Methyl-CpG-binding domain proteins (MBD) mediate functional responses of methylated DNA. MBD2 and MBD3 are components of the MeCP1 protein complex, which contains the Mi-2/NuRD complex and includes 66- and 68-kDa polypeptides. Here we identified two highly related 66-kDa proteins in a yeast two-hybrid screen with MBD2b. Based on the high degree of sequence conservation to the previously identified Xenopus p66 subunit of the Mi-2/NuRD complex, we termed these proteins hp66α and hp66β. hp66α is the human orthologue of Xenopus p66, whereas hp66β, previously identified as a component of the human MeCP1 complex, is a second member of a p66 gene family. Coprecipitation of hp66α and MBD2 demonstrates their in vivo association. Furthermore, confocal microscopy shows a nuclear colocalization of hp66α with hp66β and MBD2 in a speckled pattern. hp66α is a potent transcriptional repressor reducing gene activity about 100-fold and is ubiquitously coexpressed with hp66β in cell lines and in fetal and adult tissues. We demonstrate direct binding of both p66 family members to MBD2 as well as MBD3. Interestingly, hp66α, which binds with a higher affinity than hp66β, interacts via two interaction domains in contrast to a single interaction domain present in hp66β. These results demonstrate that two highly related mammalian p66 proteins display overlapping functions and are involved in methylation dependent transcriptional repression.

DNA methylation at the 5-position of cytosine within CpG dinucleotides has been shown in mammals to be essential for several important functions, such as cell differentiation, imprinting, and X-inactivation. Indeed, genetic diseases have been described that are caused by defects within the genes for the methyl-CpG-binding protein MeCP2 and the DNA methyltransferase Dnmt3B, respectively (1–4). The fragile X methylation of an upstream CpG de novo methylation (5, 6). Furthermore, DNA hypomethylation of the genome, as well as methylation dependent silencing of tumor suppressor genes is often found in human cancer (for review, see Refs. 7 and 8). A repressive effect on gene activity is a common theme throughout these and other functions mediated by DNA methylation. In several cases, repression could be demonstrated to be conferred by histone deacetylation (9, 10). Targeting of histone deacetylase complexes to methylated CpG dinucleotides is mediated by proteins containing a methyl-CpG-binding domain (MBD)3 (11). Four of these proteins, MeCP2, MBD1, MBD2, and MBD3, have been shown to be involved in transcriptional repression (12, 13). Indeed a transpositional repression domain has been found for MBD1, MBD2 as well as MeCP2 (12, 14, 15). Repression mediated by MeCP2 was shown to involve a component of the basal transcriptional machinery, TFIIIB, and the Sin3-deacetylase complex (9, 10, 16). Similarly, Sin3A also binds MBD2 and is involved in MBD2-mediated repression (14). In contrast to the MBD2 family members, MBD2 and MBD3 are similar even outside the MBD, although they seem to differ in function. There are two potential forms of MBD2 depending on the use of the first translation initiation codon: MBD2a and MBD2b. Until now no difference in function for either form has been identified. MBD2 binds specifically to methylated DNA (11, 14), whereas mammalian MBD3 does not bind methylated DNA in vivo or in vitro (11). In contrast to mammalian MBD3, Xenopus MBD3 has been shown to bind with high selectivity to methylated DNA (17). Complex purification provided another distinction between MBD2 and MBD3. MBD3 copurifies with the NuRD histone deacetylase complex (17, 18). MBD2 is the methyl-CpG binding component of the MeCP1 protein complex, which contains the NuRD complex and two polyamines of 66 and 68 kDa (19, 20). In other words, the MBD3 containing NuRD complex in mammals can be targeted to methylated DNA by MBD2. Such a functional difference between MBD2 and MBD3 is also evident from knockout experiments. MBD2−/− mice show a maternal behavior defect, whereas MBD3−/− mice die early during embryogenesis (21). Although MBD2 has been shown to associate with Mi-2/NuRD within the MeCP1 complex (18, 19), little is known about proteins binding directly to MBD2. Therefore, in the present study, we have searched for interaction partners of MBD2 utilizing a yeast two-hybrid screen. We identified two proteins of approximate molecular weights of 66,000. Sequence comparison of both proteins to the 66-kDa component of Xenopus Mi-2/NuRD demonstrates the existence of a novel gene family with overlapping functions.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA of hp66a was provided by the Resource Center and Primary Data Base, RZPD (IMAGE:3953055) in pGTR7 (www. bio.uni- Bonn.de), and revealed a full-length open reading frame (1899 bp) after sequencing.

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The abbreviations used are: MBD, methyl-CpG-binding domain; aa, amino acid(s); GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; UAS, upstream activation sequence; ID1 and ID2, interaction domains 1 and 2, respectively.

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pSG5 digested with MBD fragments of the corresponding Gal constructs and ligation into pGal-hp66. pGal-hp66 (1–134) and (133–633) were created by in-frame insertion of the SolI/BspHI or BspHI/XbaI fragments into the pABGAgal94 linker. pGal-hp66 (133–238) was created by digesting pGal-hp66(-133–633) with Sall/I and EcoRI, followed by religation. The C-terminal deletions of pGal-hp66 were created by digestion of pGal-hp66 with Xbal and the internal sites Ehel (aa 1–433), Bcul (aa 1–329), and Sall (aa 1–238), followed by religation. pGal-hp66(-434–633) resulted from SolI/Ehel digestion of Gal-hp66, with subsequent religation. pGal-hp66(-334–633) was constructed by subcloning the EcoRI/XbaI fragment from pSG5 into pSG5/MBD. Constructs coding for hp66-MBD proteins were expressed in Escherichia coli BL21. GST pull-downs were carried out essentially as described earlier. The GST fusion proteins were incubated with [35S]methionine-labeled hp66 proteins, produced by the T7/T3 TNt-coupled transcription/translation system (Promega) in 200 μl of binding buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 μg of ethidium bromide, 100 μg of bovine serum albumin). After 0.5 h of incubation at room temperature, they were washed six times with 1 ml of binding buffer without ethidium bromide and bovine serum albumin. The bound proteins were eluted with SDS sample buffer, fractionated on SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting. Antibodies for HDAC1 (C-19) and HDAC2 (C-8) were obtained from Santa Cruz Biotechnology.

Comparative mRNA Expression Analysis—Tissue expression patterns of transcripts from hp66a and hp66b were analyzed by semiquantitative PCR using gene-specific primers (hp66a, 5′-cagctgcaggtgctgtcgtc-3′; hp66b, 5′-gcagccagcagggatacaag-3′) and the respective 3′ BamHI, primer 5′-tagctgactgcaacctgagcaagcag-3′. The reverse transcriptase-PCR product was cut with SolI/BamHI and cloned into the SolI/BamHI site of pABGAgal94 linker. The obtained clone was sequenced.

Cell Culture and Transfections—CVI and HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum at 37 °C, 5% CO2. Transfections were done using the CaPO4 method as described earlier (24). CVI cells were cotransfected in 6-well plates (106 cells/well) using 0.85 μg of 4xUAS tk-luciferase reporter plasmid, 0.18 μg of pCMV-lacZ encoding β-galactosidase, and 0.06–1.58 μg of expression plasmids pGal-hp66a or pGal-hp66b deletions. Cells were harvested 36–72 h after transfection and assayed for luciferase and β-galactosidase activity. All transfection assays were performed in triplicate and repeated at least twice. For eukaryotic overexpression of GST and GST-MBD2b fusions together with pCEFLAU5 or pCEFLAU5-hp66a, HEK 293 cells were transfected using the CaPO4 method (see above). Cells were grown to 60% confluency and transfected with 25 μg of pCMV-GST, pCMV-GST-MBD2b, and/or pCEFLAU5 or pCEFLAU5-hp66a, respectively. Transfected cells were cultured for 48 h, harvested, and subjected to nuclear extract preparation.

The C-terminal deletions of pGal-hp66 and pCEFLAU5 fusion proteins were expressed in Escherichia coli BL21. GST pull-downs were carried out essentially as described earlier.4 Bacteria were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 20 °C. Recombinant proteins were purified with glutathione-Sepharose beads (Amersham Biosciences) and analyzed on SDS-polyacrylamide gel electrophoresis to normalize protein amounts. Equivalent amounts of GST fusion proteins were incubated with [35S]methionine-labeled hp66 proteins, produced by the T7/T3 TNt-coupled transcription/translation system (Promega) in 200 μl of binding buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, 0.5 μg of ethidium bromide, 100 μg of bovine serum albumin). After 0.5 h of incubation at room temperature, they were washed six times with 1 ml of binding buffer without ethidium bromide and bovine serum albumin. The bound proteins were eluted with SDS sample buffer, fractionated on SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting. Antibodies for HDAC1 (C-19) and HDAC2 (C-8) were obtained from Santa Cruz Biotechnology.

Nuclear Extract Preparation and Coprecipitation Assay—HEK 293 cells were transiently cotransfected with mammalian expression vectors for GST or GST-MBD2b and pCEFLAU5 or pCEFLAU5-hp66a, respectively. Cells were harvested after 48 h and nuclear extracts were prepared essentially as described previously (24). CV1 cells were cotransfected in 6-well plates (105 cells/well) using 0.88 μg of expression plasmids pGal-p66a or pCEFLAU5-hp66a, respectively. Cells were harvested after 48 h and nuclear extracts were prepared essentially as described previously (24). 0.18 μg of pCEFLAU5 and 0.18 μg of pCEFLAU5-hp66a were transfected into a new tube and diluted with 3 volumes of buffer A (20 mM HEPES, pH 7.9, 10% glycerol, 0.2% Nonidet P-40, 10 mM KCl, 1 mM EDTA). After centrifugation at 5,000 × g for 10 min at 4 °C, the supernatant was discarded and the remaining nuclei pellet resuspended in 2 volumes buffer B (420 mM NaCl, 20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA), followed by rotational incubation for 40 min at 4 °C. To remove cell debris, the extract was centrifuged at 20,000 × g for 15 min at 4 °C, the supernatant was transferred into a new tube and diluted with 3 volumes of buffer C (10 mM KCl, 20 mM HEPES, pH 7.9, 1 mM EDTA). For coprecipitation assays, 500 μg of the nuclear extracts were incubated with 50 μl of glutathione-Sepharose beads (Amersham) and washed five times with wash buffer and centrifuged in 3 volumes buffer A (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA) for 10 min at 4 °C. To remove cell debris, the extract was centrifuged at 20,000 × g for 15 min at 4 °C, the supernatant was transferred into a new tube and diluted with 3 volumes of buffer C (10 mM KCl, 20 mM HEPES, pH 7.9, 1 mM EDTA). The bound proteins were eluted with SDS sample buffer, fractionated together with the corresponding input fractions on SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting. Antibodies for HDAC1 (C-19) and HDAC2 (C-8) were obtained from Santa Cruz Biotechnology.

Expression of hp66 Gene Family in Human Tissues—In order to analyze the tissue expression patterns of hp66a and hp66b, RNA from different cancer tissue samples (293, HeLa, C33A, K562, Saos-2, A431, MCF7, HepG2, MCF10A; Invitrogen, La Jolla, CA, and NIH) was analyzed by quantitative RT-PCR using peqGold RNAPure, peqGold (25) according to methods of the manufacturer. Briefly, 5 × 106 cells were treated with 1 ml of peqGold RNAPure, heavily vortexed after addition of 0.2 ml of chloroform, and incubated at room temperature for 10 minutes. Following centrifugation at 12,000 × g for 5 minutes, the aqueous top phase was added to 0.2 ml of isopropl alcohol (1:1) and centrifuged at 12,000 × g for 1 minute. The RNA pellet was washed twice with 1 ml of 75% ethanol and dissolved in double distilled H2O. cDNAs from different human adult and fetal tissues (Human MTC Panel I and Human Fetal MTC Panel, Clontech) were used for tissue-specific expression patterns of transcripts from hp66a and hp66b. PCR amplification was performed using primers and annealing temperatures to amplify specific fragments of the human p66 Gene Family.
were used as a control for cDNA normalization. PCR products were analyzed on a 2% agarose gel and stained with ethidium bromide.

RESULTS

A Yeast Two-hybrid Screen with MBD2b Identified Two Related p66 Proteins, hp66α and hp66β—To analyze MeCP1 and MBD2 in more detail, we carried out a yeast two-hybrid screen with MBD2b as a bait. We tested a total of $5 \times 10^6$ mouse cDNA sequences and identified 15 different clones, which could be sorted into two groups. The first and larger group of cDNA sequences consisted of 13 clones, sharing an identical stretch of 57 nucleotides. Therefore, these sequences could be arranged according to their overlapping region (Fig. 1). Based on this arrangement, a sequence contig of 414 bp in length (contig 1) was generated and used to search the GenBank™ data bases for mouse and human expressed sequence tag sequences. This search revealed a high degree of homology with a mouse sequence (BC019178) and a human sequence (BF025891). In addition to this large group of overlapping cDNA clones, we found two other cDNA sequences that almost completely overlapped with each other and generated a second contig (Fig. 1).

Again, GenBank™ data base search revealed a high degree of identity with mouse and human cDNA sequences (mouse, AF411837; human, AF411836). Contig 1 is very similar to a previously identified Xenopus protein of 66 kDa (Fig. 2A), which is a component of the Mi-2/NuRD complex (17). The contig 2 sequence generated from the two-hybrid cDNAs is again similar to a p66 protein (20), but is not identical to contig

![Fig. 1. A yeast two-hybrid screen with MBD2b identifies two highly related groups of cDNA inserts. A total of 15 cDNA inserts gave rise to two different, but highly homologous contig sequences. 13 cDNA inserts with overlapping regions (minimal overlapping region indicated) could be grouped to one contig sequence (contig 1). Two additional cDNA inserts aligned to each other (contig 2), and are highly homologous to contig 1. Horizontal black bars indicate cDNA inserts. Insert ends were either determined from restriction digestion (bar with arrowhead), or from sequencing (blunt end). Gray bars delineate the length of the sequence contigs.](image1)

![Fig. 2. hp66α and hp66β are highly related but different proteins. A, alignment of xMi-2 p66 (Mi-2/NuRD) with the two protein sequences identified in the GenBank™ data base analysis with “contig 1” (BF025891) and “contig 2” (AF411836). Identical and conserved amino acids are shaded with black and gray, respectively. The two highly conserved regions (CR1/CR2) are underlined. Alignment was performed with the “Multiple Sequence Alignment” at searchlauncher.bcm.tmc.edu. B, alignment of xMi-2 p66 together with hp66α, hp66β, and the respective Drosophila (AE003547) and C. elegans (T19482) orthologues. The alignment is displayed as a dendogram after hierarchical clustering using MULTALIN (prodes.toulouse.inra.fr/multalin/multalin.html). C, schematic representation of the domain structure of hp66α and hp66β. The CR1 and CR2 are depicted as black boxes and the position of the GATA zinc finger is indicated by black bars above the diagrams. The table (top) summarizes the results from the data bank analysis. cDNAs for BF025891 and AF411836 correspond to hp66α and hp66β, respectively. Chromosomal localization was estimated using the “BLAT Search Genome” program at genome.ucsc.edu/cgi-bin/hgBlat.](image2)
1. Therefore, we directly compared the human sequence BF025891 (best fit to contig 1) with the human sequence AF411836 (best fit to the contig 2 sequence) and with the Xenopus p66 sequence (Fig. 2A). Clearly, the human cDNA sequence derived from contig 1 shows a very high degree of conservation. To clarify the evolutionary relationship between the published Xenopus p66 sequence (17), the published human p66 sequence (20), and the open reading frames identified in GenBank analysis with contigs 1 (BF025891) and 2 (AF411836), we carried out a dendogram analysis (Fig. 2B). This analysis supported our notion that the BF025891 sequence is the human orthologue of the Xenopus p66 protein. Therefore, we refer to this protein as hp66α. Consequently, AF411836 codes for a second p66 homologue we hereafter refer to as hp66β. Thus, human p66α and p66β are encoded by two different genes comprising a novel gene family. In contrast, in Drosophila as well as Caenorhabditis elegans, just a single orthologue has been described (20) that is included in the dendogram (Fig. 2B). Similar to the human genes, we identified two different cDNAs in mouse coding for two p66 gene family members (mp66α is BC019178; mp66β is AF411837). The chromosomal loci for both human sequences can be taken from the data base such that hp66α is located at 19p13.11 and hp66β is located at 1q23.1 (Fig. 2C). Inspection of well conserved sequences between the different species lead to the identification of two conserved regions (CR1 and CR2 (20)). Now in the light of the presence of homologous as well as orthologous gene members, CR1 and CR2 are not only conserved between the orthologous members in different species, but also within the two human homologous family members (Fig. 2A).

In contrast to the published Xenopus sequence (17), the protein predicted from the contig 1 sequence extended the predicted Xenopus protein by 143 amino acids at the N terminus. The authors postulated a similar (truncated) N terminus for a human cDNA sequence. Inspection of the nucleotide sequence coding for hp66α revealed a start codon 429 bp upstream of the predicted translation initiation ATG. To determine which translational start site is used, we compared the molecular weight of a protein translated in vitro of hp66α and of a deletion, hp66α-ΔN, lacking the first 133 amino acids upstream of the predicted hp66 (17). In vitro translated 35S-labeled proteins were separated by SDS-PAGE and visualized by autoradiography.

**Fig. 3.** The N-terminal extension is required for the expected molecular weight of hp66α. A, alignment of the amino acid sequence of hp66α to a previously predicted human p66α (17). The N-terminal extension is labeled with gray. Identical and homologous amino acids are boxed in black and dark gray, respectively. The alignment was performed with "Multiple Sequence Alignment" at searchlauncher.bcm.tmc.edu. B, in vitro translation of hp66α and of a deletion, hp66α-ΔN, lacking the first 133 amino acids upstream of the predicted hp66 (17). In vitro translated 35S-labeled proteins were separated by SDS-PAGE and visualized by autoradiography.

**Fig. 4.** MBD2b is associated in vivo with hp66α, HDAC1, and HDAC2. HEK293 cells were cotransfected with eukaryotic expression vectors for GST or GST-MBD2b together with the AU5 expression vector or full-length AU5-tagged hp66α, as indicated. Nuclear extracts derived from these cells were purified with glutathione beads. Precipitates (lanes 5–8) and input controls (lanes 1–4) were resolved on SDS-PAGE, transferred to a polyvinylidene difluoride membrane and probed with the antibodies indicated on the left.
ATG is used. In addition, the full-length protein migrates according to the expected molecular weight of 66,000. Because the data base contains a Xenopus expressed sequence tag clone extending in-frame toward the N terminus (20) it can be presumed that the Xenopus p66 protein has a similar N-terminal extension as the human protein.

In Vivo Association of hp66a with MBD2 and Colocalization with hp66β—The Xenopus orthologue of hp66α was found as an integral component of the Mi-2/NuRD complex (17). Similarly, sequencing of a p66 component of the human MeCP1 complex has identified hp66β (20). Because MeCP1 and Mi-2/NuRD complexes contain or bind to MBD2, respectively, it is very likely that hp66α is associated with MBD2 in vivo as well. To prove this assumption, we coexpressed GST-MBD2b constructs with hp66α fused to the AU5 tag in HEK 293 cells. Subsequent nuclear extract preparation and purification with glutathione beads allowed the comparison of proteins bound either to GST-MBD2b or to the GST domain itself as a negative control. Analysis of the bound material with an antibody against the GST domain confirmed the expression of both the control GST protein and GST-MBD2b (Fig. 4). The AU5-specific antibody identified the AU5 p66α fusion within the input fractions, whereas after purification with the glutathione beads only the GST-MBD2b containing sample retained AU5-tagged p66α (Fig. 4). In addition we tested the capacity of endogenous HDAC1 or HDAC2 to bind to expressed GST-MBD2b. Clearly, GST-MBD2b retained HDAC1 and -2 as visualized with a mixture of antibodies directed against HDAC1 and -2 (Fig. 4).

We were also interested in the subcellular localization of MBD2 and of both human p66 proteins. Immunofluorescence, followed by confocal microscopy, clearly showed that hp66α and GFP-MBD2a, but not the GFP protein alone, colocalize in a speckled nuclear pattern (Fig. 5, A–F). Although both proteins are clearly present at the same nuclear loci, as shown in the merged picture, they do not colocalize completely, indicating that both proteins also may exert their functions independently from each other. Interestingly, analyzing the colocalization of both p66 proteins in the same cell, we observed an identical distribution of both proteins, suggesting that hp66α and hp66β always appear pairwise and can be recruited, at least in part, via the MBD2 protein.

**Fig. 5.** hp66α colocalizes with MBD2 and hp66β in a speckled nuclear pattern. pCEFLAU5-hp66α was cotransfected into HEK 293 cells with pEGFP-C2 (A–C), GFP-MBD2a (D–F), or pEGFP-hp66β (G–I). Subnuclear distribution of each protein was analyzed either by direct (GFP-constructs, green) or by indirect immunofluorescence (AU5-hp66α, red), followed by confocal microscopy. A, D, and G, nuclear distribution of EGFP, GFP-MBD2a, and EGFP-hp66β, and B, E, and H, the hp66α signal within the same cell, generated with anti-AU5 antibodies against the fusion protein. C, F, and I, colocalization of hp66α with GFP-MBD2a and EGFP-hp66β, but not GFP, is shown in the merged picture.

**Fig. 6.** Gal-hp66α represses transcription. CV1 cells were cotransfected with a 4xUAS tk luciferase reporter together with Gal-DNA binding domain, Gal-hp66α or Gal-hp66α deletion constructs. Fold repression was determined relative to Gal-DNA binding domain alone at the equivalent amounts of transfected plasmid. Error bars represent variations within triplicate transfections. A, hp66α represses transcription dose dependently. Luciferase activity was measured after cotransfecting the cells with increasing amounts of Gal-DNA binding domain or Gal-hp66α (0.06–1.58 μg). B, at least two separate domains are responsible for the repressive capacity of hp66α. Schematic overview of Gal-hp66α deletion constructs (0.53 μg) and their respective repressive capacities.
hp66α Is a Potent Transcriptional Repressor—The p66 containing Mi-2/NuRD complex or the MeCP1 complex play a role in transcriptional repression. Therefore, we analyzed the transcriptional effect of hp66α fused to the GAL4 DNA binding domain. Transfection of a luciferase reporter gene, containing 4 GAL4 DNA binding sites (UAS), into CV1 cells, served as a tool to study GAL4-hp66α repression. Transfection of increasing amounts of GAL4-hp66α lead to a dose responsive increase in transcriptional repression (Fig. 6A). More than 100-fold repression by GAL4-hp66α could be observed in comparison to the unfused GAL4 DNA binding domain (Fig. 6A). This system was further used to analyze a number of different hp66α truncations (Fig. 6B). Truncation of the C-terminal 304 amino acids (GAL4-hp66α-(134 to 329)) had no effect on the magnitude of the repression. The N terminus of this protein (1–133) contains no repression activity indicating that the region from 134 to 329 is important for transcriptional inhibition. Interestingly, the remainder of hp66α-(134–633) shows a more than 33-fold repression, but does not achieve wild type activity. This region contains CR1. By testing CR1 (GAL fusion 134–238), only weak repression can be observed. These data suggest that a complex pattern of hp66α domains exist, mediating transcriptional repression. Thus, we conclude that several repressive domains act in synergy to mediate the more than 100-fold repression seen with the full-length molecule.

Both hp66α and hp66β Are Ubiquitously Expressed in Cell Lines, in Fetal and Adult Tissues—Here we show that one component of the Xenopus Mi-2/NuRD complex, the p66 protein, exists in two homologues in man and mouse. We wondered whether these p66 homologues improve the functional role of p66 by redundancy or, whether they are specialized in different aspects. A possible specificity of the two p66 homologues might contribute to tissue-specific variations of the Mi-2/NuRD complex or the MeCP1 complex. Heterogeneity of MeCP1 has been suggested from band shifts with liver and fibroblast extracts (21). Therefore, we analyzed the expression pattern of transcripts from hp66α and hp66β by semiquantitative PCR using cDNA prepared from RNA of several cell lines. The PCR primers were designed such that p66α and p66β generated fragments of different size. This enabled us to carry out single tube PCR reactions for both p66 homologues (Fig. 7A). Evaluation of the generated PCR products demonstrated a marginal variation between p66α and -β. Cell lines often do not mirror the tissues from which they are derived. Therefore, we used cDNAs from different human fetal and adult tissues (Clontech). Again, all of the tissues tested were positive for p66α and p66β, with minor differences in absolute as well as in relative amounts (Fig. 7B). Colocalization and coexpression of both p66 proteins may suggest a common role. Whether this role is solely within the Mi-2/NuRD or the MeCP1 complex remains to be shown.

hp66α and MBD2β Both Contain Two Domains Interacting with Each Other—The overlapping cDNA clones coding for fragments of p66α, which bind to MBD2b in the yeast two-hybrid assay (Fig. 1), suggested that the overlapping region contained a domain that mediates the interaction with MBD2b. To substantiate this result, we carried out in vitro GST pull-down experiments. For this purpose, we in vitro translated human full-length hp66α as well as a set of C- and N-terminal truncations (Fig. 8A). These were incubated with bacterial expressed GST-MBD2b and analyzed for binding. All of the in vitro translated constructs containing the minimal overlapping region from the yeast two-hybrid screen showed a specific binding to GST-MBD2b and no binding to the GST control, even a small fragment of just 105 amino acids (ID1 = aa 134–238). In contrast, a fragment from amino acids 1 to 133, lacking the minimal overlapping region, showed no binding (Fig. 8A). Analysis of the C-terminal region of hp66α (aa 372–633) showed specific binding as well (ID2). This region contains the CR2 sequence that codes for a GATA-zinc finger. Therefore, two fragments of hp66α harboring the conserved CR1 element or the CR2, respectively, mediated specific in vitro binding to GST-MBD2b.

To determine regions of MBD2b responsible for the interaction with hp66α, C-terminal as well as N-terminal truncations of the GST-MBD2b fusion were expressed in E. coli, purified, and controlled for similar amounts. Incubation with in vitro translated hp66α allowed the identification of domains within MBD2b responsible for binding (Fig. 5B). All of the C-terminal truncations of GST-MBD2b bound to hp66α except for the GST fusion aa 1–27. Because the MBD2b fragment from amino acids 1 to 45 mediated specific binding, it can be concluded that a region between amino acids 27 and 45 is necessary for binding, thereby defining interaction domain 1 (ID1). Indeed, a fragment from amino acids 28 to 82, which contains ID1, mediated specific binding. N-terminal truncations of GST-MBD2b showed specific binding as well, such that the fragment from
amino acids 211 to 262 was positive in binding, but not the fragment containing amino acids from 163 to 211. This argues for a second interaction domain at the very C terminus of MBD2b. These interaction domains (ID1 and ID2) co-localize with other important features of MBD2b. ID1 overlaps both with the domain responsible for binding to methylated DNA (11) and for binding to Sin3A (14). ID2 overlaps with the predicted coiled-coiled domain of MBD2 (26). Summarizing, the interaction of MBD2b and hp66/H9251 is mediated by two interaction domains on each protein.

hp66α and hp66β Differ in Their Domains Required for Binding to MBD2b and MBD3—To test possible binding specificities of MBD3 and MBD2b with respect to binding p66α or p66β, we expressed GST-MBD fusions in E. coli and analyzed binding to full-length p66 proteins (Fig. 9A). GST fusions were controlled for similar amounts of expressed proteins (Fig. 9, Coomassie-stained gel) and were either incubated with in vitro translated hp66α or hp66β. Both GST-MBDs bound p66α or p66β (Fig. 9A). A significant difference in binding affinity between the two p66 proteins was detected with about 35% binding of p66α and about 20% binding of p66β.

Because the deletion analysis above identified two MBD2b binding domains within hp66α, we tested whether both domains exist in hp66β as well. The in vitro translated ID2 of hp66α-(372–633) or an equivalent construct of hp66β-(342–593) was incubated with GST-MBD2b or GST-MBD3 and analyzed for binding. No major difference in binding of hp66α C terminus to MBD2b and -3 could be observed (Fig. 9B). Interestingly, the equivalent C-terminal region of hp66β did not bind to either MBD2b or MBD3. Because the CR2 region in both hp66 proteins is highly conserved, and the interaction with MBD2/3 is specific for the C-terminal region of hp66α, the interaction domain is probably outside the CR2 region or is specified by nonconserved residues. Thus, p66α and hp66β differ in binding to MBD2/3 with p66α showing a higher binding affinity mediated by two interaction domains, whereas hp66β binds only via the N terminus to MBD2 and MBD3.

DISCUSSION

In mammals, methylation of DNA at cytosines of CpG dinucleotides has been correlated with regulation of gene expression and is known to be involved in genomic imprinting
identified one protein, termed hp66 family outside the NuRD or MeCP1 complex is possible as well between different tissues. However, a function of the p66 gene the other homologue to the NuRD or MeCP1 complex varies is still possible, however, that the final contribution of one or proteins, presumably within a deacetylase containing complex. It expressed in various cancer cell lines as well as in human fetal and adult tissues, suggesting a common role for both p66 pro-

FIG. 9. hp66α and hp66β differ in binding to MBD2 and MBD3. In vitro translated hp66α and hp66β protein (A) and the respective C-terminal ends, hp66α-(372–633) and hp66β-(342–593) (B), were subjected to GST pull-down assays using purified GST-MBD2b and GST-MBD3. After electrophoresis, the gel was stained with Coomassie Blue, to ensure equal usage of protein in the pull-down reactions (lower panel), followed by autoradiography (top panel). The input shown is 10% of the in vitro translated product used in the assay. The percentage of binding indicated below the lanes was determined by densitometry. The Coomassie-stained band (⁎) in the input lane is bovine serum albumin.

(27) and X-chromosome inactivation (28). Histone deacetylase complexes, such as MeCP1 and Sin3A, are thought to be targeted to regions of methylated DNA and regulate transcription by altering the nucleosomal structure (18, 29, 30). The MeCP1 complex represses transcription through nucleosomal remodeling and histone deacetylation resulting in a condensed chromatin structure (19). The complex is composed of 10 subunits, including the methyl-CpG-binding proteins MBD2, MBD3, and the Mi-2/NuRD complex with 2 proteins of 66 and 68 kDa. Recently, the p66/p68 components of the MeCP1 complex were identified as a single protein differing by a proposed modification (20). Here we demonstrate that two members of a p66 gene family exist in mouse and man. Using a yeast two-hybrid screen we identified two proteins, both with homologies to the 66-kDa protein of Mi-2/NuRD and show that hp66α in a new homologue to the previously identified p66 component of the MeCP1 complex (20). The second protein, termed hp66β, turned out to be identical to the p66 protein from MeCP1.

We demonstrate here that both members of the p66 gene family colocalize to nuclear speckles and that MBD2 and hp66α show a matching speckled distribution within the nucleus. Furthermore, GST-MBD2b expressed in eukaryotic cells is associated with AU5-tagged hp66α and histone deacetylases 1 and 2. Additionally, hp66α and hp66β are ubiquitously coexpressed in various cancer cell lines as well as in human fetal and adult tissues, suggesting a common role for both p66 proteins, presumably within a deacetylase containing complex. It is still possible, however, that the final contribution of one or the other homologue to the NuRD or MeCP1 complex varies between different tissues. However, a function of the p66 gene family outside the NuRD or MeCP1 complex is possible as well and requires further investigation. Functionally, hp66α is a potent transcriptional repressor inhibiting reporter activity in a dose-dependent manner up to 140-fold. Several deletion constructs of hp66α indicate that a synergistic action of several repressive domains is responsible to achieve full repressive capacity. Repression by the homologous protein hp66β has been shown to depend on CR1 (20), which in the case of hp66α is clearly not the only repression domain. Because the identification of both p66 proteins in a yeast two-hybrid screen already suggests direct binding to MBD2, we further verified the binding characteristics of both proteins, hp66α and hp66β. Indeed, utilizing in vitro GST pull-down assays we could demonstrate direct association of both proteins with MBD2 and MBD3. Thus, members of the p66 gene family may play a role not only structurally within the Mi-2/NuRD complex, but also may function as a molecular bridge to MBD2 within the MeCP1 complex. In addition, MBD2-MBD3 heterodimerization (31) may link the Mi-2/NuRD complex to MeCP1. Other proteins binding directly to MBD2 are the zinc finger protein MIZF (32) and Sin3A (14). The latter suggests that the function of MBD2 may also involve other histone deacetylase complexes as well, like the Sin3A complex (for review, see Ref. 33). Interestingly we show here that the C-terminal domain of hp66α and hp66β differ in respect to binding MBD2 or MBD3. Although both C termini contain the conserved region 2 (CR2), hp66α is able to bind MBD2 or MBD3 via its C terminus, whereas the equivalent region of hp66β does not. Additionally the CR2 of hp66β has been shown to be involved in targeting hp66β and MBD3 to speckles within the nucleus (20). Together, these data suggest that nuclear targeting to speckles and binding to MBD2 or MBD3 are two different functions of the C-terminal domains of hp66α and hp66β.

Interestingly, many of the factors involved in chromatin remodeling, deacetylation, and transcriptional repression in the context of methylated DNA have been found in pairs. The HDAC core complex, found also in the Sin3A complex, is comprised of a pair of the histone deacetylases HDAC1 and -2, together with their associated proteins RbAp48/46. In addition, the simultaneous purification of the Mi-2/NuRD components Mi-2α (CHD3) and Mi-2β (CHD4), which both confer nucleosomal remodeling and ATPase activity and are associated with histone deacetylases, has been described (34). Similar observations have been made for the 70-kDa subunits of Mi-2/NuRD, which have been identified either as metastasis associated protein 1 (MTA1) (35) or its homologues MTA1-like (=MTA2) (18). MTA1 has been originally identified as being overexpressed in metastatic carcinomas (36). Its homologue, MTA2, besides the association with NuRD, where it directs the assembly of an active histone deacetylase core complex (18), was also found to specifically interact with and to target deacetylation of p53 (37). Here we show that in humans, p66, another component of the Mi-2/NuRD complex (17, 20) comes in two homologues, p66α and p66β, as well. It is interesting to note that in
**p66 Gene Family**

*Drosophila*, as an example for a lower eukaryotic organism, this kind of duplex organization cannot be observed. All NuRD subunit orthologues have been identified and reported to be also present in a functional, active *Drosophila* Mi-2/NuRD complex, comprised of the single factors dMi-2, dMTA-like, dRPD3, p55 (=RhAP48/46), and dMBD-like (38). In addition to providing functional redundancy, the pairwise duplicated factors in mammals may allow for a larger spectrum of regulatory responses. Similarly, hp66α and hp66β, despite remarkable similarities in repressive activity, ubiquitous expression, and nuclear distribution, differ in their binding domains and affinities to Mi-2/NuRD.

Colocalization of hp66α and hp66β in a speckled nuclear pattern, *in vivo* binding of Mi-2 to hp66α and HDAC1 and -2, and identical expression profile of both p66 proteins in adult and fetal tissues suggests that both proteins exert their function at identical sites within the genome. Because recent studies showed *in vivo* binding of p66 (hp66) to Mi-2 and Mi-3 (20), it can be speculated that both hp66α and hp66β complete the set of duplicated proteins within the MeCP1 complex. However, future experiments will determine whether hp66α is contained within MeCP1 or if it is involved in different functions, as compared with hp66β.

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