein-DNA interaction (8, 21). Since our protein-protein interaction studies did not suggest that CR2 has a role in the association of p66 with other MeCP1 components (Fig. 2 and data not shown), we decided to investigate the role of CR2 in directing the cellular localization of p66. We reasoned that if this region is involved in targeting the MeCP1 complex by either direct binding to DNA or indirect association with a DNA-binding protein, deletion of this region should change the staining patterns of p66 and components of the MeCP1 complex. After unsuccessful attempts to use the affinity purified p66 peptide antibody to examine the endogenous p66 protein, we transfected Flag- or green fluorescent protein (GFP)-tagged p66 constructs into NIH 3T3 and HeLa cells. Fluorescent staining and immunostaining revealed that expression of both constructs resulted in similar nuclear staining patterns in both cell lines (Fig. 5A, panel a, and data not shown). Comparison with the DAPI staining pattern of the same cell (Fig. 5A, panel d) indicated that GFP-p66 does not exclusively colocalize with heterochromatin. Deletion of the extreme C-terminal sequence did not change the speckled staining pattern of p66 (Fig. 5A; compare panels a and b). However, deletion of CR2 dramatically changed the nuclear staining pattern of GFP-p66 from speckled to diffuse in most transfected cells (Fig. 5A; compare panels b and c). This result supports an important role of CR2 in directing p66 to specific nuclear loci.

Having established a role of CR2 in directing p66 to specific nuclear loci, we asked whether it also has a role in the localization of the other MeCP1 components. Since our protein-protein interaction studies indicated that p66 interacts with MBD3 (Fig. 2), we asked whether p66 can direct MBD3 localization. Consistent with the findings of previous studies (4), GFP-MBD3 showed a diffuse nuclear localization pattern (Fig. 5B, panel a). However, cotransfection of Flag-p66 and GFP-MBD3 changed the GFP-MBD3 localization pattern from diffuse to speckled (Fig. 5B; compare panels a and c). Importantly, GFP-MBD3 and Flag-p66 completely colocalized (Fig. 5B, panel e). Thus, we conclude that p66 has a role in directing MBD3 localization.

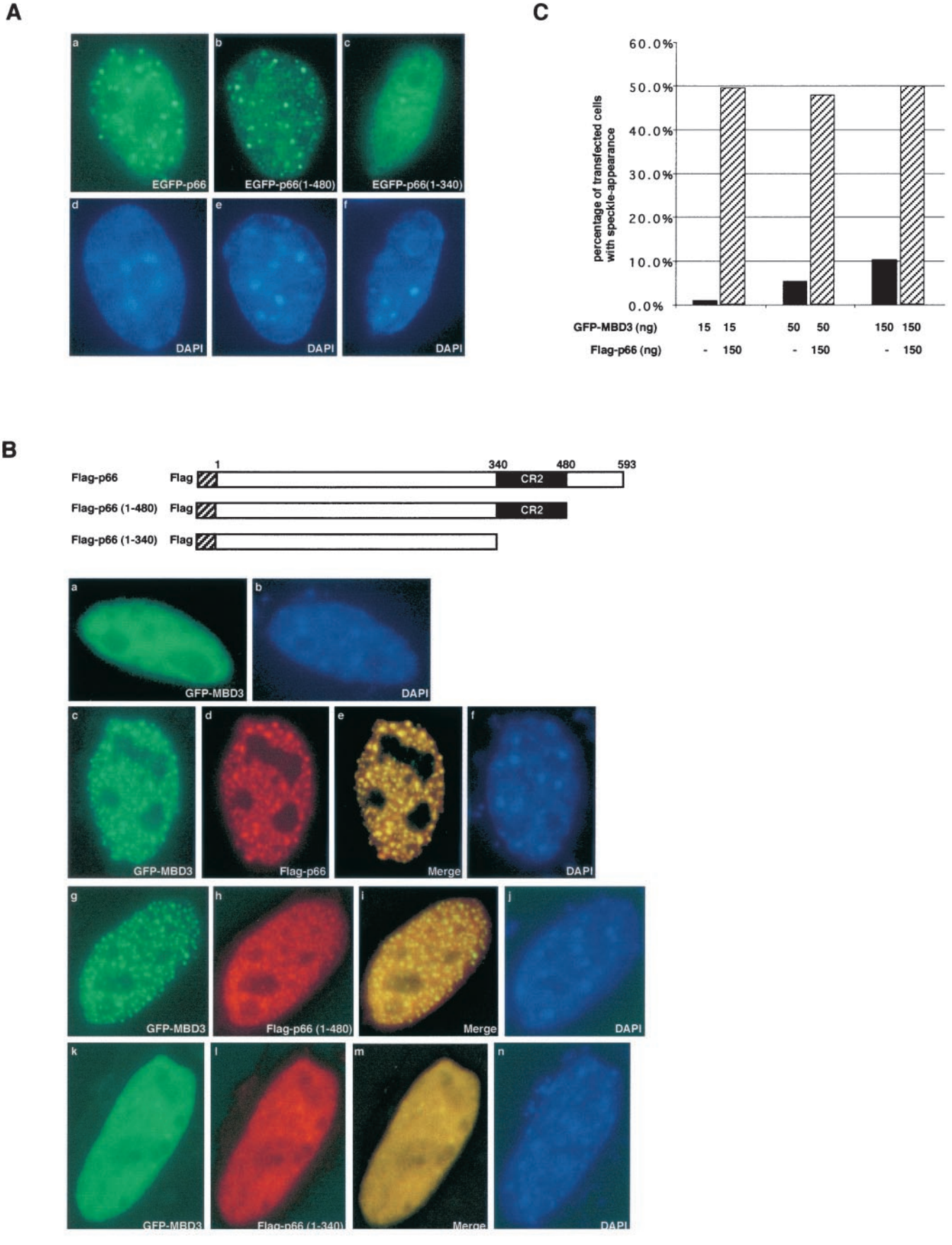
To determine whether CR2 of p66 is required for directing MBD3 to specific nuclear loci, we compared the effects of two p66 mutants with or without CR2 on GFP-MBD3 localization. Like the wild-type Flag-p66, the CR2-containing mutant Flag-p66(1–480) was capable of directing GFP-MBD3 to specific nuclear loci (Fig. 5B; compare panels c through f and g through j). However, the CR2 deletion mutant Flag-p66(1–340) lost its ability to direct itself as well as GFP-MBD3 to specific nuclear loci, resulting in a diffuse nuclear staining pattern (Fig. 5B, panels k through m). Thus, collectively, these results support a potential role of CR2 in targeting both p66 and MBD3 to specific nuclear loci.

Previous studies indicated that GFP-MBD3 has a diffuse nuclear staining pattern in cells when expressed at a low level but accumulates in nuclear foci in cells when expressed at a high level (4). To address the possibility that coexpression of Flag-p66 simply increased the GFP-MBD3 level, resulting in nuclear speckles, different amounts of GFP-MBD3 were transfected with or without the presence of Flag-p66. Calculation of the cell population with a speckled GFP-MBD3 pattern indicated that increasing the GFP-MBD3 level only slightly increased the speckled cell population (Fig. 5C). However, coexpression of Flag-p66 dramatically increased the speckled cell population (Fig. 5C). In addition, the different staining patterns of the CR2-containing mutants and the CR2 deletion mutants also argue against the possibility that our observation is due to a dosage effect. It is worth noting that while the data presented were obtained using NIH 3T3 cells, similar results were also obtained using HeLa cells (data not shown). Therefore, we conclude that CR2 of p66 plays an important role in targeting p66 and MBD3 to specific nuclear loci.

DISCUSSION

The MeCP1 complex was originally described as a methyl-CpG-binding activity (7). Although this activity was identified more than 10 years ago, the molecular composition of the complex has been elusive. It has recently been found that the methyl-CpG-binding domain containing protein MBD2 is a component of the MeCP1 complex, which also includes the histone deacetylases HDAC1 and HDAC2 (9). In an effort to define the molecular composition of the MeCP1 complex, we purified the MeCP1 complex by monitoring MBD2 and its methyl-CpG-binding activity (3). We found that the MeCP1 complex is composed of 10 polypeptides, including MBD2, all the 7 NuRD components, and 2 novel proteins of 66 and 68 kDa (3). This complex is able to repress transcription of methylated DNA through preferential binding, remodeling, and deacetylation of methylated nucleosomes (3). To further characterize the MeCP1 complex, we have now identified and characterized the p66 and p68 components. Thus, all 10 of the MeCP1 components have been identified.

Mass spectrometry analysis of p66 and p68 indicates that p68 is a modified form of p66. Deletion studies indicate that the modification resides within a serine-rich region between amino acids 110 and 165. Preliminary studies indicate that the modification is not likely to be phosphorylation, since it is resistant to phosphatase treatment. The molecular nature and function


A
a
EGFP-p66
b
EGFP-p66(1-480)
c
EGFP-p66(1-340)
d
DAPI
e
DAPI
f
DAPI
C
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
percentage of transfected cells with speckle-appearance
GFP-MBD3 (ng) 15 15 50 50 150 150
Flag-p66 (ng) - 150 - 150 - 150
B
1
340
480
593
Flag-p66 Flag CR2
Flag-p66 (1-480) Flag CR2
Flag-p66 (1-340) Flag
a
GFP-MBD3
b
DAPI
c
GFP-MBD3
d
Flag-p66
e
Merge
f
DAPI
g
GFP-MBD3
h
Flag-p66 (1-480)
i
Merge
j
DAPI
k
GFP-MBD3
l
Flag-p66 (1-340)
m
Merge
n
DAPI

of this modification are currently under investigation. Proteins with significant sequence homology to human p66 can be found in diverse multicellular organisms including *C. elegans, Drosophila*, *Xenopus*, and mice, indicating that the function of p66 is evolutionarily conserved. Sequence analysis of the p66 homologs identified two highly conserved regions, CR1 and CR2. Protein-protein interaction and deletion studies indicate that CR1 is not only involved in MBD3–p66 interaction in vitro (Fig. 2); it is also required for incorporation of p66 into the MeCP1 complex in vivo (Fig. 3). Consistent with its role in incorporation of p66 into the MeCP1 complex, CR1 is required for the transcriptional repression activity of p66 (Fig. 4).

To determine the biochemical function of p66 in the MeCP1 complex, a baculovirus expressing p66 was generated. We formed subcomplexes by coinfection of Sf9 cells with a p66-expressing baculovirus and baculoviruses expressing other components of the MeCP1 complex, followed by immunoprecipitation. Analysis of the histone deacetylase activities of the different subcomplexes indicates that p66 has no effect on histone deacetylase activity (data not shown). Since all of our efforts to establish a role of p66 in modulating the known biochemical properties of the MeCP1 complex had negative results, we explored the possibility that p66 functions as a targeting subunit. We found that deletion of CR2 changed the nuclear localization pattern of p66 from speckled to diffuse (Fig. 5A). Consistent with a potential role of p66 in targeting the MeCP1 complex to specific loci, coexpression of p66 changed the GFP-MBD3 nuclear pattern from diffuse to speckled (Fig. 5B). Importantly, the ability of p66 to direct GFP-MBD3 to specific nuclear loci depends on CR2 (Fig. 5B). Collectively, our data support a potential role of p66 in targeting the MeCP1 complex to specific nuclear loci.

The MBD2 component of the MeCP1 complex can bind specifically to methylated DNA (3, 4, 9). It is possible that p66 might play an important role in determining which methylated DNA MBD2 binds. A notable feature of CR2 is that it contains a GATA-type zinc finger found in several GATA transcription factors (8, 21). However, p66 is not likely to be capable of binding to the GATA sequence, because several amino acids known to be required for GATA sequence binding are not conserved in the p66 zinc finger (10). It is possible that CR2 may recognize a DNA sequence similar to the GATA sequence. Alternatively, it is also possible that CR2 may be involved in interaction with a DNA binding protein. Further work is needed to understand how CR2 is involved in determining the localization of p66 as well as that of MBD3.

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FIG. 5. CR2 of p66 affects the cellular localization of p66 and MBD3. (A) CR2 of p66 is critical for its cellular localization. Panels a through c show the various EGFP-p66 deletion constructs expressed in NIH 3T3 cells. Panels d through f are the corresponding DAPI stains. (B) CR2 of p66 is critical for directing MBD3 to specific nuclear foci. (a and b) GFP-MBD3 expression and DAPI staining, respectively. (c, d, e, and f) A representative cell coexpressing GFP-MBD3 and wild-type p66. (g, h, i, and j) A representative cell coexpressing GFP-MBD3 and the p66 C-terminal deletion mutant. (k, l, m, and n) A representative cell coexpressing GFP-MBD3 and the p66 CR2 deletion mutant. In all the transfections, 150 ng of plasmid DNA encoding GFP-MBD3 was transfected either alone or in combination with 150 ng of wild-type or mutant Flag-p66 plasmid. The Flag-tagged p66 stained with Flag antibody has a red color. Schematic diagrams of all the p66 constructs are shown above the images. (C) The effect of p66 on MBD3 localization is independent of the MBD3 level. Different amounts of plasmid DNA encoding GFP-MBD3 were transfected either alone or together with Flag-p66 plasmid into NIH 3T3 cells. More than 300 transfected cells were counted in each transfection. Bars represent percentages of cells with a speckled appearance. The amount of plasmid DNA transfected is given below the graph. p66 and MBD3 were derived from humans and mice, respectively. Similar results were also obtained when the experiments were performed with HeLa cells (data not shown).

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