CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2

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ABSTRACT

DNA methylation at HpaII (CmCGG) sites inhibits expression of a human proenkephalin-CAT fusion gene when it is transiently expressed in CV-1 cells or stably expressed in C6-glioma cells. The inhibitory effects of HpaII methylation have been mapped to a site within the human proenkephalin promoter located at position -72 relative to the start site of transcription. This region spans a cAMP and phorbol ester inducible enhancer and methylation at this position inhibits both basal transcription and transcription induced by either cAMP or TPA. The HpaII site is located within an element which binds the transcription factor AP-2. In vitro methylation at this HpaII site inhibits the binding of AP-2. These results suggest that CpG methylation inhibits proenkephalin gene expression by directly interfering with the binding of a positively acting transcription factor previously shown to be essential for maximal basal, cAMP, and TPA inducible transcription.

INTRODUCTION

Transcriptional control of gene expression involves the interaction of a large number of cellular trans-acting factors with cis-acting DNA elements to promote transcription (1, 2). Temporal and tissue-specific regulation of gene expression may depend upon the activity of various DNA binding proteins. The activity of these proteins may in turn be determined by a variety of cellular signals, protein/protein interactions, as well as the structure and accessibility of the gene in its local chromatin environment (3-7). Modification of DNA structure by DNA methylation may play an important role in controlling DNA/protein interactions and result in the activation or repression of gene expression. The comparison of DNA methylation patterns before and after induction of gene expression, or between expressing and nonexpressing cell types, correlates the loss of methylation at specific CpG sites within promoter regions with gene expression (3-8). In general, an inverse correlation between gene expression and DNA methylation at CpG dinucleotides has been established for a variety of vertebrate and viral genes (4, 5). In addition,

in vitro cytosine methylation at specific CpG residues within a variety of cellular and viral genes, followed by reintroduction of the methylated genes into tissue culture cells, dramatically inhibits gene expression (9, 13-20). Although the mechanism of this effect has not been determined, recent evidence supports two different models. DNA methylation at CpG dinucleotides located within the binding sites of positive acting transcription factors may alter protein/DNA interactions (3, 35-37). Alternatively, methylation may indirectly effect transcription by altering chromatin structure (34).

The human proenkephalin promoter region contains 10 times the number of CpG dinucleotides expected at random and some CpG sites have been shown by analysis of genomic DNA to be methylated in a tissue specific fashion (21). Recently, a cAMP and phorbol ester inducible enhancer has been localized within the promoter region (22). A detailed point mutation analysis of the inducible enhancer region has resolved the enhancer into multiple DNA elements located within a 50 base pair region of DNA (-64 to -114) upstream of the transcription initiation site (23). Furthermore, each of these elements act synergistically to activate transcription in the presence of cAMP or phorbol esters. Four different DNA binding proteins have been shown to interact with these elements *in vitro* (23). One of these proteins, AP-2, which also interacts with the SV-40 (24) and metallothionein (25) enhancers, binds to a site within the proenkephalin enhancer located by DNase I footprinting between -85 and -65 base pairs upstream of the start site of transcription (23, 24, 27). Mutations within this site reduce both basal and cAMP and TPA induced transcription approximately 5 fold, although this element in the absence of sequences located immediately upstream is unable to stimulate correctly initiated transcription (23). This indicates that the AP-2 binding element acts synergistically with upstream elements to activate transcription (27).

In this report we show that *in vitro* methylation of CpG dinucleotides within the proenkephalin promoter at HpaII sites (CCGG) dramatically inhibits both basal and cAMP induced transcription when reintroduced into tissue culture cells by transfection. DNA mapping and transfer experiments localize the effects of HpaII methylation to a site located within a binding site for the transcription factor AP-2. Furthermore, DNase I

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footprinting experiments demonstrate that DNA methylation at this HpaII site inhibits the binding of AP-2 to its recognition site correlating the *in vivo* effects of HpaII methylation with the *in*

vitro binding of AP-2. DNA methylation at HpaII sites and the effects of mutation within the AP-2 site (27) produce functionally similar effects on basal, cAMP and TPA inducible transcription.

Figure 1. a) Schematic diagram of the human proenkephalin and mouse POMC/CAT fusion plasmids. Construction of these plasmids has been described previously (22). The location of HpaII sites which differ between plasmids are indicated. The start sites of transcription are indicated by arrows. **b)** DNA methylation at HpaII sites inhibits expression of a human proenkephalin/CAT fusion gene when expressed transiently in CV-1 cells. The promoter region containing a single HpaII site transfers HpaII sensitivity to the POMC promoter. The -58 to -155 region of the human proenkephalin promoter/enhancer was fused to the -84 truncated POMC promoter by insertion of a 97 base pair BssHII fragment into a MluI site in pPOMCAT-84. The 97 bp BssHII fragment contains a single HpaII site located at position -74. DNA methylation, transfection, preparations of cell extracts, and enzyme activities were carried out as described in the Materials and methods section. Plasmids were methylated and transfected into CV-1 cells as described in the Materials and methods section. Cell extracts were prepared 48 hours post transfection and CAT and B-galactosidase activities measured from the same extract. CAT expression was determined as described in Materials and methods and was normalized to the B-galactosidase activity. Three (pRSVCAT, pENKAT-12, pPOMCAT-84) or two (pENKPOM-2) separate experiments using different preparations of mock and HpaII methylated plasmid DNA were carried out and representative data shown above.

Together these results suggest that DNA methylation may control the level of human proenkephalin gene expression *in vivo* by altering DNA/protein interactions at the AP-2 binding site.

MATERIALS AND METHODS

Materials

All methylases, restriction and DNA modifying enzymes used in the construction of plasmids and footprinting probes were from New England Biolabs. 14-C-chloramphenicol was from Amersham and Butyryl-CoA was from Pharmacia.

In vitro methylation of DNA fragments

Plasmids and purified DNA fragments were treated with HpaII methylase at 37°C for 2–6 hours in the presence of 10 mM EDTA and 80 μ M S-adenosylmethionine as recommended by the suppliers. The extent of methylation was determined by subsequent cleavage with HpaII and Msp I restriction enzymes. Mock methylated controls were incubated as described above except enzyme was omitted. Only completely methylated DNA preparations were used in transfection and footprinting experiments. Hemimethylated DNA was prepared by primed DNA synthesis using a ³²P-end-labeled oligonucleotide complementary to nucleotides +48 to +70 as primer and single stranded M13 DNA containing a fragment of the human proenkephalin gene extending from -193 to +150 as template. The primer/template annealing mix was split in half and double stranded DNA was synthesized in the presence of dCTP or 5-methyl-dCTP using DNA polymerase I (Klenow fragment). This reaction produced a double stranded DNA probe labeled at +70 which contained either cytosine or 5-methyl-cytosine incorporated into the coding DNA strand.

Cell culture

C6-Glioma and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). HeLa cells were grown in spinner culture using Joklik's modified Eagle's medium supplemented with 7.5% horse serum.

Transient and stable analysis of gene expression

Transfection of CV-1 cells was performed as previously described (22). For transient expression CAT plasmids (5 μ g) were cotransfected with 10 μ g pRSVBGAL (28) and 10 μ g pUC18. Following transfection with CaPO₄ precipitated DNA and glycerol shock, cells were incubated for 16 hours in media containing 10% FCS. Cells were induced by incubation with CPT-cAMP (0.2 mM) and IBMX (0.5 mM) for 6 hours, and harvested for protein extracts. CAT activities were measured as described previously (22) or by a modification of the standard assay (29). Butyryl CoA (3 mM) was used instead of acetyl CoA and reaction products were extracted with 400 μ L of a 2 to 1 mixture of pristane/xylene instead of TLC chromatography. Under these conditions butyrylated chloramphenicol was preferentially extracted into the organic phase and 300 μ L was counted directly in scintillation fluid. B-Galactosidase activities were determined as previously described (22, 30) and used to normalize CAT activities within a given experiment. Stably expressing cell lines were produced by CaPO₄ cotransfecting 8×10^5 C6-Glioma cells in a 10 cm dish with 4 μ g pRSVNEO (28) and 20 μ g indicator CAT plasmid. One day following transfection cells were transferred to selective media (500 μ g/ml G418) and grown for two weeks. Under these conditions of cotransfection between 25 and 50% of the G418 resistant colonies express the nonselected CAT plasmid. 400 to 1,000 independent

G418 resistant colonies from each transfection were treated with trypsin and pooled into two plates containing identical numbers of cells. Cells were induced by incubation with 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) (0.2 mM) and isobutylmethylxanthine (IBMX) (0.5 mM) for 2 to 4 hours and harvested for analysis of RNA or incubated for 6 hours and harvested for CAT assays. Total cytoplasmic RNA was isolated by lysis of cells with 0.5% NP-40, removal of nuclei by centrifugation (13,000 rpm for 10 min. in microfuge), multiple phenol/chloroform extractions, and ethanol precipitation of RNA. Forty μ g samples of RNA from control and cells treated with regulators were hybridized with a single stranded DNA probe spanning hENK sequences -193 to +70, treated with S1 nuclease as previously described (22), loaded onto 8% sequencing gels, and exposed to film for 16 to 48 hours in the presence of an intensifying screen.

Preparation of nuclear extracts and affinity purified proteins

Nuclear extracts were prepared from HeLa cells as described by Dignam (31). Large scale nuclear extracts were prepared from HeLa S3 cell (Dignam et al., 1983) grown in spinner culture in 7.5% horse serum. Purification of AP-2 by affinity chromatography was carried out as described by Kadonaga (32) using a synthetic oligonucleotide spanning human proenkephalin sequences between -85 and -62. AP-2 fractions used in DNase I footprinting experiments were purified by three successive passes over the affinity column and contained approximately 1 footprint unit/100ng protein (1 footprint unit is defined as the amount of protein required to completely protect the human proenkephalin AP-2 binding site in the standard DNase I protection assay using 10 fmoles of DNA probe).

DNase I footprinting

DNase I footprinting was carried out as described by Lee (33). Typically 10 fmoles 32-P-labeled DNA probe (end-labeled on the coding strand at position +1 by the 'filling in' reaction using alpha-32-P dCTP) was incubated on ice for 15 minutes with purified protein. Samples were then incubated at room temperature for 1 min. and treated with DNase I for 1 min. at 25°C. Samples were phenol/chloroform extracted, ethanol precipitated, and analyzed by 7% acrylamide/urea gel electrophoresis.

RESULTS

Methylation at HpaII sites inhibits a proenkephalin/CAT fusion gene

As shown in Figure 1b in vitro methylation of the plasmid pRSVCAT at all HpaII sites has no significant effect on CAT expression after transfection and expression in CV-1 cells. In contrast, methylation of pENKAT-12 at HpaII sites inhibits CAT expression 4 to 6 fold. Similar levels of repression of CAT expression by HpaII methylation have been observed after transfection and transient expression in mouse L cells and C6-glioma cells (data not shown). Since pRSVCAT contains all of the bacterial sequences (pBR322 and CAT) found in pENKAT-12 the differential effects of methylation can be localized to either the proenkephalin 5' or 3' sequences controlling CAT expression. In all transient expression experiments methylated and mock methylated pENKAT-12 is cotransfected with pRSVBGAL, a plasmid containing the Rous Sarcoma Virus (RSV) promoter/enhancer linked to the bacterial B-galactosidase

gene (see Materials and Methods). CAT activities measured from transfected cell extracts are then normalized to the B-galactosidase activity determined from the same extract to compensate for variations in DNA uptake and expression.

Mapping and transfer of methylation sensitivity to a heterologous promoter

To localize the HpaII sites responsible for methylation sensitivity, we transferred hENK 5'-flanking sequences to a POMCAT fusion gene, POMCAT-84. The plasmid, POMCAT-84, contains the mouse proopiomelanocortin promoter sequences from -84 to +234 linked to the CAT gene (see Figure 1a). The POMC sequences contain a single HpaII site located at +144. POMCAT-84 expresses basal CAT activity 2 to 4 fold lower than pENKAT-12, and is not inhibited by HpaII methylation (Fig. 1b). Because this plasmid differs from pENKAT-12 only by replacement of the human proenkephalin promoter with the mouse POMC promoter region (-84 to +107), and is not sensitive to HpaII methylation we conclude that the replaced hENK sequences confer HpaII methylation sensitivity. To further define the HpaII sites conferring methylation sensitivity, hENK gene sequences -155 to -58 were fused to the POMC promoter at position -84 (pENKPOM-2). This plasmid shows a 2 to 4 fold increase in basal expression and confers sensitivity to HpaII methylation (Fig. 1b) suggesting that the 97 bp of hENK 5'-flanking sequences are sufficient to confer methylation sensitivity to the POMC promoter. The transferred region of the hENK gene contains a single HpaII site located at position -72, indicating that methylation at this HpaII site is sufficient to inhibit CAT expression directed from the ENK/POMC fusion promoter.

Effects of HpaII methylation on cAMP inducible transcription

The inhibitory effects of in vitro DNA methylation on CAT expression directed by the hENK promoter map to the region of DNA previously shown to contain a cyclic AMP and TPA inducible enhancer (22, 23). In addition the effects of HpaII methylation act in an orientation independent fashion (data not shown) suggesting that the inhibitory effects of DNA methylation might be the result of interference or inhibition of enhancer function. To test this possibility, we examined the effects of HpaII methylation on cyclic AMP inducible transcription. As shown in Figure 2a both basal expression and cAMP and TPA inducible expression are inhibited approximately 5 fold by cytosine methylation at HpaII sites. It should be noted that the relative fold induction by the methylated plasmids is unchanged as both basal and inducible expression are reduced.

In addition to studying the effects of HpaII methylation on transient expression in CV-1 cells we also examined the effects of HpaII methylation on proenkephalin gene expression when methylated plasmid was stably integrated into the genome of C6-glioma cells. HpaII methylated or mock methylated pENKAT-12 (20 μ g) was cotransfected with pRSVNEO (5 μ g) into C6-glioma cells, selected in the presence of 500 μ g/ml G418, and equal numbers of resistant colonies were grown together in mass culture. To determine the effects of methylation on gene expression, we examined CAT and RNA levels from methylated and mock methylated pooled stable clones. As shown in Figure 2b, both CAT activity and correctly initiated RNA levels (Fig. 2c) were inhibited by HpaII methylation. Roughly the same number of NEO resistant colonies were obtained from methylated and mock methylated plasmids.

Figure 2. a) HpaII methylation inhibits both basal and cAMP inducible enhancer function. CV-1 cells transfected with mock or HpaII methylated plasmids were treated with 200 μ M CPTcAMP and 500 μ M IBMX 16 hours post transfection. Cells were harvested and extracts prepared 6 hours after addition of cAMP agonists. b) HpaII methylation inhibits both basal and cAMP inducible expression when stably introduced into rat C6-glioma cells. C6-glioma cells (8×10^5 /10 cm dish) were cotransfected with mock-methylated and HpaII methylated pENKAT-12 or pENKAT-84 (20 μ g) together with pRSVNEO (4 μ g), selected in 500 μ g/ml G418, and approximately 400–500 colonies pooled, equal numbers of cells plated and treated in the presence and absence of 200 μ M CPT-cAMP and 500 μ M IBMX for 6 hours. CAT activities were determined as described in Materials and methods. c) Analysis of correctly initiated RNA from ENKAT-12 stably transfected C6-glioma cells. C6-glioma cells stably transfected with mock methylated or HpaII methylated pENKAT-12 were treated in the presence (+) or absence (–) of CPT-cAMP/IBMX for 2 hours, total cytoplasmic RNA isolated, hybridized to a proenkephalin probe, treated with S1 nuclease, and digestion products analyzed by polyacrylamide/urea gel electrophoresis as described previously (22). The arrow indicates correctly initiated proenkephalin transcripts.

HpaII methylation and incorporation of 5-methylcytosine inhibits the binding of AP-2

To examine the mechanism of action of HpaII methylation we next tested whether HpaII methylation affected the binding of proteins to the region spanning the HpaII site located 72 base pairs upstream of the start site of transcription. DNase I footprinting experiments using crude HeLa cell nuclear extracts (data not shown) indicated that the binding of a nuclear factor to the –85 to –65 region of the enhancer was inhibited by HpaII methylation. Previously, we have demonstrated that this region

is protected by the transcription factor AP-2 (23, 24, 27). We next tested whether the binding of affinity purified AP-2 was inhibited by HpaII methylation. As shown in Figure 3a and 3b the binding of affinity purified AP-2 to the –85 to –65 region of the proenkephalin enhancer is inhibited by HpaII methylation but not by Hha I methylation. To further characterize the presence of 5-methylcytosine within the AP-2 binding site, at positions other than the HpaII site, we prepared hemimethylated DNA which contained 5-methylcytosine substituted for cytosine at each position along one strand of the AP-2 binding site (see Figure

Figure 3. HpaII methylation and incorporation of 5-methylcytosine inhibits binding of the transcription factor AP-2. **a)** A DNA restriction fragment (ApaI to XmaI) spanning the human proenkephalin promoter enhancer region (–440 to +1) was labeled at position +1 and +2 by filling in a XmaI restriction site with 32-P dCTP using reverse transcriptase. This produces a probe in which the coding strand is labeled at position 1 and 2. Approximately 1 nanogram of labeled fragment was incubated with the indicated volume (μ l) of affinity purified AP-2 protein, (–) refers to the omission of AP-2 protein. DNase I footprinting reactions and DNA methylation were carried out as described in the Materials and methods section and analyzed by 7% polyacrylamide/urea gel electrophoresis. Protected regions of the DNA (DNase I footprint) are indicated at the left of the panel. **b)** DNase I footprinting using a DNA probe labeled on the opposite strand. A DNA fragment (EcoRI to SmaI) was labeled at position –193 by filling in the EcoRI site using 32-P dATP and reverse transcriptase. **c)** Control and hemimethylated end-labeled DNA probes were prepared by primed DNA synthesis using a 32-P-labeled oligonucleotide spanning human proenkephalin sequences +48 to +70 as primer and single-stranded M13 DNA containing a fragment of the human proenkephalin gene spanning sequences from –185 to +405. The primer/template annealing reaction was split in two with half the reaction extended in the presence of dCTP and the other half extended in the presence of 5-methyl-dCTP. Reaction products were purified by phenol/chloroform extraction and ethanol precipitation. Approximately 100 fmole of dCTP or 5-methyl-dCTP DNA was incubated with the indicated volume (μ l) of affinity purified AP-2 as described in Materials and methods.

4). This DNA was prepared by primer extension using a 32-P labeled oligonucleotide spanning human proenkephalin sequences +48 to +70 as primer and single-stranded M13 DNA containing a fragment of the human proenkephalin gene spanning –185 to +150 as template. As shown in Figure 3c, incorporation of 5-methyl-dCTP, but not dCTP, completely blocked the binding of affinity purified AP-2 to its recognition site.

DISCUSSION

Although the inhibitory influence of DNA methylation on gene expression has been well documented for a variety of genes (4),

the molecular mechanisms by which DNA methylation effects gene expression are still poorly understood. Most evidence suggests that modification of DNA by methylation serves to generate a local chromatin configuration that renders the gene inaccessible to the transcriptional machinery and hence inactive (4, 7). This may result from direct inhibition of sequence specific DNA binding factors or from indirect effects on chromatin structure. Recently several groups have reported that CpG methylation has no effect on the DNA binding of the transcription factor, Sp-1, to its recognition site (34–35). In contrast, CpG methylation appears to block binding of transcription factor E2F or MLTF to the adenovirus E2 promoter and major late

Figure 4. Summary of the effects of DNA methylation and protein/DNA interactions within the human proenkephalin enhancer/promoter. Position of restriction and DNA methylation sites are indicated in the top section of the figure. The BssHII restriction fragment which transfers HpaII methylation sensitivity and cAMP inducible enhancer function to the POMC promoter is indicated directly below. The sequence of the human proenkephalin gene spanning -111 to -53 is shown below. The position of inducible elements and protein binding sites spanning the inducible enhancer region, determined by single base substitution mutations and DNaseII footprinting, are indicated below. The hemimethylated DNA probe used in DNaseII footprinting experiments contained 5-methyl-C incorporated into the lower strand, the positions of incorporation through the AP-2 binding site are indicated with asterisks.

promoters (36). In addition, binding of as yet unidentified proteins to the tyrosine aminotransferase gene (3) and binding of ATF/CREB-like proteins to the CRE (37) are inhibited by CpG methylation. These results suggest that the DNA binding affinity of certain transcription factors may be regulated by CpG DNA methylation.

The results presented here demonstrate that methylation at a single HpaII site within the proenkephalin promoter inhibits both basal and cAMP inducible transcription. This HpaII site lies within a region of DNA previously shown to act as a cAMP inducible enhancer which consists of at least three tightly-spaced adjacent DNA elements; ENKCRE-1, ENKCRE-2, and an AP-2 binding element (Figure 4) (23). Each element is essential for maximal basal, cAMP, and TPA inducible transcription (23, 26) and each functions synergistically with the other elements. For example, the AP-2 binding element or the ENKCRE-1 element which themselves are unable to promote basal or inducible transcription will greatly augment cAMP or TPA inducible transcription in the presence of a functional ENKCRE-2 element (23, 26). Mutations which inactivate the AP-2 site yet leave the ENKCRE-1 and ENKCRE-2 elements intact result in a 4 to 6 fold reduction in cAMP and TPA inducible transcription and a several fold reduction in basal transcription (26). The effects of inactivating the AP-2 element by mutation are very similar to the effects of HpaII methylation within the element. Hence, both mutation and DNA methylation within the AP-2 binding site produce functionally similar inhibitory effects on the *in vivo* activity of the human proenkephalin cAMP and phorbol ester inducible enhancer.

In vitro binding of the transcription factor, AP-2, to the proenkephalin enhancer is inhibited by CpG methylation at the HpaII site or by incorporation of 5-methylcytosine within the AP-2 site. Together these results suggest that HpaII methylation acts to inhibit proenkephalin gene expression *in vivo* by reducing the affinity of a positively acting transcription factor, AP-2, with its DNA recognition site located within the cAMP and TPA inducible enhancer.

The role of CpG methylation in the developmental and tissue-specific regulation of human proenkephalin gene expression has yet to be determined. Competition between the binding of transcription factors to CpG containing recognition sites and methylation dependent inhibition of factor binding is an attractive mechanism to produce stable alterations in gene expression both during development, and in the mature organism. Displacement of transcription factor binding by tissue-specific methylation at sites spanning the inducible enhancer could result in either positive or negative effects on transcription depending on the activity of the displaced factor. Furthermore, tissue-specific differences in DNA methylation could serve to regulate factor interactions and hence the mode of transcriptional regulation in cells expressing the same set of transcription factors.

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